Enzyme Technology

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Springer

ENZYME TECHNOLOGY

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ASIATECH PUBLISHERS, INC.
New Delhi

ISBN 978-1-4419-2124-6 ISBN 978-0-387-35141-4 (eBook)

9 8 7 6 5 4 3 2 1

springeronline.com

Published by N.K. Muraleedharan for Asiatech Publishers Inc.
E-20, Arya Nagar Apartment, Plot No. 91, I.P. Extension, Patparganj, Delhi 110092.
Phone: +91-11-22724601 • e-mail: asiatech_pb@yahoo.com
www.asiatechpublishersinc.com

Foreword

The generic subject of enzyme technology has a largely industrial and commercial purpose. Consequently it behoves researchers to operate within a culture of awareness of the constraints implicit on industrial practice. These are often reflected in allowable costs leading to the primacy of yield and limitations on the use of recovery, separation and purification processes to achieve saleable product. Because of the nature of the products, the process conditions and the raw materials, industrial safety is a significant feature. Also, as many of the products are used in food and food related applications, product safety is often paramount. Appreciation is also required of the product targets such as formulation, purity, activity, dispense, handling, stability, storage, specificity, safety, uses and conditions of use.

In contrast, the industrial practitioner needs an acute awareness of the underpinning science and the range of practical choices that can be explored to create a process and product, which both exploits the available science and meets the industrial and commercial constraints. Such 'process synthesis' represents one of the most elegant, intellectually challenging and ultimately satisfying achievements of any technology, and together with the desirable product characteristics is often referred to as 'product engineering' to emphasise the primacy of saleable product material as its objective.

In 'Enzyme Technology' the Editors have set out to provide a snap-shot of current practice and research which will assist both the researcher and the industrial technologist meet their respective goals. This has been achieved by providing an extensive basis of industrial enzymology, examples of industrial success covering the production and uses of many industrial enzyme products, and by illustrations of evolving practice. These 'developed areas' are complemented by descriptions of speciality process practices and descriptions of pioneering work concerned with improving the enzymes themselves, using protein engineering and other techniques, for use in industrial conditions with commercial objectives, and in the creation of new diagnostic and therapeutic products.

Enzyme technology is applied by companies, large and small, on a worldwide basis as part of their industrial production, often a very small part. The Editors have brought together this widespread custom and practice through a catholic range of authors, to give a worldview of a fascinating subject. To achieve coherence they have guided the contributors towards a presentational 'template' to provide a balance of information, style and format – and in this they are to be congratulated.

Overall, you will find a text which not only benefits researchers and practitioners but also provides a kaleidoscope of images of enzyme technology for those entering the area.

Bernard Atkinson

Preface

Enzymes are now a material well established in the field of biotechnology. This book covers different aspects related with the enzymes such as the producing microorganisms, their mode of cultivation, downstream processing, industrial production, properties and applications, with a special emphasis on industrial enzymes.

The book comprises 36 chapters written by internationally reputed authors in the field, which are classified and presented in four parts. Part I deals with general and fundamental aspects of enzyme technology and has nine chapters in it. Chapter one gives an overview of subject matter, beginning with the historical developments on the applications of enzymes since ancient times even before when their functions and properties were understood. The chapter provides brief details on the demand and business of enzymes, giving the names of the companies involved in the business, classification and nomenclature system of enzymes, and enzyme action, safety and therapy. Chapter two describes in details the general properties of enzymes such as chemical nature, structure, cofactors, enzyme specificity, measurement and expression of enzyme activity, stability and denaturation and factors affecting enzyme activity such as reaction time, amount of enzyme, substrate concentration, temperature, pH, ionic strength, pressure, inducers and inhibitors. Third chapter in this part is on enzyme kinetics and modeling of enzymatic systems. It provides theories of enzyme kinetics- free and immobilized. It describes alternative formulations of enzyme kinetics such as fractal and virial approaches, reversible enzyme inhibitors and substrate inhibition, and also deals with the mathematical models in enzyme kinetics, citing examples of modeling of enzyme reactions such as dipeptide synthesis, computation analysis of substrate binding, diagnostic tests, etc. The next chapter, i.e. fourth one is also on fundamental aspects dealing with the thermodynamics of enzyme catalyzed reactions. It first provides information on general principles such as thermodynamics of reactions involving specific chemical species and sum of chemical species- biochemical reactions. Then it gives details on what information that thermodynamics provides could be useful for industrial applications. The chapter also discusses experimental methods, physiological tables and cycles and estimation methods and quantum chemical calculations. Chapters fifth and sixth of the book are on biocatalysis; former one is on regeneration of cofactors for enzyme biocatalysis in which authors have discussed the importance and regeneration of various cofactors such as NAD^+, NADH, $NADP^+$, NADPH, ATP, sugar nucleotides, CoA, PAPS, etc for effective biocatalysis. The sixth chapter deals on biocatalysis in organic media. Although enzymes when added in organic solvents may lose their intrinsic activity because of denaturation, dehydration, inhibition, or chemical modification, they have been successfully used in biphasic media containing water and organic solvents. It discusses several related issues with the subject matter and also the potential application of using enzymes in organic solvents such as for kinetic resolution of enantiomers, asymmetric synthesis, peptide synthesis, glycoside synthesis and esterification, etc. Chapter seventh of the book describes the application of knowledge generated as described in the chapters as above for the biotransformation (biocatalysis) using crude enzymes and

whole cells. It discusses information about existing biocatalysts, their genetic modification and screening for novel biocatalysts. It also provides information on general procedures for isolation and selection of microorganisms, biocatalysts treatment and operation, and biotransformation such as asymmetric synthesis and steroids and terpenes biotransformation. The chapter finally discusses specific case studies dealing with biotransformation for flavour compounds such as methyl ketones, alkylpyrazines, etc. Chapter eighth of the book is again on application describing enzymes as tools for the stereospecific carbon-carbon bonds formation in monosaccharides and analog synthesis. It deals with DHAP and pyruvate aldolases, thiamine pyrophosphate dependent enzymes, transketolase, etc. In this chapter a glimpse has also been put on exploring the biodiversity to find new catalysts. The ninth and last chapter of this part describes enzymes engineered for new reactions, aiming at novel catalysis for organic synthesis. Enzyme-catalyzed synthesis in a non-aqueous reaction medium is a standard synthetic tool in chemistry. Biotechnological opportunities offer unique opportunity in redesigning and modifying enzymes for a targeted application. The chapter discusses protein engineering strategies to attain these.

Part II of the book has fifteen chapters, which provide information about specific industrial enzymes such as alpha amylase, glucoamylase, glucose isomerase, cellulase, pectinase, lipase, protease, xylanase, inulinase, phytase, tannase, peroxidases, chitinase, invertases and mannanases. Each of the chapter provides state-of-art information for specific enzyme, its sources such as plant, animal and microbial and then discusses details about microbial sources, production methods and strategies, purification and characterization. Each chapter also provides details on how to assay the enzymes using different methods.

Part III of the book is on bioreactors, downstream processing and applications of enzymes and has five chapters. First chapter in part, i.e. 25th chapter of the book describes bioreactor analysis and application for enzyme production and enzymatic processes. It discusses major types of the bioreactors (fermenters) used in submerged fermentation and solid-state fermentation. It also discusses operating parameters such as power consumption, mixing, shear stress, equilibrium phases, mass transfer, etc. Next chapter in this part is on isolation and purification of the enzymes suing any kind of bioreactor or fermentation mode/system. It describes processes of filtration and centrifugation for the removal of insolubles, extraction and purification for the solubles by ultrafiltration, liquid-liquid extraction, and recovery and purification of intracellular products. It provides great deals of chromatographic techniques for the purification of enzymes. The next chapter, *viz.* Chapter 27 is on industrial applications of enzymes, which provides details on enzymes production and markets such as in food industry, feed industry, paper and pulp industry, textiles and leather industry, detergents, personal care, energy (fuel alcohol), industrial waste treatment, etc. Chapter 28 of the book is on the immobilized enzymes for different purposes. It describes the properties of enzymes influenced by immobilization, principles, methods and examples of immobilization and chemical coupling of enzymes. The last chapter in this part is on protein engineering of industrial enzymes. It describes several industrial enzymes, ration design methods such as site-directed mutagenesis, and other random methods for their improvements.

The fourth and last part of the book deals with specific enzymes and their applications and has seven chapters, out of which five chapters are on thermozymes, cold-adapted enzymes, ribozymes, hybrid enzymes, diagnostic enzymes and therapeutic enzymes. Each of these chapters provides details on the microbial sources and application of the specific enzyme, their production and properties. The last chapter of the part and book, i.e. Chapter 36 is on inteins: enzyme generating protein splicing. It provides details on protein splicing pathway, control and applications, and inteins function and evolution.

We thank authors of all the articles for their cooperation and also for their preparedness in revising the manuscripts in a time-framed manner. We also acknowledge the help from the reviewers, who in spite of their busy professional activities, helped us by evaluating the manuscripts and gave their critical inputs to refine and improve the articles. We warmly thank Mr. NK Muraleedharan and the team of Asiatech Publishers, Inc. for their cooperation and strong efforts in producing this book.

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General Introduction

Ashok Pandey and Sumitra Ramachandran

1. INTRODUCTION

Enzymes are natural catalysts, which permit endogenous biological reactions to occur rapidly through well-defined pathways. They accelerate the rate of reactions, without being lost in the process. They occur in almost all creatures of the nature, from a minute microorganism to well-advanced human beings. They are located in the cells, cytoplasm, mitochondria, tissues, body fluids, etc. Enzymes are composed of one or more polypeptides organized in a specific three-dimensional structure. The efficiency of an enzyme's activity is often measured by the turnover rate, which measures the number of molecules of compound upon which the enzyme works per molecule of enzyme per second. Carbonic anhydrase, which removes carbon dioxide from the blood by binding it to water, has a turnover rate of 10^6. That means that one molecule of the enzyme can cause a million molecules of carbon dioxide to react in one second (Bell & Bell, 1988). Some of the outstanding features of the enzyme include high substrate specificity, specificity in promoting only one biochemical reaction with their substrate ensuring synthesis of a specific biomolecular product without the concomitant production of byproducts, stereospecificity and regiospecificity, which they express in catalysis. Enzymatic reactions occur within a narrow temperature range and an optimal pH. Effective catalysis also depends crucially upon maintenance of the molecule's elaborate three-dimensional structure. Any change occurring in the crucial factors such as pH or temperature may result in loss of structural integrity, which could lead to a loss of enzymatic activity.

Many enzymes require the presence of certain non-protein compounds for their action which helps in accelerating the enzyme action. These non-protein components tightly bound to the protein are called prosthetic groups. On the other hand, if the non-protein compounds are not firmly attached to the enzyme protein, but exist in free state in the solution contacting the enzyme protein only at the instant of enzyme action, they are called co-enzymes. Prosthetic groups usually are located in the active site of an enzyme molecule where catalytic events take place. It is also the place where the substrate and coenzymes bind just before reaction takes place. The entire enzyme system consisting of the enzyme protein and the coenzymes or prosthetic group is called the holoenzyme and the protein portion is sometimes called apoenzyme.

2. HISTORICAL DEVELOPMENTS

Enzymes have been exploited since ancient period, long before their functions and properties were understood. Only at the beginning of 19th century, the potential of enzymes in the process of fermentation

was understood. The term enzyme comes from zymosis, the Greek word for fermentation. In 1860, Loius Pasteur recognized that enzymes were essential to fermentation. However, he also assumed that their catalytic action was inextricably linked with the structure and life of the yeast cell. German chemist Edward Buchner in 1897 showed that cell-free extracts of yeast could ferment sugars to alcohol and carbon dioxide and denoted his preparation as zymase. This important achievement was the first indication that enzymes could function independently of the cell. In 1926 American biochemist J. B. Sumner was the first to isolate and crystallize an enzyme. He isolated urease in pure crystalline form from jack bean and suggested a contradictory statement to the previously prevailing opinion that the enzymes are protein molecules. The protein nature of the enzymes was firmly established when enzymes such as pepsin, trypsin and chymostrypsin were successfully crystallized during the period 1930-1936. Until 1980, it was believed that all enzymes are proteins. However, it is now known that proteins do not have a monopoly on biocatalysis as RNA molecules could also act as enzymes. Over the past three decades there have been rapid developments in the use of enzymes as catalysts.

3. APPLICATIONS AND BUSINESS OF ENZYMES

Enzymes from different sources (plants, animals and microorganisms) have wide application in different industries such as food, pharmaceuticals, leather, detergents, textiles, paper and pulp, waste management, etc. While it is expected that some 25,000 enzymes exist, only 2100 have been identified and listed so far. Food industry is the largest consumer of enzymes and approximately 45% of bulk share goes to it. Detergents are the second stake holder in enzymes consumption. While most of the enzymes for commercial application are obtained from the microorganisms, including bacteria, fungi and yeast, some are still better obtained through plant sources (Table 1). Similarly some others are obtained through animal sources (Table 2). Among all the industrial enzymes, hydrolytic enzymes account for 85%. The market size was approximately US$ 1.6 billions in 2002 and has witnessed ~12% annual growth over a

Table 1. Plant sources of enzymes

Source	Enzyme	Application
Jack bean	Urease	Diagnostic
Papaya	Papain	Baking, tanning, dairy
Pineapple	Bromelain	Baking
Horseradish	Peroxidase	Diagnostic
Wheat	Esterase	Ester hydrolysis
Barley	β-amylase	Baking, maltose syrup
Soybean	β-amylase	Baking, maltose syrup

Table 2. Animal sources of enzymes

Source	Enzyme
Calf, bovine, lamb	Diastase, pre-gastric esterase, pepsin, trypsin
Hen eggs	Lysozyme
Human urine	Urokinase

period of last one decade. It is expected that the market will continue to grow fast and reach US$ 3 billions by 2008. Table 3 shows the names of companies involved in the business of biotechnology (www.marketresearch.com).

An important and fast growing segment in the enzyme industry is of therapeutic enzymes, which are used to prepare health care products, e.g. penicillin acylase, or could be used directly also in the treatment of disorders, e.g. asparaginase. Some examples of pharmaceutical products prepared by the use of therapeutic enzymes include semi synthetic penicillins, cephalosporin, steroids, chiral compounds, etc. Table 4 shows some important applications of the therapeutic enzymes in the treatment of diseases. Table 5 shows some of the therapeutic enzymes produced commercially through microbial sources.

Table 3. Major companies involved in the business of enzyme production/marketing

1. Abgene (UK)
2. Agen Biomedical Limited (Australia)
3. Agouron Pharmaceuticals, Inc. (USA)
4. Alltech Biotechnology Centre (Ireland)
5. Altus Biologics, Inc. (USA)
6. American Home Products Corp. (USA)
7. Amersham PLC (UK)
8. Amrad Corporation Limited (Australia)
9. Annahdah Medical Company Limited (Saudi Arabia)
10. Asahi Breweries Limited (Japan)
11. Associated British Foods PLC (UK)
12. Astrazeneca PLC (UK)
13. Atofina SA (France)
14. Aventis Pharma S.A.E (Egypt)
15. Axys Pharmaceuticals,Inc.(USA)
16. Bachem AG (Switzerland)
17. BASF Aktiengesellschaft (Germany)
18. Bayer AG (Germany)
19. Baxter International, Inc. (USA)
20. Becton, Dickinson And Company (USA)
21. Beryl Laboratories (India)
22. Bio-Technology General Corp. (USA)
23. Biocatalysts Ltd (UK)
24. Bioclone Australia Pty, Limited.(Australia)
25. Biocon India Limited (India)
26. Biocryst Pharmaceuticals,Inc.(USA)
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33. Biosynth AG (Switzerland)
34. Biosynth International,Inc. (USA)
35. Biotec ASA (Norway)
36. Biozyme Laboratories (UK)
37. Boehringer Ingelheim GmbH (Germany)
38. Bosnalijek D D (Bosnia and Herzegovina)
39. British Biotech PLC (UK)
40. Burns Philp & Co. Ltd. (Australia)
41. Calbiochem-Novabiochem Corporation (USA)
42. Celgene Corporation (USA)
43. Celltech Group PLC (UK)
44. Cephalon, Inc. (USA)
45. China Medical University, Pharmaceutical Plant (China)
46. Chiron Corp. (USA)
47. Celltech Chiroscience (UK)
48. Chr.Hansen A/S (Denmark)
49. Clorox Company (USA)
50. Connetics Corporation (USA)
51. Csiro Molecular Science (Australia)
52. Danisco A/S (Denmark)
53. Degussa AG (Germany)
54. Diagnostic Products Corp. (USA)
55. Diversa Corp. (USA)
56. DNA Plant Technology Corp. (USA)
57. Dr. Eberle GmbH & Cie.(Germany)
58. DSM N.V. (The Netherlands)
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Table 3 .. Cont.

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69. Forest Laboratories, Inc. (USA)
70. FZB Biotechnik GmbH(Germany)
71. Genencor International,Inc. (USA)
72. Genencor International Oy (Finland)
73. Genzyme Corporation (USA)
74. Gilead Sciences, Inc. (USA)
75. Glaxosmithkline (UK)
76. Godo Shusei Company Ltd. (Japan)
77. Greentech SA (France)
78. Halcyon Proteins Pty Ltd. (Australia)
89. Hayashibara Biochemical Laboratories, Inc. (Japan)
80. Henkel Kgaa
81. Human Genome Sciences,Inc. (USA)
82. ICOS Corporation (USA)
83. ID Biomedical Corp. (Canada)
84. Idexx Laboratories, Inc. (USA)
85. Imperial Ginseng Products Ltd. (Canada)
86. Incyte Genomics, Inc. (USA)
87. Intergen Company (USA)
88. Jelfa S.A. (Przedsiebiorstwo Farmaceutyczne) (Poland)
89. Kare Group of Companies (India)
90. Kemat Belgium SA-NV (France)
91. Kim Jeong Moon Aloe Co, Ltd. (Korea)
92. Labmaster OY (Finland)
93. Leciva AS (Czech Republic)
94. Life Science Research Israel Ltd. (Israel)
95. Lifeway Foods, Inc. (USA)
96. Liposome Co., Inc. (USA)
97. Maps (I) Ltd. (India)
98. Martek Biosciences Corporation (USA)
99. Medimmune Inc. (USA)
100. Medisense, Inc. (USA)
101. Medopharm (India)
102. Meiji Seika Kaisha Ltd (Japan)
103. Nagase & Company Limited (Japan)
104. Nagase (Europa) GmbH (Germany)
105. Neose Technologies,Inc. (USA)
106. New Brunswick Scientfic Co., Inc. (USA)
107. Nichimen Europe (Germany)
108. Novartis AG (Switzerland)
109. Novartis Farmaceutica SA (Spain)
110. Novo Nordisk A/S (Denmark)
111. Novo Nordisk Ltd., (UK)
112. Novozymes A/S (Denmark)
113. Nutrition Care Pharmaceuticals Pty Ltd. (Australia)
114. Ortron Co.poration Pty Ltd.(Australia)
115. Palsgaard A/S (Denmark)
116. Pentapharm AG (Switzerland)
117. Perkinelmer, Inc. (USA)
118. Pfizer Inc. (USA)
119. Pfizer Chemicals (Australia)
120. Pfizer Egypt SAE (Egypt)
121. Pharmacia Corporation (USA)
122. Protea Industrial Chemicals (South Africa)
123. Psiron Ltd. (Australia)
124. Purdue Pharma LP (USA)
125. Rakuto Kasei Ltd. (Israel)
126. Randox Laboratories Ltd. (United Kingdom)
127. Respironics Inc. (USA)
128. Rhodia SA (France)
129. Rohm & Haas Co. (USA)
130. Sannitree Ltd. (South Africa)
131. Sanosil Ltd. (Switzerland)
132. Senn Chemicals AG (Switzerland)
133. Sepracor, Inc. (USA)
134. Serono SA (Switzerland)
135. Shamrock Pharmaceuticals Pvt Ltd.
136. Shire Biochem Pharmaceuticals Inc. (Canada)
137. Sigma Aldrich Quimica SA (Spain)
138. Solvay SA (Belgium)
139. Synaco Chemicals SA (Belgium)
140. Takeda Chemical Industries Ltd. (Japan)
141. Targeted Genetics Corp. (USA)
142. Tecra International Pty.Ltd.(Australia)
143. Textan Chemicals (P) Ltd.
144. Thoratec Corporation (USA)
145. Town End (Leeds) PLC (UK)
146. Unizyme Laboratories A/S (Denmark)
147. Vaudaux-EppendorfAG (Switzerland)
148. Vikrant Pharmaceuticals Ltd.(India)

Table 4. Applications of therapeutic enzymes for the treatment of diseases

Acute inflammation
Back pain
Celic disease
Colitis
Cystic Fibrosis
Food allergies
Gastric duodenal ulcer
Gout
Premenstrual syndrome

Table 5. Microbial therapeutic enzymes already being produced commercially

Enzyme	Application
Penicillin acylase	Semi synthetic penicillins
Serratio-peptidase	Anti-inflammatory agent
Asparaginase	Treatment of leukemia
Ribonuclease	Treatment of asbestos related cancer

4. NOMENCLATURE AND CLASSIFICATION

Several hundred enzymes were discovered by 1950 yet there was no systematic way in classifying them. Since many enzymes had a misleading and uninformative name, there prevailed a confusion regarding their naming. Formal recommendations were subsequently prepared under the auspices of the International Union of Biochemistry (Cornish-Bowden & Cardenas 1987). As nomenclature and classification are interlinked, they are discussed and dealt together.

- Suffix -ase, are given for single enzymes, i.e. single catalytic entities while systems containing more than one enzyme are named on the basis of the overall reaction catalysed by them, followed by the word system. e.g., fatty acid synthase system
- Enzymes are classified on the basis of the chemical reaction catalysed, which specifically distinguishes them from the other enzyme.
- Enzymes are divided into groups on the basis of the type of reaction catalysed together with the name(s) of the substrate(s). This also forms the basis for code numbers.

In 1961 the first Enzyme Commission devised a system for classification of enzymes, in which each enzyme was assigned a code number. These code numbers, prefixed by EC are now widely in use, which contain four elements separated by points, with the following meaning:

(1) First number shows to which of the six main divisions (classes) the enzyme belongs,

(2) Second number gives the subclass,

(3) Third figure indicates the sub-subclass,

(4) Fourth digit is the serial number of the enzyme in its sub-subclass.

4.1. Class 1 - Oxidoreductase

Enzymes, which catalyse oxidoreduction reactions, are classified in this class. The substrate that is oxidized is regarded as hydrogen donor. The second figure in the code number of the oxidoreductases indicates the group in the hydrogen (or electron) donor that undergoes oxidation, e.g. 1 denotes a -CHOH- group. But the case is not true with 11, 12, 13, 14, 15. The third figure, except in subclasses EC 1.11, EC 1.13, EC 1.14 and EC 1.15 indicates the type of acceptor involved, e.g. subclass 1 denotes NAD(P).

4.2. Class 2 - Transferases

Transferases are enzymes, which bring about transfer of certain groups from one organic compound to another, e.g. a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor).

$$X\text{-}Y + Z\text{-}H = X\text{-}H + Z\text{-}Y$$

The second figure in the code number of transferases indicates the group transferred; a one-carbon group in EC 2.1, an aldehydic or ketonic group in EC 2.2, and so on. The third digit gives further information on the group transferred; e.g. subclass EC 2.1 is subdivided into methyltransferases (EC 2.1.1), hydroxymethyl- and formyltransferases (EC 2.1.2) and so on. However, in EC 2.7, the third figure indicates the nature of the acceptor group.

4.3. Class 3 - Hydrolases

These enzymes catalyse the hydrolysis of substrates having large molecular weight. They cleave C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Since hydrolysis itself can be regarded as transfer of a specific group to water as the acceptor, it might be classified as transferases. However, in most cases, the reaction with water as the acceptor was discovered earlier and is considered as the main physiological function of the enzyme. This is why such enzymes are classified as hydrolases rather than as transferases. The second figure in the code number of the hydrolases indicates the nature of the bond hydrolysed; EC 3.1 are the esterases; EC 3.2 the glycosylases, and so on. The third figure normally specifies the nature of the substrate, e.g. in the esterases the carboxylic ester hydrolases (EC 3.1.1), thiolester hydrolases (EC 3.1.2), phosphoric monoester hydrolases (EC 3.1.3); in the glycosylases the O-glycosidases (EC 3.2.1), etc. Exceptionally, in the case of the peptidyl-peptide hydrolases the third figure is based on the catalytic mechanism as shown by active centre studies or the effect of pH.

4.4. Class 4 - Lyases

Lyases are enzymes that catalyse the addition or removal of some chemical groups of a substrate by mechanism other than oxidation, reduction or hydrolysis. They differ from transferases in that the chemical group liberated is not merely transferred to another, but liberated in the free state. These enzymes cleave C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. Decarboxylase, aldolase, dehydratase are enzymes, which catalyse the elimination of carbondioxide, aldehyde, and water, respectively. The second figure in the code number indicates the bond broken: EC 4.1 is carbon-carbon lyases, EC 4.2 carbon-oxygen lyases and so on. The third figure gives details on the group eliminated, e.g. carbon dioxide in EC 4.1.1, water in EC 4.2.1. and so on.

4.5. Class 5 - Isomerases

These are enzymes, which catalyse the interconversion of isomers. According to the type of isomerism, they may be called racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases or cycloisomerases. The interconversion in the substrate is sometimes brought about by an intramolecular oxidoreduction (EC 5.3). There occurs no oxidized product as hydrogen donor and acceptor is the same molecule. However, they may contain firmly bound NAD(P). Therefore, they are not classified as oxidoreductases. The subclasses are formed based to the type of isomerism, the sub-subclasses to the type of substrates.

4.6. Class 6 - Ligases

Ligases are enzymes catalysing the joining together of two compounds coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The second figure in the code number indicates the bond formed: EC 6.1 for C-O bonds (enzymes acylating tRNA), EC 6.2 for C-S bonds (acyl-CoA derivatives), and so on. Sub-subclasses are in use only in the C-N ligases.

From time to time, some enzymes have been deleted, while some others have been renumbered. The actual naming and reclassification is always done by the International Union of Biochemistry. Whenever reclassification results in deletion of an enzyme, the old number is not allocated to new enzyme, but is permanently discarded. Entries of reclassified enzyme transferred from one position to another are followed by a comment indicating the former number for the sake of reference.

In BRENDA enzyme database, which was initiated in 1987, the enzymes are classified according to the Enzyme Commission list of enzymes and later supplements. According to EC numbers some 3400 "different" enzymes are covered. Frequently very different enzymes are included under the same EC number. During the integration of new data into the data base a combination of computer-oriented and human controls are applied to ensure a high level of data quality. This databank provides comprehensive information about each enzyme. For example, information covered under nomenclature for each enzyme provides its EC number, systematic name, recommended name, synonymes and CAS Reg. No. Similarly, information about reaction and specificity provides details on catalysed reaction, reaction type, natural substrate, substrate spectrum, product spectrum, inhibitor(s), cofactor(s) prosthetic group(s), metal compounds/salt, turnover number, specific activity, K_m value, pH optimum and range, temperature optimum and range. Details given under enzyme structure include its molecular weight, subunits, and glycoprotein and lipoprotein. Information on the isolation and preparation gives its source (organisms/ tissue and localization), purification, crystallization, and if cloned and renaturated. It provides comprehensive details about the stability for pH, temperature, oxidation, organic solvents and storage (http://www.brenda.uni-koeln.de).

5. ENZYME ACTION AND KINETICS

The first to carry out kinetic studies with pure enzyme, trypsin was a British Physical Chemist John Alfred Valentine Butler. The specificity of enzyme action is explained in terms of precise fitting together of enzyme and substrate molecules. This is referred as Fischer's lock and key hypothesis. This is the primary requirement for the efficient enzyme catalysis. The groups on the polypeptide chain of the enzyme, solvent (water) and groups on cofactor are considered essential for the enzyme catalysis. Many enzymes require metal ions for activity, e.g. Mg^{2+} in ATP-dependent enzymes. Some other metal ions act as activators and are directly involved in the catalysis. They also contribute to nucleophile catalysis

by binding to OH^- ions and supplying them to the reaction centers. Effective action requires correct geometry. The reacting groups must be correctly positioned and oriented to interact simultaneously with the substrate. Any molecule that binds tight to a transition state than it does to the reactants or products will effectively stabilize it with respect to reactants and products and thus will act as catalyst.

Enzymes can also serve to couple two or more reactions together, so that a thermodynamically favourable reaction can be used to drive a thermodynamically unfavorable one. One of the most common examples is enzymes, which use the dephosphorylation of ATP to drive some otherwise unrelated chemical reaction. Several enzymes also work together in a cyclic (specific) order, creating metabolic pathways (e.g., the citric acid cycle). In a metabolic pathway, the product of one enzyme serves as the substrate for another enzyme and the reactions continue till the formation of end-product(s). These end-product(s) are often uncompetitive inhibitors for one of the first enzymes of the pathway (usually the first irreversible step, called committed step), and regulate the amount of end-product(s) produced.

6. ENZYME SAFETY

Safety of the enzymes applied in the food, pharmaceutical, textile, detergent industry, etc. should be ensured. Safety refers to lack of pathogenicity of the organism, absence of toxicity of the enzymes, or the product of the enzymatic reaction, absence of antibiotics and relative absence of microbial contaminants. Enzymes derived from non-approved organisms require extensive testing for various toxicity tests. No enzyme has been found to be toxic, mutagenic or carcinogenic by itself as might be expected from its proteinaceous structure. However, enzyme preparations cannot be regarded as completely safe as potential chemical toxicity from microbial secondary metabolites such as mycotoxins and aflatoxins may occur in some cases or some contaminant derived from the enzyme source or produced during its processing or storage may also be present. Enzymes have far fewer side effects and unknown possible reactions than other compounds, supplements, or medications. This feature makes them extremely safe. Some of the enzymes are classified as a safe food in the United States and have GRAS status (Generally Regarded As Safe). Such enzymes include α-amylase, β-amylase, bromelain, catalase, cellulase, ficin, α-galactosidase, glucoamylase, glucose isomerase, glucose oxidase, invertase, lactase, lipase, papain, pectinase, pepsin, rennet and trypsin. Since enzymes are not in the realm of the Food and Drug Administration (FDA), specific digestive enzyme blends are neither FDA approved or unapproved.

Some of the safety problems associated with the use of free enzymes may be overcome by using immobilised enzymes. This is an extremely safe technique, so long as the materials used are acceptable and neither they, nor the immobilised enzymes, leak into the product stream. Safety evaluations of many enzymes were carried out since several years and enzyme preparations were reported to be safe. Some of the enzymes, which have been evaluated for safety include β-glucanase derived from *Trichoderma reesei* (Coenen et al 1995), *Thermomyces lanuginosus* xylanase enzyme (SP 628) and the *Aspergillus aculeatus* xylanase enzyme (SP 578) (Bergman & Broadmeadow 1997), *Thermomyces lanuginosus* xylanase expressed by *Fusarium venenatum* (Pedersen & Broadmeadow, 2000), amino peptidase enzyme preparation derived from *Aspergillus niger* (Coenen & Aughton 1998), glucanase preparation intended for use in food (Elvig & Pedersen 2003), lactase enzyme preparation derived from *Kluyveromyces lactis* (Coenen et al 2000), lipase produced from *Rhizopus oryzae* (Flood & Kondo 2003).

7. ENZYME THERAPY

Apart from various other applications, enzymes also exhibit therapeutic property. Enzyme therapy is FDA approved in the treatment of certain health conditions. These include:

- cardiovascular disorders
- gastrointestinal conditions, particularly pancreatic insufficiency and related disorders (Mohan et al 1998)
- replacement therapy for specific genetic disorders and deficiencies (Eng et al 2001)
- cancer treatment (Leipner & Saller 2000)
- debridement of wounds (Adamian & Gliantsev 1992)
- removal of toxin substances from the blood (Desser et al 2001)

The basis of enzyme therapy is based on one gene-one enzyme hypothesis, which states that each gene is responsible for directing the building of a single, specific enzyme and that not all the genes code for enzymes; they may instead direct the building the structural proteins such as the collagen in human skin or keratin in hair.

8. CONCLUSIONS AND PERSPECTIVES

Enzymes have long been used as useful additives in several industrial applications, such as detergents, food, textiles, leather, and pulp and paper. Three largest segments of the markets for enzymes are in food applications, viz. starch and sugar processing, dairy, and bakery applications. While currently the bulk of the business for enzymes is in the industrial sector, the new areas are emerging in biotechnology. Recently a number of biotech companies have become active in this field, developing new and interesting enzymes for specialty applications such as fine and specialty chemicals, health care and personal care products. The business of enzyme industry is perhaps of most secretive nature and it is hard to get reliable information and estimates on the production techniques, nature of substrates, purification, etc. Thus, in enzyme industry where investments in research and development are considered one of the corner stones of profitability, companies remain under pressure to focus on activities to derive benefits from their core strengths.

Use of genetically modified microorganisms to obtain hyper-producers and to develop the desired specificity in the enzymes through molecular tools offer potential benefits but with increasing regulatory concerns and the increasing public inclination toward non-GMO food, such practices need to be adapted with careful consideration. Also, since the long-term effects of genetic modification have not yet been well, rather fully understood, there is opposition to it. Thus, the challenges for the companies are significant.

One of the aspects of biotechnological research and development is targeted towards the advanced understanding of the function of enzymes and its broadened application. Better insights into the framework of synthesis of genetically engineered enzymes, design of novel enzyme(s), etc, will open and explore opportunities for the manufacture of sophisticated products. The challenge for the enzyme technologists lies in exploiting the unprecedented level of knowledge in utilization and application of more enzyme complex systems.

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General Properties

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1 INTRODUCTION

Enzyme mediated reactions are the basis of all the living beings. They offer efficient biocatalytic conversion potentials to technologies ranging from agricultural to pharmaceutical industries. History of enzymes dates back to 17th century that describes yeast catalyzed transformation of juices into alcoholic beverages. Many years (over 100 years) later, A. Lavoisier in 1789 showed that sugar was converted to carbon dioxide (CO_2) and alcohol. Enzymes occur widely throughout the biological systems and complex network of reaction brought about by enzymes are basis for the continuity of living world that has evolved over millennia to ensure the survival and reproduction of organisms and diversity of the life. Almost every chemical reaction that takes place in living things is catalyzed by enzymes; an animal cell may contain up to 4,000 different enzymes (Krogh 2002). Enzymes are generally membrane bound or fully soluble in biological systems. Enzymes occurring inside a cell are called intracellular, while those occurring out of a cell are often called extracellular. Extracellular enzymes are usually robust and tolerate range of conditions compared to intracellular ones. Extracellular enzymes may exist in immobilized form with viable catalytic activity for thousands of years, e.g., phosphatases in soils. Artificially immobilized enzymes have been used for various purposes ranging from environmental clean-up to industrial biocatalysis because of their robustness.

An enzyme is a catalytic protein and it remarkably lowers activation energy of a given reaction. Although all enzymes are proteins, many enzymes contain non-protein components as well, for example, carbohydrates, phosphates, metallic ions, lipids or organic moieties, etc. (Whitaker 1972).

Enzymes have practical applications from activities as diverse as industrial production of alcohols and drugs to chemical warfare and detergents. Understanding how enzymes function in different reaction environment helps to modify their function for industrial use. Enzymes are utilized for diverse application ranging from food, feed and agriculture to paper, leather and textile industries. Because of rapid developments on the technology fronts and issues of health, energy, environment and raw materials, various sectors/industries are embracing the enzyme technology (Pant et al. 1994a, b; Onifade et al. 1998; Kamini et al. 1999; Pant & Warman 2000; Acamovic 2001; van Beilen & Li 2002). Advances in enzyme technologies, which provide innovative ways to utilize enzymes in bioprocesses, have direct impact on efficiencies of bioprocess engineering (Gross et al. 2001; Panke & Wubbolts 2002) and resource conservations.

Preparations of artificial bi- and poly-functional enzymes by gene fusion have great potential in biochemical analysis, metabolic engineering and enzyme process technology (Bulow & Mosbach 1999).

Recent developments indicate that thermoenzymes, from thermophilic microorganisms, are catalysts of great industrial interests (Bruins et al. 2001). Similarly, genetic engineering has helped to overcome the barriers of transitions of enzyme technology from laboratory to market place ranging from enzyme therapy to industrial biosynthesis. However, enzyme technology although provides opportunities in many industries, development and commercialization of novel or useful techniques are not easily attainable (Wong 1993), and substantial research and development is still needed in the field.

2 CHEMICAL NATURE, STRUCTURE AND CLASSIFICATION OF ENZYMES

An enzyme is mainly a protein chain folded upon itself, forming a macromolecular assemblage with a well defined structure (Rosevear et al. 1987). Some of the typical physico-chemical characteristics of enzymes include homogeneity, absorbance index, active site concentrations, isoelectric points, ultra-violet (UV) absorbance, florescence, sub-unit compositions, amino acids composition and their sequences, peptide maps, etc. Amino acids are building blocks of proteins. Since all the enzymes are proteins, they are made up of amino acids, too. Only 20 amino acids are found in proteins (Krogh 2002). All amino acids contain a carboxyl and amino groups; however, different amino acids have different side chain, i.e., they differ at 'R' position (Fig 1). Carbohydrates, lipids, porphyrins or flavins may also be covalently bound in this structure as well as other biochemicals, and metal ions may also be held as complexes (Rosevear et al. 1987).

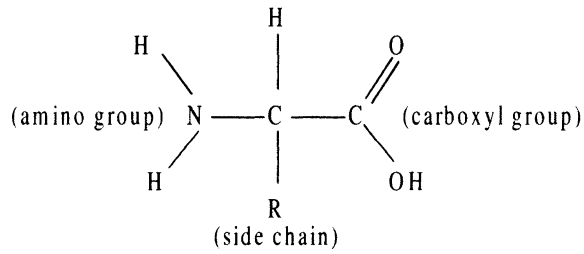

Fig. 1. Structural components common to all amino acids.

Many enzymes have one polypeptide chain and one active site (per polypeptide chain). However, many active sites may also be found in multi-chain enzymes and some of which may be associated with regulatory mechanisms. Thus, an enzyme is basically a fragile globular protein, and its each molecule attains same shape as all the others. Although numbers of structural conformations are possible from bond rotations, the native conformation is the most stable one for the given molecule (Anfinsen 1972).

The concept of enzyme specificity and steric relationship, i.e., "lock and key" analogy between an enzyme and its substrate was first developed by Emil Fisher, a German Chemist in 1894. Fisher's concept of stereo-specificity still holds in that an enzyme acts upon only on closely related compounds. However, there are enzymatic reactions that not only supports lock and key analogy, but also the Koshland's induced fit model. According to the induced fit model, (i) the geometry of protein changes as the substrate binds to the active site, (ii) a delicate orientation of catalytic groups is required for transformation, and (iii) the substrate induces such orientation by binding to the active site (Koshland 1960).

Basis for naming of enzymes are complex and misleading in some instances. However, International Commission of Enzymes arranged them in organized fashion so that it would be easier to follow. In 1863, Payen and Persoz named enzyme 'diastase' because of its ability in separating soluble dextrin from insoluble encase of starch grains. Perhaps, that set the unorganized pattern of enzyme naming. The name of the enzyme often has nothing to do with the type of reaction they catalyze, such as pepsin and trypsin. Similarly, numbers of enzymes are named after their discoverer, methods of isolation/purification, colour, and genus or species from which they are isolated/purified. Moreover, substrate utilization has also been used with adding '-ase' to the stem of the substrate, e.g., maltase (which acts upon maltose) so as the amylase (which acts upon amylose), phosphatase (which acts upon phosphorus), etc. However, such naming system is neither perfect nor limitless. As the more and more substrates of an enzyme are identified, the more difficult it becomes, i.e., which substrate to use for the naming of a particular enzyme. Moreover, the naming based on substrate that enzyme acts upon does not offer any information about the nature of the reaction it catalyzed as well as the location of catalysis such as their bond specificities.

As the advances in enzyme purification/isolation have been made, it has become clear that enzyme catalyzing the same reaction, but isolated/purified from different sources differ in their proportionate constituents and molecular mass. For example, alcohol dehydrogenase from yeast has 150,000 Da molecular mass, four sub-units and four Zn(II), whereas that from horse liver has 70,000 Da molecular mass, two sub-units and four Zn(II) (Whitaker 1972). Again, as the enzyme technologies advances, multiple molecular forms of enzymes are found, in turn giving further complications in enzyme nomenclature.

A well defined enzyme has three designations, a systematic name, a trivial name and a number (Bowden 1996). A systematic name of an enzyme consists 1st part as the name of the substrate (for more than one substrate reactions, the substrates' names are separated by a colon), the 2nd part indicating nature of the reaction ending –ase; however, if there are two different reactions involved, the 2nd reaction is denoted by adding a participle in parentheses [e.g., sarcosine: oxygen oxidoreductase (demethylating)]. The commonly used name is a trivial name, such as amylase, cellulase, phosphatase, etc. The code number for an enzyme, however, contains four elements that are permanent and come from the classification scheme. The scheme for numbering an enzyme is based on class, sub-class and sub-sub-class. There are generally six classes of enzymes with numbers of sub-classes and sub-sub-classes (Table 1). This classification, however, ignores or does not properly classify multiple molecular form of an enzyme (isoenzymes) and certain difficulties arise while naming enzymes acting upon polymeric substrates and site specific deoxyribonucleases (Cornish-Bowden 1996).

3 COFACTORS

Many enzymes need assistance from certain chemical entities called cofactors to attain their catalytic activity (Engle 1996). The complete enzyme system, which includes both the protein and non-protein components, is defined as holoenzyme. The protein component of the active enzyme system is referred as apoenzyme, while that of non-protein is called cofactor. The cofactors vary from simple inorganic ions to complexity of B_{12}. Without the cofactor, apoenzyme will have no activity, meaning cofactors are essential for enzymes to be active. Cofactors are engaged in binding substrate with enzyme and/or

Table 1. A concise systematic classification of enzymes

Class	Principal sub-class (acting upon)	Reaction type	Enzyme nomenclature
1. Oxidoreductases	1.1 CH-OH group	Oxidation/reduction	Donor: acceptor oxidoreductase (EC 1.1.1.1, alcohol:NAD^+ oxidoreductase)
	1.2 aldehyde or oxo group		
	1.3 CH-CH group		
	1.4 CH-NH_2 group		
2. Transferases	2.1 transferring one-carbon group	Group-transfer	Donor: acceptor group transferase (EC 2.1.3.2, carbomyl-phosphate: L-aspartate carbamoyltransferase)
	2.3 acyltransferases		
	2.4 glycosyltransferases		
	2.6.1 transaminases		
	2.7 transferring phosphorus-containing groups		
3. Hydrolases	3.1 esterases	Hydrolysis	Substrate hydrolase (EC 3.1.1.7, acetylcholine acetylhydrolase)
	3.2 glycosidases		
	3.4 peptidases		
4. Lyases	4.1 carbon-carbon lyases	Elimination	Substrate group lyase (EC 4.1.1.1., 2-oxo-acid carboxylase)
	4.2 carbon-oxygen lyases		
	4.3 carbon-nitrogen lyases		
5. Isomerases	5.1 racemases and epimerases	Isomerization	Substrate reactionase (EC 5.3.1.1, D-glyceraldehyde-3-phosphate ketol-isomerase)
	5.3 intramolecular oxidore ductases		
	5.4 intramolecular transferases (mutases)		
6. Ligases	6.1 forming carbon-oxygen bonds	Bond synthesis coupled to hydrolysis	X-Y ligase (product-forming) [EC 6.1.1.1, tyrosine-$tRNA^{Tyr}$ ligase (AMP-forming)]
	6.2. forming carbon-sulfur bonds		
	6.3 forming carbon-nitrogen bonds		

transformation of substrate to products and can be grouped into metal-ions and organic cofactors; organics are further sub-categorized into coenzymes, prosthetic groups and co-substrates, depending on their repetitive turnover in a functioning system (Whitaker 1972; Engle 1996).

Availability of active forms of cofactors may play a dictating role in process yields (San et al. 2002), indicating the importance of manipulation of levels of cofactors from their industrial use. A case in point, ratio of NADH/NAD^+ can be altered by using different oxidation state of C sources so as to achieve desired metabolic goals (San et al. 2002). Many enzymes are just comprised of protein units, but some require the presence of cofactors. Metal cofactors such as Mn^{2+}, Mg^{2+} and Fe^{2+} can increase the enzyme activity of proteases by 20% (Zvidzai & Zvauya 2001). Similarly, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) can be used as cofactor for the maximum production of erythritol in *Torula carrallina*, a yeast strain, catalyzed by erythrose reductases (which converts erythrose to erythritol) (Lee et al. 2003). Reductive dehalogenation either is catalyzed by a specific dehalogenase or may be mediated by free or enzyme-bound transition metal cofactors (porphyrins, corrins), and because of their enantiomer selectivity, some dehalogenases are used in industrial biocatalysis of chiral compounds (Fetzner & Lingens 1994).

The cofactor specificity of many enzymes can become liability for artificial metabolic pathways. For example, the preference for NADPH over NADH shown by corynebacterium 2, 5-diketo-D-gluconic acid (2, 5-DKG) reductase may not be ideal for industrial biosynthetic production of vitamin C (Banta et al. 2002). However, site-directed mutation in cofactors-binding pocket can be utilized to shift the preference of cofactor (Banta et al. 2002).

Enzyme catalyzed oxidation-reduction reactions often require cofactors (e.g., desulfurization of oil by biocatalysis), which need to be regenerated for the continuation of reaction. Metabolic conditions can usually be designed using whole cell biotransformations to promote regeneration of cofactor (Setti et al. 1997). In certain cases, cofactor regeneration can make the use of an enzyme requiring cofactors expensive for industrial applications. For example, oxidoreductase require a cofactor, which functions as an electron carrier if use in stoichiometric quantities. The cost of cofactors would make synthetic uses of redox enzymes rather expensive for industrial uses (Leonida 2001). Thus, oxidoreductases are considered as uneconomical for industrial applications because of their dependence on cofactor or prosthetic groups for their activities, and difficulties in regenerating the cofactors. Industrial biocatalysis has been utilized mainly as agrochemical and pharmaceutical precursors for a long time, however, as the artificial cofactor regenerating mechanisms are developed its use is constantly evolving in different arenas.

4 ENZYME SPECIFICITY

A number of inorganic catalysts such as coal and platinum show low specificity over the reaction substrate they catalyse, and a small amount of the selected catalyst is enough to carry out a chemical reaction (Montgomery et al. 1994). The enzymatic catalysis has the interesting characteristic to be specific. The enzyme specificity is one of the most important biological phenomena without which the ordered metabolism of living matter would not exist, and life would be impossible (Sigman 1990). Enzymes are specific in action, bringing substrates together in favourable orientations to promote the formation of a transitory state, called enzyme–substrate complex [ES]. The properties and spatial arrangements of amino acids residues that form the active site of an enzyme are responsible for its specificity, and determine which molecules can bind and be substrate for this enzyme (Hames & Hooper 2000). The

active site is a three-dimensional entity that binds the substrate, and promotes its conversion into product. It is usually a small part of the whole enzyme molecule containing the residues that directly participate in the making and breaking of bonds (Hames & Hooper 2000; Berg et al. 2002).

Substrate specificity is generally determined by changes in a few amino acids in the active site. This specificity always results from multiple weak interactions between the enzyme and its specific substrate, like van der Waals interactions, electrostatic interactions, hydrogen bonding and hydrophobic interactions (Voet et al. 2000; Nelson & Cox 2002). Some enzymes such as alcohol dehydrogenase and hexokinase have group specificity and may act on several different substrates to catalyse a reaction involving a particular chemical group. Other enzymes such as glucokinase exhibit absolute specificity being able to act only on one particular substrate (Palmer 1999).

In enzymes without non-protein components, essentially the groups placed in the radicals of some amino acids (OH function of serine, phenol function of tyrosine, COOH function of aspartic and glutamic acid etc.) are involved in the active site to catalyse reactions. In holoenzymes the substrate is linked to the protein portion, called apoenzyme and the non-protein portion, called prosthetic group or co-substrate, carries out the catalytic reaction (Scriban 1984).

Enzyme catalysed reactions are also product-specific as well as substrate-specific, i.e., while uncatalyzed reactions often generate a wide range of products, the reactions catalysed by enzymes produce only the target product. In addition to chemical specificity, enzymes have stereo chemical specificity. They are able to identify the correct isomer of a substrate that exists in two stereo chemical forms to undergo the reaction (Palmer 1999). For example, desaminases are active only on L-amino acids, and totally inactive on the optic isomers D (Illanes 1994). The only enzymes that can act on both stereo isomeric forms of a substrate are those responsible for the inter-conversion of L and D forms, like alanine racemase (Palmer 1999). However, the specificity is not always absolute; for example, glucose isomerase that catalyses the conversion of glucose to fructose is more effective to transform xylose into xylulose (Illanes 1994).

Some points must be considered for the investigation of enzyme specificity: the enzyme should be as pure as possible and in any case should be free from any other enzyme acting on similar substrates; the substrates also should be as pure as possible and free from any other substances on which the enzyme can act; isomers should be tested separately; the enzymes should be used at reasonably low concentration, to avoid reactions due to traces of contaminating enzymes; it is normal to select a reference substrate, generally the most readily attacked biological substrate, and to work out the optimal conditions for it and use the same conditions for the other possible substrates (Sigman 1990).

5 MEASUREMENT AND EXPRESSION OF ENZYME ACTIVITY

On the kinetic basis, the enzymes are catalytic agents that increase the conversion rate of substrate into product. As without enzyme the reaction rate is generally negligible, the quantification of enzyme activity is based on the measurement of the reaction rate (Acevedo et al. 2002). Considering the following reaction:

$$S \xrightarrow{E} P$$

the reaction rate can be estimated based on the disappearance of the substrate or on the appearance of the product:

$$v = dP / dt = - dS / dt \quad (1)$$

The progress curves of most enzyme reactions have the profile showed in Figure 2, in which the reaction rate decreases with time from an initial maximum value. Many factors can contribute to this falling off: the reaction products may inhibit the enzyme; the degree of saturation of the enzyme with the substrate can fall with substrate concentration decrease; the reverse reaction can become more important as the concentration of products increases or inactivation of enzyme occurred by pH or temperature changes (Sigman 1990).

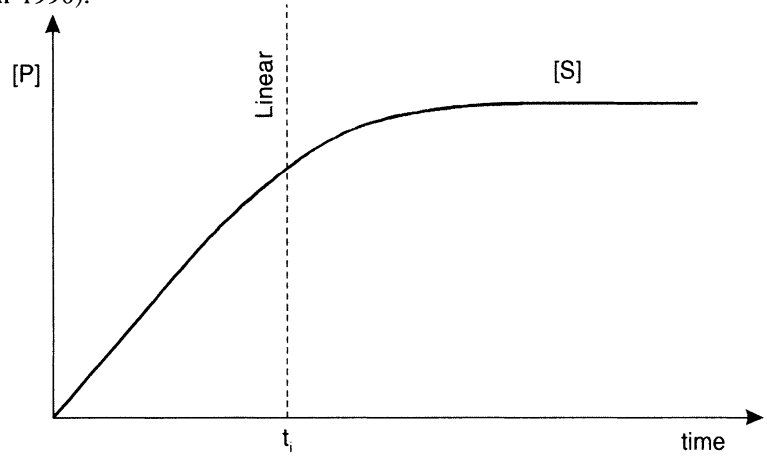

Fig. 2. Product concentration [P] versus reaction time for a defined substrate concentration [S]

To standardize the measurement and to ensure being free from the influence of the factors described above, the enzyme activity value is identified with the initial velocity of the reaction (Illanes 1994). The enzyme activity can be estimated as demonstrated in equation (2):

$$EA = v_t \rightarrow_0 = (dP / dt)_t \rightarrow_0 = - (dS / dt)_t \rightarrow_0 \quad (2)$$

It is important to emphasize that the enzyme activity obtained by this way represents the maximum catalytic power of the enzyme for the environmental conditions used. It must also be stressed that in some cases, the estimation of the enzyme activity based on the measurement of the initial velocity is not indicated. This is in the case of enzymes acting on heterogeneous substrates, like cellulases (Illanes 1994; Acevedo et al. 2002).

Two types of methods can be used to follow catalysed reactions: sampling and continuous methods. In the sampling methods, the observations are not made in the reacting mixture, but on samples withdrawn at different times, giving a number of separate points on the progress curve. In the continuous methods, the observations are made on the mixture while it is reacting, giving a continuous curve (Sigman 1990). An important aspect of enzyme assay is the use of a blank that has the objective of indicating any product formation due to other factors that are not related to the enzymatic reaction.

If the reaction is a simple one, either substrate or product may be used to estimate enzyme activity, but in cases of two stage reactions, in which an intermediate between substrate and product can be accumulated, only the substrate disappearance gives a correct measure of the first reaction (Sigman 1990).

Enzyme catalysed reactions can be monitored spectrophotometrically, as many of the substrates or products of enzymes absorb visible or non-visible light. This is a common, simple, sensible and cheap method of assaying enzymes and can be used for sampling or continuous monitoring, giving a complete progress curve (Wiseman 1985; Illanes 1994). The absorbance (A) at a certain wavelength (L) may be related to the concentration of a problem substance (C) by the Lambert Beer equation:

$$A = \varepsilon \,.\, L.\, C \tag{3}$$

where ε is the extinction coefficient.

A number of reactions do not involve substrates or products that absorb light at a suitable wavelength. In such cases, it is often possible to assay the enzyme that catalyses this reaction by linking it to a second enzyme reaction that does involve a characteristic absorbance change. A common example is the enzyme glucose oxidase, which is largely used in clinical tests to determine the glucose content in blood as well as in analytical tests in research laboratories to monitor glucose concentration during fermentation processes. The action of this enzyme does not produce a change in absorbance upon conversion of substrate to products, but a second enzyme, peroxidase can act on the hydrogen peroxide generated in this reaction. This enzyme is able to convert a colourless compound into a colour one whose absorbance can be measured, and directly related to the glucose concentration (Hames & Hooper 2000).

Cofactors generally present a characteristic range of absorption and are largely used for enzyme activity determination even directly or by means of coupled reactions (Illanes 1994). Two of the most common molecules used for absorbance measurement in enzyme assays are the coenzymes *reduced nicotinamide adenine dinucleotide* (NADH) and *reduced nicotinamide adenine dinucleotide phosphate* (NADPH), each absorbing ultraviolet light at 340 nm (Hames & Hooper 2000).

The enzyme activity, as proposed by the International Union of Biochemistry, is expressed as International Units (U) of enzyme activity. One unit is defined as the amount of enzyme that catalyses the transformation of one micromole of substrate per minute under defined environmental conditions. These conditions are: saturated substrate concentration, optimal pH and temperature (Acevedo et al. 2002). An alternative to the international unit is the katal (kat) that replaces micromoles per minute by moles per second (Wiseman 1985; Illanes 1994).

6 ENZYME STABILITY AND DENATURATION

The most important property of an enzyme is its catalytic capacity or activity. The catalytic capacity depends on the protein structure, i.e. the native structure of the protein that results from numerous weak interactions (Illanes 1994). It is, therefore, obvious that a multitude of physical and chemical parameters can and do cause perturbations in the native protein's geometrical and chemical structure with concomitant reductions in activity (Bailey & Ollis 1986). This phenomenon is called enzyme denaturation. Depending on the magnitude of the denaturating agent, the quaternary (if it exists), tertiary or secondary structure may be affected. The quaternary structure alteration is often reversible; the change in the tertiary structure is often irreversible leading to a total or partial activity loss; and the alteration of the secondary structure is irreversible, leading to a coagulation effect and total inactivation of the enzyme (Illanes 1994).

Enzyme stability is largely affected by temperature, pH and ionic strength. A temperature increase leads to an increase in the vibration energy that may cause the break of the hydrogen bonding, and destruction

of non-polar interactions. On the other hand, it is important to emphasize that for low temperatures, that do not often have denaturant effect, the repetition of solid-liquid transitions (congelation-decongelation) promotes important losses of enzymatic activity (Scriban 1984). The electric charge of the proteins' amino acid residues depends on the protons concentration in the mixture. The pH values promoting accumulation of negative or positive charges may destabilise the enzyme structure due to the repulsion forces. Organic solvents less polar than water may be denaturant agents, modifying the standard formation of hydrogen bridge and non-polar interactions, and to stick out the electrostatic forces of interaction between the amino acid residues, diminishing the dielectric constant of the mixture. Low values of ion strength leads to a decrease in the enzyme–solvent interactions and to an increase in the ion interactions inside the chain, destabilizing the structure. That is why it is not advisable to manipulate enzymes in low ionic strength solutions like water (Illanes 1994).

After identifying the individual parameter, which influences enzyme denaturation, it is important to recognise that it is not the individual factors, but their combinations that determine the rates of enzyme deactivation. The sensibility of a certain protein to denaturation at high temperatures can vary with solution pH, and the influence of various pH-temperature combinations may be completely different from one protein to another (Bailey & Ollis 1986).

These alterations in protein structure and function have many important practical implications. Considering the enzyme production by fermentation, proteins are often recovered from culture broth by precipitation, an operation that involves changing the configuration or the chemical state of the protein by pH or ionic strength adjustment. Later protein purification steps may use antibodies as highly specific sorbents, a process that can fail if the antibodies deactivate, and lose their ability to identify and absorb a specific protein (Bailey & Ollis 1986). Most of the research literature on enzyme kinetics is devoted to initial rate data and analysis of reversible effects on enzyme activity. Especially, when continuous flow reactor is used, enzyme activity declines, which is a critical characteristic. In these situations, the economic feasibility of the process may depend on the useful lifetime of the enzyme (Bailey and Ollis 1986).

Figure 3 shows an example of the influence of temperature on the stability of the extracellular and periplasmic exo-inulinase from *Kluyveromyces marxianus* ATCC 36907. The enzymes were submitted to temperatures between 30 and 70°C during 60 min and enzyme activity was evaluated at the optimal conditions for these enzymes. The extracellular enzyme showed to be more stable with increasing temperature than the periplasmic one.

7 FACTORS INFLUENCING ENZYME ACTIVITY

While the use of enzymes in industrial scales is wide-spread from food industries to pharmaceutical ones, the understandings of factors influencing enzyme activity are crucial for the control and manipulation of products of enzymatic reactions. Some of the factors that influence desired enzymatic reactions can be manipulated with relative ease, while some of them are not. The accuracy of the determination of enzyme activity can be achieved only on the basis of defined conditions of measurement. The conditions of measurement need to take a number of factors into account, among them the measurement of temperature, pH, substrates and enzymes. To define the optimal conditions for enzyme action, it is necessary to carry out a number of experiments varying the value of the studied parameter knowing that often exist interdependence between them. Nevertheless, the concept of optimum is ambiguous especially for pH and temperature because they depend on the reaction time. The values reported as optimum for

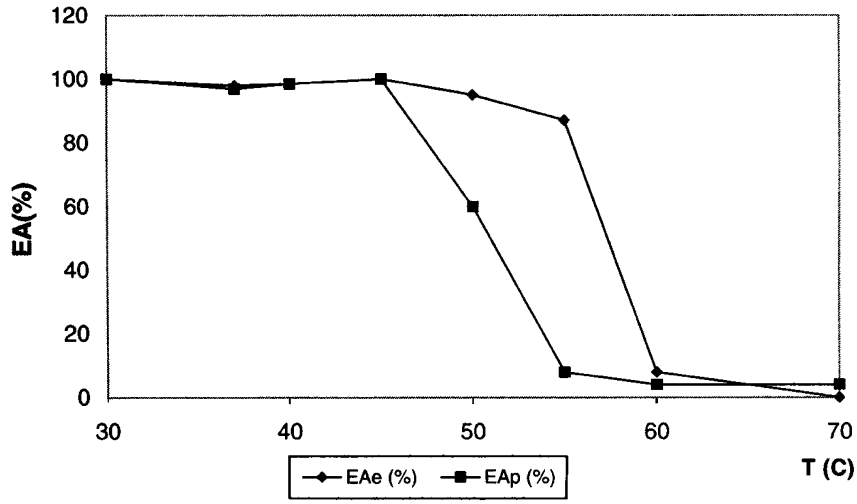

Fig. 3 Influence of temperature on the stability (% of enzyme activity) of the extracellular (EAe) and periplasmic (EAp) exo-inulinase from *Kluyveromyces marxianus* ATCC 36907 (Schneider 1996)

these parameters are often analytical optimums obtained within short period of reaction time and do not represent the operational optimums of long reactions times. Brief reviews of various factors that can affect any given enzymatic reactions are provided below.

7.1 Reaction time

Reaction time may affect enzymatic reactions many ways ranging from quality to quantity of the reaction products (Mu et al. 1998; Yadav & Gupta 2000; Chen et al. 2002; Chang et al. 2003; Garcia et al. 2003). Reaction time sometimes can be substantially reduced for a given enzymatic reaction by adding more enzyme activity. In some instances, even the same enzyme from different sources, however, behave differently (Rostometal 1998). Reaction time affects the enzyme catalysis of various reactions (Mantha et al. 2002; Markovic et al. 2002). Similarly, reaction time can be crucial in assessing viability of an enzyme for its industrial applications, a case in point, requirements of long reaction time, and high cost of the use of white-rot-fungi or lignolytic enzymes from them, are some of the drawbacks in degradation/ detoxification of organochloride compounds (present in various industrial effluents) despite their promising catalytic potential (Freire et al. 2000).

Zhang et al. (2000) reported high influence of reaction time on interesterification of oil blend between palm stearin and coconut oil during the production of margarine. Shieh and Lou indicated that reaction time was among one of the most important variables in enzymatic synthesis of citronellyl butyrate. Similarly, Mu et al. (1998) showed that reaction time was the most critical factor in the production of specifically structured triglycerols by lipase-catalyzed interesterification in a continuous reactor.

7.2 Amount of enzyme

For a defined substrate concentration, the study of initial reaction velocity dependence on enzyme concentration (Fig 4) shows a hyperbolic profile. The initial reaction velocity increases with enzyme

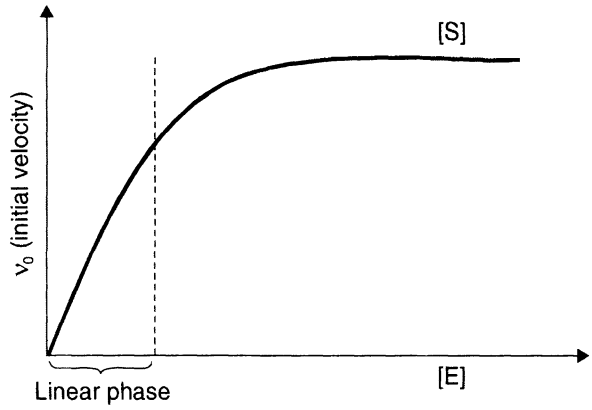

Fig. 4 Effect of enzyme concentration [E] on the initial velocity (v_0) for an enzyme catalysed reaction

concentration until a certain point from which the total amount of substrate is found in the form of [ES] complex and the initial reaction velocity is constant and maximal. For kinetic studies, it is necessary to keep in the linear phase of this curve, i.e., at low enzyme concentrations. It means that the amount of enzyme must be extremely low when compared to the substrate concentration to warrant that the enzyme-substrate complex [ES] formation do not modify or only slightly modify the substrate concentration (Scriban 1984).

7.3 Substrate concentration

One of the most important factors affecting the velocity of an *in vitro* enzyme catalysed reaction is the substrate concentration [S]. However, it is difficult to study the effect of substrate concentration due to the fact that [S] varies with reaction time while substrate is converted into product. With increasing substrate concentration, the rate of its conversion becomes greater until it approaches its optimum value (Nelson & Cox 2002).

7.3.1 Henri and Michaelis and Menten hypothesis

Kinetic models to explain these findings were postulated by Victor Henri in 1903. Henri proposed that the combination of an enzyme and its substrate to form the enzyme-substrate complex [ES] is an obligatory step of the enzymatic catalysis. Ten years later (1913), Michaelis and Menten recognised the importance of using initial velocity v_0 rather than any velocity v (Nelson & Cox 2002).

Considering a single-substrate enzyme-catalysed reaction where there is only one substrate-binding site per enzyme, we can write the following general equation (White et al. 1976; Palmer 1999; Nelson & Cox 2002) :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (4)$$

If we consider only the initial period of the reaction, where the reaction rate is maximum (Fig 2), the product concentration is negligible and the formation of [ES] from product decomposition can be

ignored. Under these conditions, equation 4 can be simplified:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} [ES] \xrightarrow{k_3} E + P \tag{5}$$

Based on the Michaelis-Menten hypothesis, the conversion of [ES] into E + P is too slow when compared to the break down of [ES] given E + S. This means that $k_3 << k_2$ and that E and [ES] are in equilibrium:

$$k_1 [E] [S] = k_2 [ES] \text{ or } \frac{[E] [S]}{[ES]} = \frac{k_2}{k_1} = K_s \tag{6}$$

where K_s is the dissociation constant of [ES].

The conversion of [ES] into E + P, thus the rate limiting step, and the velocity equation may be written as follows:

$$v_0 = k_3 [ES] \tag{7}$$

The total amount of enzyme present in the reacting mixture $[E_0]$ is the sum of the amount of free [E] and bound [ES] enzymes:

$$[E_0] = [E] + [ES] \tag{8}$$

Substituting [E] derived from equation (8) into (6) and isolating [ES]:

$$[ES] = \frac{[E_0] [S]}{[S] + K_s} \tag{9}$$

If we substitute [ES] derived from equation (7) into (9), we obtain:

$$v_0 = \frac{k_3 [E_0] [S]}{[S] + K_s} \tag{10}$$

If substrate concentration is increased ($S_1 < S_2 < S_3 < \ldots\ldots < S_{n-1} < S_n$), the initial velocities could also increase. However, the higher the [S] the lower the increase of v_0, until reaching the stage in which no increase is observed (Fig. 5). At this point, the enzyme-substrate complex [ES] is at maximum, i.e., all the enzyme is completely saturated with the substrate and is present in the [ES] form. The initial limiting velocity is reached:

$$v_{max} = k_3 [ES]_{max} = k_3 [E_0] \tag{11}$$

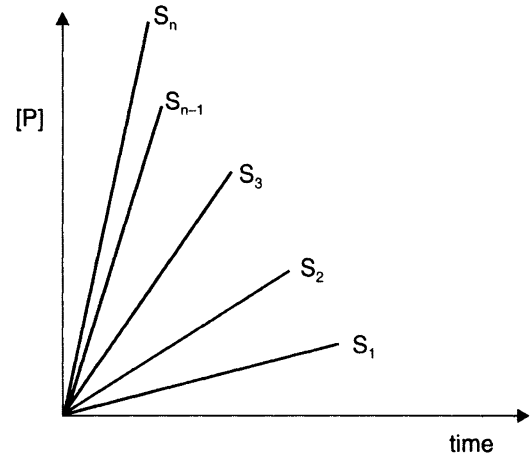

Fig. 5 Product formation [P] versus reaction time for different substrate concentrations

Substituting the expression k_3 $[E_0]$ in equation (10) by the value obtained in equation (11), we have:

$$v_0 = \frac{v_{max}\,[S]}{[S] + K_s} \tag{12}$$

7.3.2 Briggs and Haldane hypothesis

Considering the Michaelis-Menten equation as a very restrictive, Briggs and Haldane (1925), proposed the existence of a steady-state, in which the rate of [ES] formation would be equal to its decomposition rate to both directions, i.e., products formation and break down with substrate release (Scriban 1984; Palmer 1999). Based on this, the following equations could be proposed:

$$k_1\,[E]\,[S] = (k_2 + k_3)\,[ES] \text{ or } \frac{[E]\,[S]}{[ES]} = \frac{(k_2 + k_3)}{k_1} = K_m \tag{13}$$

where K_m is another constant.

Remembering that $[E_0] = [E] + [ES]$ and substituting [E] by its value obtained in the equation (13), we have:

$$[E_0] = \frac{(k_2 + k_3)\,[ES]}{k_1\,[S]} + [ES] \quad \text{or} \quad [E_0] = [ES]\ \left(1 + \frac{(k_2 + k_3)}{k_1}\ \frac{1}{[S]}\right)$$

$$[E_0] = [ES]\ \left(\frac{k_1\,[S] + (k_2 + k_3)}{k_1\,[S]}\right) \quad \text{or} \quad [ES] = \frac{[E_0]\,.\,k_1\,[S]}{k_1\,[S] + (k_2 + k_3)}$$

If we divide nominator and denominator by k_1, we have:

$$[ES] = \frac{[E_0] \cdot [S]}{[S] + \frac{(k_2 + k_3)}{k_1}} \qquad (14)$$

And substituting [ES] obtained in equation (14) in the equation (7), we obtain:

$$v_0 = \frac{k_3 \cdot [E_0] \cdot [S]}{\frac{(k_2 + k_3)}{k_1} + [S]} \qquad (15)$$ General equation of velocity

Finally, when the substrate concentration is too high compared to the enzyme concentration, the initial velocity tends to $k_3 \cdot [E_0]$. At this moment all the enzyme is present as enzyme-substrate complex, and the general equation may be simplified.

If we substitute in the equation (15), the values of k_3 ($k_3 = v_{max} / [E_0]$) defined in the equation (11) and K_m ($K_m = (k_2 + k_3) / k_1$) defined in the equation (13), we obtain:

$$v = \frac{v_{max} \cdot [S]}{K_m + [S]} \qquad (16)$$ Michaelis-Menten equation

This equation means that the velocity of an enzyme-catalysed reaction is a hyperbolic function of substrate concentration as showed in Figure 6.

A simple kinetic experiment is the measurement of the initial reaction velocity, called v_0, when [S] is largely higher then the enzyme concentration equivalent to [E]. If reaction time is short enough, the changes in substrate concentration will be insignificant, and [S] can be considered constant. At relatively low substrate concentrations, v_0 increases almost linearly with the increase of [S]. At high substrate concentrations, v_0 increases less and less with the increase of [S] until reaching a point over which v_0 increases are insignificant - the optimal or maximal velocity (v_{max}) is reached. When the initial velocity is equal to a half of the maximal velocity, the substrate concentration is equal to the Michaelis-Menten constant (K_m).

7.4 Temperature

Enzyme catalysed reactions, like all chemical reactions increase in rate with rises in temperature (Wiseman 1985). The temperature coefficient of the reaction rate can be as large as 10% per degree centigrade or more. This means that for a temperature rise equal to 1°C, enzyme activity increases about 10%. A linear relation generally exists between the logarithm of the rate constant (k), and the reciprocal of the absolute temperature (T) (Bergmeyer 1983). This effect can be described by the Arrhenius relationship

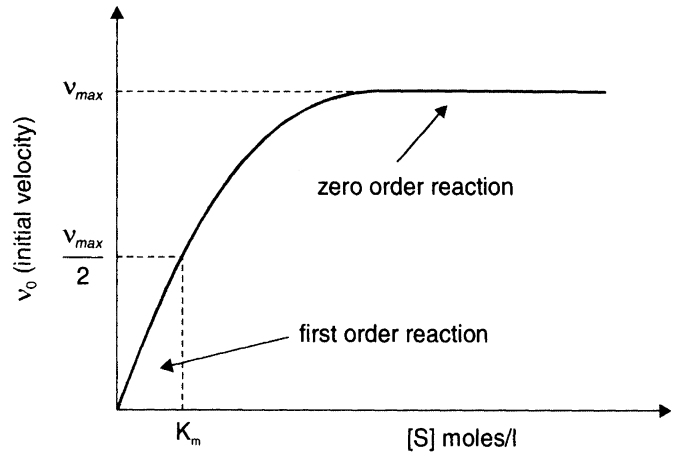

Fig. 6 Effect of substrate concentration [S] on the initial velocity (v_0) for an enzyme catalysed reaction.

(Equation 17). Arrhenius proposed that the activation energy (E_a) of a kinetic process could be obtained by the same way of enthalpy variation (ΔH) in an equilibrium process (White et al. 1976).

$$2.3 \log k = A - E_a / RT \text{ or } k = A \cdot e^{-Ea/RT} \quad (17)$$

A = Arrhenius constant or frequency factor

R = gas-law constant

In an Arrhenius plot (Fig. 7), log k is graphed against 1/T to give a straight line with a slope of $-E_a/2.3R$. The Arrhenius dependence on temperature is satisfied for the rate constant of many enzyme-catalysed reactions (Scriban 1984).

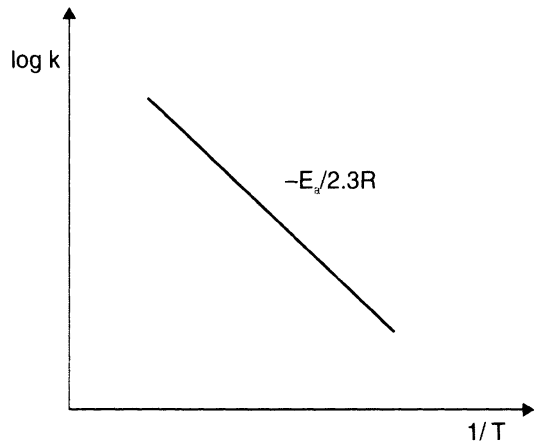

Fig. 7 Arrhenius plot.

Temperature generally has dual effects on the enzyme reaction. The increase in the temperature level raises the reactivity of the enzyme substrate complex and may also inactivate the enzyme due to the alteration of the native three-dimensional structure, as described in 2.6 (White et al. 1976; Scriban 1984; Acevedo et al. 2002). While the first effect does not depend on the reaction time, the second one is strongly influenced by time. It is not surprising that the optimal temperature of an enzymatic process also depends on the operation time, and its definition must take into account this double effect of temperature: activity and stability with time (Illanes 1994).

For many proteins, denaturation begins to occur at 45 to 50°C and is severe at 55°C (Bailey & Ollis 1986; Doran 1998). Only a few enzymes can be heated to above 100°C and still retain activity. Adenylate kinase is one example of enzyme that can retain activity even after having been maintained at pH 1.0 and 100°C. On the other hand, some enzymes are less stable when cooled (Wiseman 1985).

The enzymes present a range of temperature, sometimes narrow, in which the catalytic activity is maximal (Scriban 1984). Figures 8 and 9 show how the range of optimal enzyme activity can vary from one enzyme to another. Figure 8 presents the range of optimal temperature for extracellular and periplasmic exo-inulinases obtained from *Kluyveromyces marxianus* ATCC 36907, which have large range of optimal temperature. On the other hand, Figure 9 shows that β-D galactosidase produced by *Kluyveromyces marxianus* CDB 002 has a very narrow range of optimal temperature.

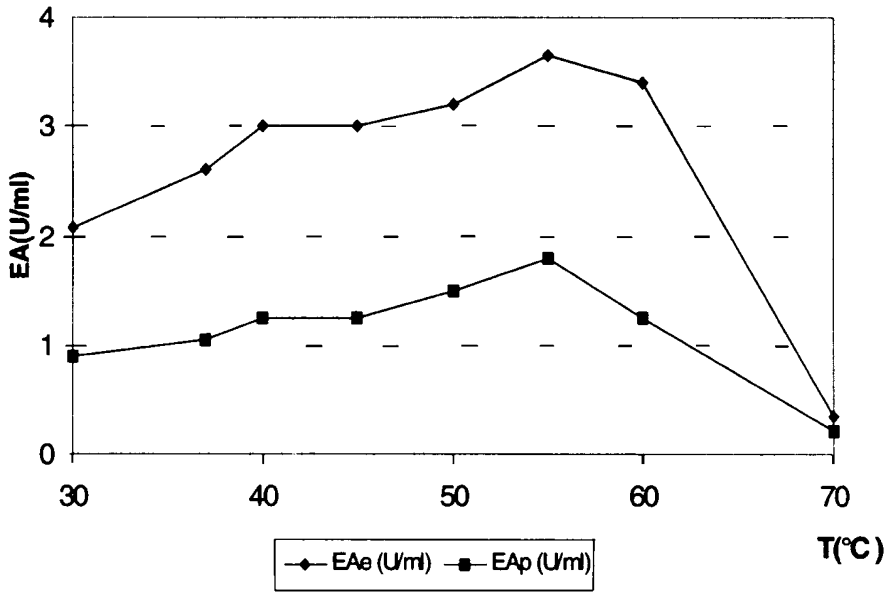

Fig. 8 Range of optimal temperature for extracellular and periplasmic exo-inulinases obtained from *Kluyveromyces marxianus* ATCC 36907 (Schneider 1996).

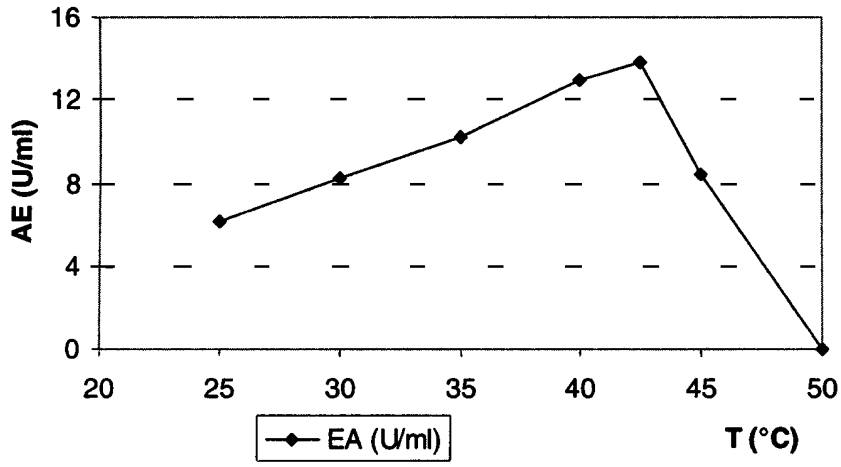

Fig. 9 Range of optimal temperature for β-D galactosidase produced by *Kluyveromyces marxianus* CDB 002.

7.5 pH

All enzymes have an optimum pH range for their activities, which is often very narrow. The optimum pH does not depend only on the nature and ionic strength of the buffer, in general also depends on the temperature and substrate concentration (Bergmeyer 1983; Scriban 1984). A definite optimum pH for enzyme activity is usually observed because, like other proteins, enzymes have a number of ionisable groups so that pH changes may modify the conformation of the enzyme, the binding of the substrate, and the catalytic activity of the groups present in the active site of the enzyme (Wiseman 1985). It also means that pH affects enzyme stability as descried in 2.6. Enzyme stability loss depends on the length of time the enzyme has been maintained at the unfavourable pH such that the optimum operational pH is generally a compromise between the effects on enzyme activity and enzyme stability (Wiseman 1985). The optimum pH varies largely among the enzymes. For example, the optimum pH of pepsin that acts in the acid medium of stomach is about 1.5, while the optimum pH of arginase, the enzyme that breaks the amino acid arginine, is about 9.7. But for the majority of enzymes the optimum pH is between 4 and 8 (Montgomery et al. 1994).

Proteins are constructed from amino acids residues that posses basic, neutral or acidic groups. Consequently, the intact enzyme may contain both negatively or positively charged groups at any given pH. These groups are apparently in general, part of the active site of the enzyme since acid and base catalysis has been related to several enzyme mechanisms. In order to have an appropriate catalysis the ionisable groups in the active site must often have a particular charge. This means that the catalytically active enzyme exists in only one particular ionisation state, may be a large or small fraction of the total enzyme present, depending upon the pH (Bailey and Ollis 1986).

To obtain a useful form to represent such pH effects on enzyme kinetics, we will consider the hypothesis (Bailey & Ollis 1986; Borzani et al. 2001):

- the enzyme is present in the mixture under three different ionisation states (E, E^{1-} and E^{2-});
- only E^{1-} is catalytically active;
- the following equilibrium exists where k_1 and k_2 are the equilibrium constants:

$$E \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} E^{1-} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} E^{2-} \qquad k_1 \quad k_2 \tag{18}$$

- the concentrations of E, E^{1-}, E^{2-} and $+H^+$ are e, e^{-1}, e^{-2} and h^+, respectively;
- e_0 is the total enzyme concentration: $e_0 = e + e^{-1} + e^{-2}$ (19)

We can write the following relations:

$$\frac{h^+ e^{-1}}{e} = k_1 \tag{20}$$

$$\frac{h^+ e^{-2}}{e^{-1}} = k_2 \tag{21}$$

and determine the fraction of the total enzyme present, which is active (y^{-1}):

$$y^{-1} = e^{-1} / e_0 \tag{22}$$

or:

$$y^{-1} = \frac{1}{1 + h^+/k_1 + k_2/h^+} \tag{23}$$

Figures 10 and 11 present the range of optimal pH for extracellular and periplasmic exo-inulinases obtained from *Kluyveromyces marxianus* ATCC 36907, and for β-D galactosidase produced by *Kluyveromyces marxianus* CDB 002. As observed for the temperature effect, the optimal pH range for exo-inulinase action is much larger than that obtained for β-D galactosidase.

7.6 Ionic strength

Catalytic activity of enzymes can be affected by ionic strength of the reaction media (Hicks et al. 2003). Wagner and Nicell (2002) suggest that reduction in conversion of phenol in wastewater by horseradish peroxidase and H_2O_2 occurs because of the increased in ionic strength of the solution. Ionic strength affects affinities between the enzymes and their respective substrates, specifically those involving electrostatic bindings. While some enzymes are active/stable at high ionic strength, such as CoB (a thermophilic protein) (Mana-Capelli et al. 2003) and endopeptidase (from Bromelia balansae) (Pardo et al. 2000), some are not. Similarly, same enzyme but from different sources may have different optimal ionic strength, e.g., electron transport flavoprotein (ETF) from human have low optimal ionic strength (about 10 meq), while that from *Paracoccus denitrificans* have high (>75 meq) (Roberts et al. 1999).

7.7 Pressure

High pressure (≥ 10 Kbr) may irreversibly denature enzymes. The denaturation of enzyme under high pressure may involve solvation of both the charged groups of hydrophobic interior and non-polar amino

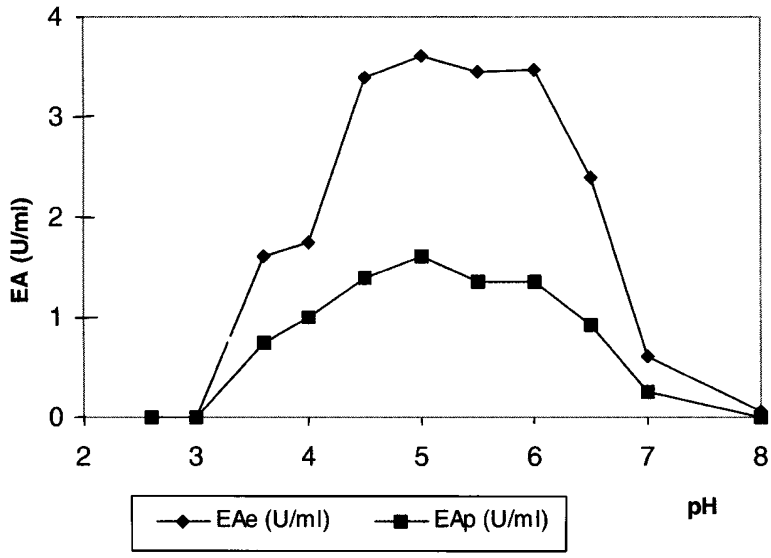

Fig. 10 Range of optimal pH for extracellular and periplasmic exo-inulinases obtained from *Kluyveromyces marxianus* ATCC 36907 (Schneider 1996).

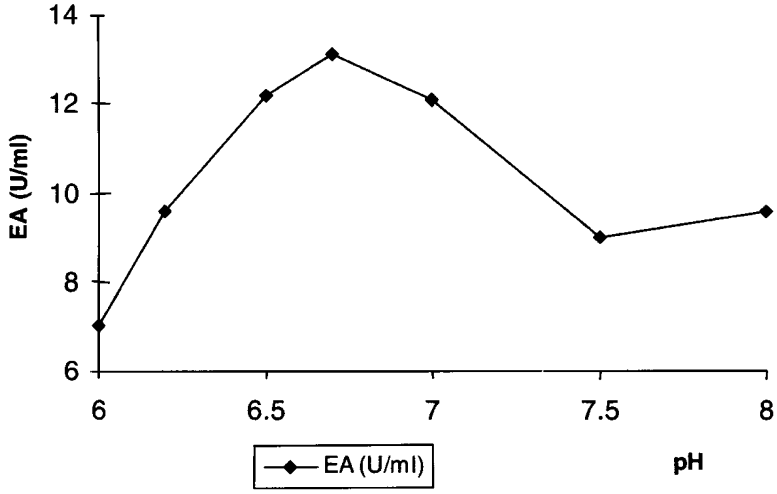

Fig. 11 Range of optimal temperature for β-D galactosidase produced by *Kluyveromyces marxianus* CDB 002.

acids (Suelter 1985). Oligomeric enzymes/proteins are more susceptible to pressure-induced denaturation than monomeric ones (Jaenicke 1981; Suelter 1985). Thus, structural instabilities can be induced in enzymes during ultracentrifugation due to hydrostatic pressure.

Protective effects of polyhydric alcohols on stability and activity of lipase against the thermal and pressure denaturation increase with their concentrations, and C chain length (Noel & Combes 2003).

The application of hydrostatic pressure to enzymes placed in surfactant nanocontainers (e.g. reverse micelles) could be advantageous for increasing the enzyme stability and modulating the enzyme activity (Kohling et al. 2002). Elevated pressure can exert substantial effects on mesophilic (adapted to atmospheric pressure) as well as on piezophilic (adapted to high pressure) organisms. Thus, high pressure would affect modulation of enzyme activities along with DNA replications, and cell divisions (Bartlett 2002). Certain enzyme activities, such as pectin methyl esterase in orange juice, however, can be inactivated or killed by pressure treatment (Basak et al. 2001). Interesting enough, Mori et al. (2001) even suggests that certain enzyme catalyzed reaction (e.g., esterification by lipid coated lipase) could be switch on or off by adjusting pressure or temperature.

7.8 Inducers and inhibitors

Enzymes are proteins with specific shapes, thus can be denatured and influenced by various factors including pH, temperature, pressure, ionic strength, reaction time, etc. Presence of inhibitors and inducers also affect enzyme catalyzed reactions. Any substance that increases the velocity of an enzyme catalyzed reaction by any mechanisms is an inducer, and any substance that reduces the velocity of the reaction is an inhibitor of that enzyme. Enzyme inducers and inhibitors have wide range of applications ranging from elucidation of enzymatic reactions to pharmacology.

Inhibitors can be categorized into competitive, non-competitive and uncompetitive inhibitors depending on the nature of inhibitions. A competitively inhibited reaction is dependent upon the relative concentrations of the substrates and inhibitors.

$$\text{E (Enzyme)} + \text{I (Inhibitor)} \longleftrightarrow \text{EI}$$

$$K_i = \frac{[EI]}{[E][I]} \tag{24}$$

Where, K_i is the dissociation constant for the EI.

Compounds similar to the substrate can thus combine with the enzyme at the active site, and inhibit the catalytic reaction. However, the inhibitory effects can be reversed by increasing the concentration of the substrate.

In non-competitive inhibition, however, the inhibition only depends on the concentration of inhibitor, i.e., the inhibitor combines with the enzyme at other than the active site, but that leads to an adequate alteration in enzyme's conformation so as to prevent substrate being combined.

$$[E] + [I] \longleftrightarrow EI$$

and

$$[ES] + [I] \longleftrightarrow ESI$$

Both EI and ESI are inactive!

$$K_i^{EI} = \frac{[EI]}{[E][I]} \tag{25}$$

Similarly,

$$K_i^{ESI} = \frac{[ESI]}{[ES][I]} \tag{26}$$

Where, K_i^{EI} and K_i^{ESI} are the dissociation constants for the EI and ESI, respectively.

In case of uncompetitive inhibition, as in the non-competitive ones, the inhibition cannot be reversed by increasing substrate concentration.

$$K_i = \frac{[ESI]}{[ES][I]} \tag{27}$$

Where, K_i is a dissociation constant for ESI.

Such inhibitors are pronounced mostly in enzymatic reactions with two or more substrates.

There are inducers as well as inhibitors of enzyme activity. Cytochrome P-450-enzyme activity can be induced by 1, 8 naphthalic anhydride, ethanol and certain herbicides (e.g., pyrazosulfuron-ethyl, bensulfuron-methyl), while the activity can be inhibited by piperonyl butoxide (Yun et al. 2001). Similarly, 1-mono-O-acyl-3-O-(α-D-) sulfoquinovosyl-glyceride), a sulfo-lipid, inhibits the activity of mammalian DNA polymerase, and is also a potent apoptosis inducer (Murakami et al. 2003).

Many inducers and inhibitors such as ethylene, and di- and trichlorophenoxyacetic acids are extensively used to control growth, reproduction and maturation of plants. Inhibitors, at cellular level, may alter cell permeability, transport mechanism, ATP and cellular constituent formations or enzyme biosynthesis (Whitaker 1972; Engel 1996). Inhibitors may react with apoenzyme, cofactors, substrates, inducers or intermediate products of the enzymatic reaction. Some of the specific inhibitors, e.g., EDTA, cynide, sulfide, CO inhibit many enzymes containing Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, etc. as essential cations (Whitaker 1972; Engel 1996).

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Enzyme Kinetics and Modeling of Enzymatic Systems

Emmanuel M. Papamichael and Leonidas G. Theodorou

1. INTRODUCTION

Enzymes are proteins acting as catalysts, i.e. they increase the rate of a reaction without modifying its overall standard Gibbs-energy change (NC-IUB 1983). The section of biochemistry, which deals with enzymes, is termed "enzymology", whose a branch is known as "enzyme kinetics" relevant to the study of enzyme mechanisms, their reaction rates and the conditions which affect these rates. Another useful term is "substrate" i.e. a reactant other than catalyst in a catalytic reaction. The rate of an enzymatic reaction is expressed as the change in concentration of one of its substrates or products versus time, and it is a function of numerous parameters including enzyme and substrate concentrations, the period of time in which the reaction is allowed to proceed, the pH-value and the temperature of the reaction medium, the concentration of any other compound which affects the reaction rate (for example activators and/or inhibitors), and the possible diffusion of reactants and/or products.

Mathematical models along with computer techniques have been proved valuable tools in searching for optimal operating conditions and/or creating optimal microenvironments for enzymes in order to optimize their effectiveness. Recently, the study of various kinds of models of enzymatic reactions attain of great interest in research, as well as in the industrial application of these biocatalysts (the enzymes), for optimal operation points, and to enhance our knowledge about the process. Mechanistic models and model-equations incorporate the information concerning each particular enzymatic reaction or system, and have been proved as effective tools in estimating the process variables. Modeling has been found an important role in enzymatic reactions (Wandrey 1993, Hogan & Woodley 2000, Straathof 2001) despite their complex nature. In that context, we will examine various means, including reasonable approximations, in order to specify the enzymatic reactions to a workable level while trying to minimize the effects introduced by both the experimental errors and the approximations.

The rate equation of an enzymatic reaction is a mathematical expression, which illustrates the catalytic process in terms of rate constants and reactant concentrations, and essentially it represents the experimental data by a realistic model-equation and a mechanism. In addition, the kinetic mechanisms of enzymatic reactions provide evidence of how enzymes and their substrates could combine (Brown 1902) to accomplish catalysis, by explaining the molecular machinery of enzyme action. Therefore, to understand how enzymes function as catalysts, and how their catalytic function is regulated, the knowledge of their kinetic mechanism is required. Likewise, the development of such mechanism of enzymatic reactions, among other things, could provide information on the nature of their transition state, the geometry of the enzyme's active site, the substrate specificity, the acidic and/or basic groups associated with catalysis, the possible allosteric properties and the mode of regulation, etc.

2. THEORIES ON ENZYME KINETICS

Henri was the first who published the general rate equation (1) (Henri 1902) for reactions involving enzymes. Henri's equation formulated the observation that the initial rate of an enzymatic reaction is directly proportional to the concentration of enzyme preparation, and it is increased nonlinearly with increasing the substrate concentration up to a limiting maximum value. The derivation of equation (1) was based on the following reasonable assumptions:

- The enzyme E (Segel 1975) reacts rapidly with its substrate S to form an enzyme-substrate complex ES (Brown 1902); however, $[S]_t >> [E]_t$ (where t stands for total) should be satisfied so that the formation of ES does not alter the substrate concentration. This latter is associated with the assumption of *quasi-steady-state approximation* (QSSA) established after an initial short transient state, where the reactant concentrations vary slowly and an excess substrate concentration is the main requisite; the transient state must be brief (Wong 1965, Laidler 1995) and it is obtained for $[S]_t/[E]_t$ ratios greater than 100.
- Enzyme, substrate, and ES are involved in enzymatic reactions. After the equilibrium is reached, ES dissociates to E and S much faster than it breaks down directly by forming free enzyme and products, limiting also the overall rate of the enzymatic reaction.
- Initial rates of enzymatic reactions should be measured during their early stages when any reverse reaction is insignificant, according to QSSA or to rapid equilibrium assumption.

Consequently, the overall reaction should be written as in Scheme I, where [S] represents the substrate concentration, and the reaction rate is given by the Henri equation (1). In equation (1) $v = -d[S]/dt$ or $v = d[P]/dt$, represents the initial velocity. In practice, $v = -\Delta[S]/\Delta t$, or $v = \Delta[P]/\Delta t$, provided that the disappearance of S (or the appearance of P) is linear with time during the assay procedure, and no more than 5% of the $[S]_t$ is utilized; k_p is the rate constant for the breakdown of ES to E and P, $Ks = k_{-1}/k_1$ is the dissociation constant of the ES complex, and K is a constant, characteristic of the particular enzyme preparation.

$$v = \frac{K[S]}{1+\frac{[S]}{K_s}} \tag{1}$$

Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_p} E + P$$

Ten years later Michaelis and Menten modified Henri's formulation and published (Michaelis & Menten 1913) a slightly different rate expression, equation (2), where in most cases, $K_s = k_{-1}/k_1$, as before. Besides, the physical meaning of constant K of equation (1) can be elucidated now, which it is equal to $k_p[E]/K_s$. However, according to Scheme I, $v = k_p[ES]$ and if $k_p[E]_t$ is termed as V_{max}, i.e. the limiting maximal velocity observed if all the enzyme in the reaction is bound to ES, then the familiar Michaelis-Menten equation (2a) is obtained; $[E]_t$, the total enzyme concentration is equal to [E] + [ES]. Commonly, equation (2a) is referred as the Henri-Michaelis-Menten (H-M-M) equation.

$$v = \frac{k_p [E]_t [S]}{K_S + [S]} \qquad (2), \quad v = \frac{V_{max} [S]}{K_s + [S]} \tag{2a}$$

The derivation of the H-M-M equation could be obtained from rapid equilibrium considerations (Dixon & Webb 1971) or by applying the QSSA (Briggs & Haldane 1925) to the reaction Scheme I, where ES accumulates to an almost constant level, the so-called "steady-state" level (Bodenstein 1913). After a short initial transient or pre-steady-state period ES is formed at the same rate at which it is decomposed. The QSSA level would be very close to the equilibrium level if $k_p << k_{-1}$. On the other hand, if k_p is comparable to k_{-1} or larger, then ES decomposes to free enzyme and products so fast that it never can attain a level that would be in equilibrium with E and S. The rate at which P is formed will be proportional to the steady-state concentration of ES. Another reasonable assumption is that the reverse of reaction in Scheme I should be neglected because during the early stage of reaction the concentration of products is essentially zero. Therefore, the rate of ES formation should be given by equation (3), whereas the rate of its decomposition should be given by equation (3a); at the steady-state level the sum of these two rates should be zero, according to equation (4).

$$\left(+\frac{d[ES]}{dt}\right) = k_1 [E][S] \qquad (3), \qquad \left(-\frac{d[ES]}{dt}\right) = (k_{-1} + k_p)[ES] \tag{3a}$$

$$k_1 [E][S] = (k_{-1} + k_p)[ES] \qquad (4), \qquad [E] = \frac{(k_{-1} + k_p)[ES]}{k_1 [S]} = [E]_t - [ES] \tag{4a}$$

By taking into account the mass-balance equations $[E]_t = [E] + [ES]$ and $[S]_t \approx [S]$ and by rearranging equation (4), we obtain equation (4a). By solving equation (4a) to [ES], and substituting to velocity-dependence $v = k_p[ES]$, we obtain equation (5); by multiplying both terms of the right part fraction of equation (5) by [S], we obtain equation (5a).

$$v = \frac{k_p [E]_t}{1 + \frac{(k_{-1} + k_p)}{k_1 [S]}} \qquad (5), \qquad v = \frac{k_p [E]_t [S]}{[S] + \frac{(k_{-1} + k_p)}{k_1}} \tag{5a}$$

The relation $\frac{(k_{-1} + k_p)}{k_1}$ is called K_m, the Michaelis constant, whereas $k_p[E]_t$ is called V_{max}; then, we obtain, the H-M-M equation (2a) again, in the form of equation (6). The only difference between equations (2a) and (6) is the replacement of K_s by K_m. However in equation (6), K_m represents a dynamic or a pseudo-equilibrium constant, depending on the magnitude of k_{-1} versus k_p. For $k_{-1} >> k_p$ $K_m = K_s = k_{-1}/k_1$, as before; however if $k_{-1} << k_p$ then $K_m = k_p/k_1$. Although, the described above general procedure was applied for a simple reaction involving one enzyme having a single catalytic site, one ligand (substrate), and one enzyme-substrate complex, however, it can be used to obtain velocity equations for all kinds of steady-state systems, including those involving multiple ligands.

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{6}$$

For equation (6), a plot of v against [S] is a rectangular hyperbola passing through the origin, with asymptotes $v = V_{max}$ and [S] = $-K_m$ (Fig 1); furthermore, it can be easily deduced that K_m equals the [S], for which the half of V_{max} is obtained. Actually, it is restricted to measure v for finite positive values of [S], and thus it is impossible to measure V_{max} and K_m accurately from such a plot, as asymptotes cannot be approached closely enough. In cases where [S] << K_m or [S] >> K_m, equation (6) reduces to $v = \frac{V_{max}[S]}{K_m}$ or to $v = V_{max}$, and equal to $k_a[E]_t[S]$ (2^{nd} order reaction) or to $k_b[E]_t$ (1^{st} order reaction), respectively. Consequently, we could assign two important kinetic parameters $k_b = k_{cat}$ and $k_a = k_{cat}/K_m$; k_{cat} represents the number of enzymatic reaction cycles per unit time (s^{-1}), whereas k_{cat}/K_m represents the overall effectiveness, and both are referred to the enzyme - substrate pair.

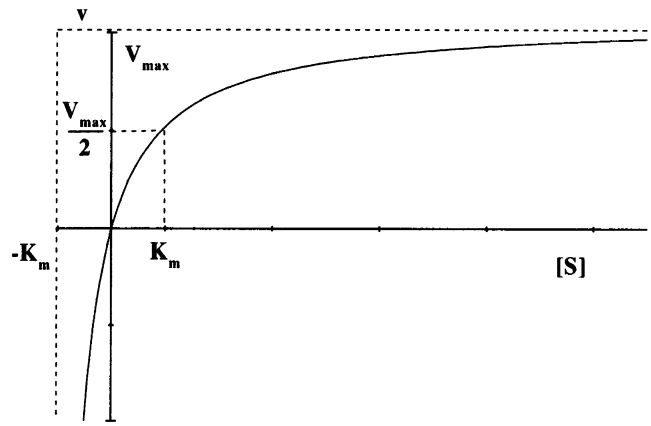


Figure 1: The H-M-M hyperbola, and its characteristic features. The solid line is drawn according to equation (6), where V_{max} = 10.7, and K_m = 1.06.

The obvious awkwardness of the H-M-M equation made necessary for Michaelis and Menten to use equation (7), whose plot (Fig 2) has a point of inflexion at $p[S] = pK_m$, and which is obtained straightforwardly from equation (6), in order to achieve estimates of V_{max} and K_m (Dixon & Webb 1971). Although, this method could be statistically acceptable, however it has not found favour among enzymologists (Eisenthal & Cornish-Bowden 1974).

$$v = \frac{V_{max}}{10^{(p[S]-pK_m)} + 1} \tag{7}$$

Currently, any non-linear model equation, as it is the H-M-M equation, can be used for fitting experimental data and obtain parameter estimates due to the available computers and software. However, most of enzyme kineticists, during decades have preferred to use some, among many, linear transformations of

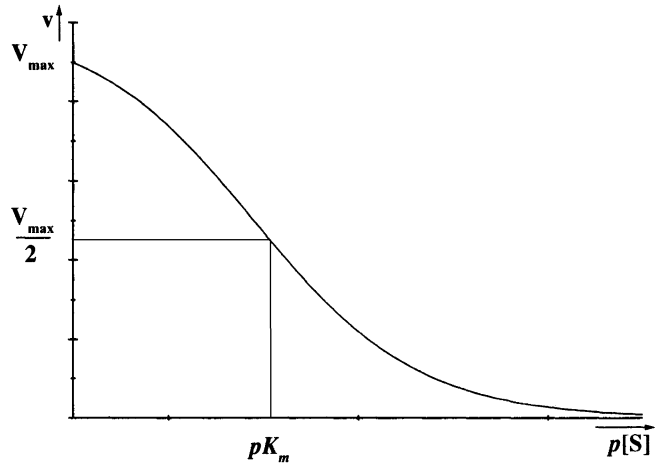


Figure 2: A graph introduced by Michaelis and Menten, so as to obtain estimates of V_{max} and K_m. The solid line is drawn according to equation (7), where V_{max} = 10, K_m = 8.9125.

the H-M-M equation for fitting purposes according to their scale in mathematics and statistics and/or the available instrumentation. The following linear transformations of the H-M-M equation have been mostly utilized in the past.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]} \quad \text{(Lineweaver-Burk)} \quad (8) \quad \text{(Copeland 2000)}$$

$$\frac{[S]}{v} = \frac{1}{V_{max}} [S] + \frac{K_m}{V_{max}} \quad \text{(Hanes-Wolff)} \quad (9) \quad \text{(Copeland 2000)}$$

$$v = V_{max} - K_m \frac{v}{[S]} \quad \text{(Eadie-Hofstee)} \quad (10) \quad \text{(Copeland 2000)}$$

$$\frac{V_{max}}{v} - \frac{K_m}{[S]} = 1 \quad \text{(Direct linear plot)} \quad (11) \quad \text{(Eisenthal \& Cornish-Bowden 1974)}$$

Hanes (1932) drew attention to the statistical dangers, which attend any linear transformation of the H-M-M equation, but his warning has been almost ignored until the appearance of works of Johansen and Lumry (1961) and Wilkinson (1961) who examined seriously the statistical properties of linear plots. These authors, along with Dowed and Riggs (Dowed & Riggs 1965) found equations (8), (9) and (10) as statistically improper, especially when large errors of unknown source were involved in the experimental measurements, as it was the case in enzyme kinetics [Mannervik 1982]. Alternatively, a different transformation was proposed i.e. a straight line in (V_{max}, K_m) space shown in equation (11) (Eisenthal & Cornish-Bowden 1974). Thus V_{max} and K_m are linearly related for given values of v and $[S]$, even though v and $[S]$ themselves are not (Eisenthal & Cornish-Bowden 1974). For each observation ($[S]_i$, v_i), there

exists a straight line in (V_{max}, K_m) space, with intercepts at -*[S]* and *v*, on the K_m and V_{max} axes, respectively, and relates all values of V_{max} and K_m, which satisfy the H-M-M equation exactly for the particular values of *[S]* and v. It follows that the co-ordinates of the point where all lines intersect provide the only values of V_{max} and K_m, which satisfy the H-M-M equation for every observation. For erroneous observations, as it is the case in practice, straight lines are intersected by two, yielding n(n-1)/n intersections (Eisenthal & Cornish-Bowden 1974). Excellent estimates are obtained for V_{max} and K_m by considering the median of all intersections, which unlike the mean is not strongly affected by the usual parametric statistical requirements; if n(n-1)/n is even number, then the mean of the two median values is taken. Fig. 3 illustrates both cases, what is in theory and what it happens in practice.

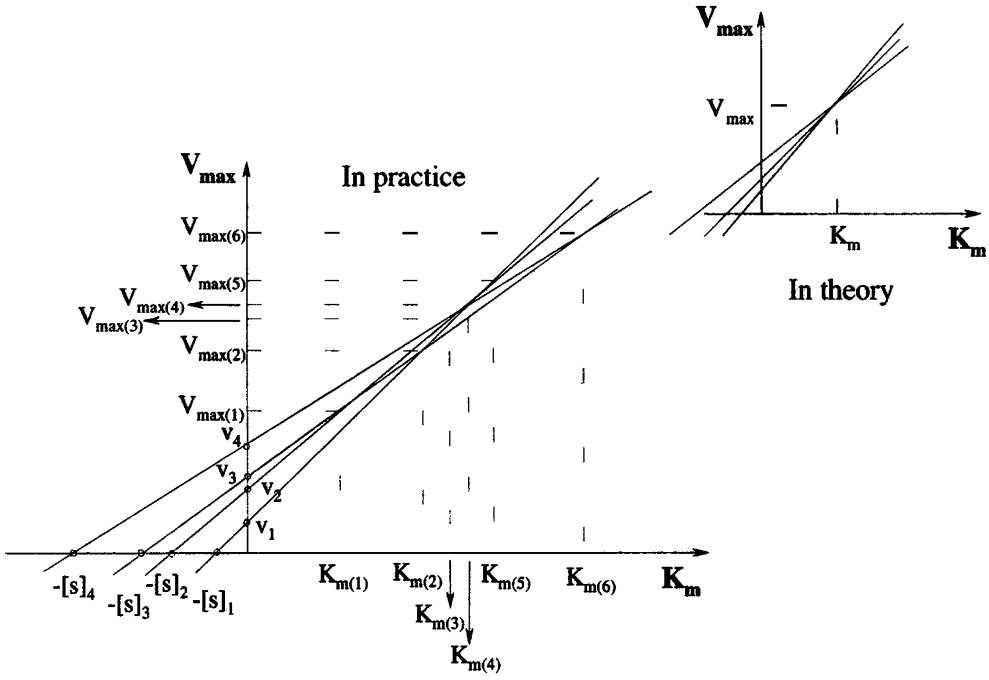


Figure 3: Two graphs of the direct linear plot introduced by Eisenthal and Cornish-Bowden. In theory, the coordinates of the intersection point correspond to the estimates of V_{max} and K_m; in practice straight lines are intersected by two, yielding n(n-1)/n intersections, where the coordinates of the median intersection point correspond to the estimates of V_{max} and K_m.

However, we should yield model equations also when $[E]_t$ is comparable to $[S]_t$, as for example, in case of *in-vivo* enzymatic reactions. In this incident we should take into account another mass-balance equation concerning the substrate concentration: $[S]_t = [S] + [ES]$. By applying the same technique as previously for the H-M-M equation, i.e. the QSSA, the quadratic equation (12) is formed, whose negative root solution should be taken to form equation (13); the positive root solution of equation (12) should be neglected because the situation requires that [ES] = 0 when either $[S]_t = 0$ or $[E]_t = 0$ (Segel 1975).

$$[ES]^2 - \{[E]_t + [S]_t + K_m\}[ES] + [E]_t\,[S]_t = 0 \tag{12}$$

$$v = V_{max}\left[\frac{([E]_t + [S]_t + K_m) - \sqrt{([E]_t + [S]_t + K_m)^2 - 4[E]_t[S]_t}}{2[E]_t}\right] \tag{13}$$

Not often, the H-M-M equation cannot fit satisfactorily experimental data from enzymatic reactions. In these cases, it is not uncommon to assume some reasonable simplifications to succeed the best fit; however, it is recommended (NC-IUB 1983) to use rational equations having the general form of equation (14), where n ≥ m, whereas the meaning of parameters a_1, a_2,...a_n, and b_1, b_2,...b_m, depends on the reaction itself and the experimental conditions.

$$v = \frac{a_1[S] + a_2[S]^2 + a_3[S]^3 + ... + a_n[S]^n}{1 + b_1[S] + b_2[S]^2 + b_3[S]^3 + ... + b_m[S]^m} \tag{14}$$

$$v = \frac{V_{max}\,[S]^n}{K_H^n + [S]^n} \tag{14a}$$

We should note that the most acknowledged specific case of equation (14) is equation (14a) known as the Hill equation (Hill 1913), which is applied in cases of polymeric enzymes containing *n* equivalent binding sites usually located on *n* different monomers (subunits). One more assumption is necessary to generate equation (14a), including those employed for the H-M-M equation, i.e. only one substrate molecule is bound per subunit, according to Scheme II. In equation (14a) K_H^n no longer equals the substrate concentration that yields half-maximal velocity except for $n = 1$, when it reduces to the H-M-M equation.

Scheme II

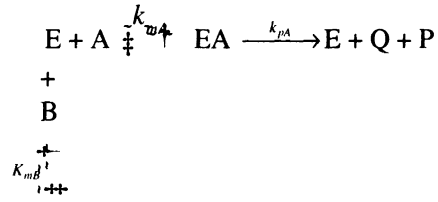
$$E + nS \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_n \xrightarrow{k_p} E + nP$$

Occasionally, a preparation may contain two or more enzymes (or multiple forms of the same enzyme), which catalyze the same reaction; then, the velocity should be the sum of the velocities contributed by each enzyme. As an example, for two enzymes, the velocity is given by the so-called double Michaelis-Menten equation (15) (Hsu & Tseng 1989).

$$v = \frac{V_{max_1}\,[S]}{K_{m_1} + [S]} + \frac{V_{max_2}\,[S]}{K_{m_2} + [S]} \tag{15}$$

It is not uncommon a single enzyme to act simultaneously on two different substrates present in the reaction medium. If the products of these substrates can be distinguished from each other, the system can be treated easily. However, if products are identical, or appear as a result of the assay method used, then the situation becomes complex. For example, suppose that a non-specific phosphatase catalyses the reactions and where **A** and **B** are two phosphate esters, Q and R are two distinct alcohols, and P is inorganic phosphate. In presence of A and B the equilibria are according to Scheme III. If the rate of formation of P is measured, then the v_t is the sum of two reactions, and equation (16), or its equivalent equation (16a), describe the reaction Scheme III. Thus one enzyme, which can react with two substrates, would yield results identical to those obtained by two enzymes.

Scheme III



$$v_t = \frac{V_{max\,A}\dfrac{[A]}{K_{mA}} + V_{maxB}\dfrac{[B]}{K_{mB}}}{1 + \dfrac{[A]}{K_{mA}} + \dfrac{[B]}{K_{mB}}} \tag{16}$$

$$v_t = \frac{V_{maxA}[A]}{K_{mA}\left(1 + \dfrac{[B]}{K_{mB}}\right) + [A]} + \frac{V_{maxB}[B]}{K_{mB}\left(1 + \dfrac{[A]}{K_{mA}}\right) + [B]} \tag{16a}$$

2.1 Kinetics of immobilized enzymes

In biochemistry and biotechnology it is common to deal with enzymes immobilized on various artificial or natural (biological) (Papamichael & Lymperopoulos 1998) solid matrices, where the immobilized biocatalysts are found in dynamic equilibrium with a liquid (water) phase in a heterogeneous system. The size of solid matrices of immobilization varies and sometimes approaches that of the colloid dispersion. In addition, a "microenvironment" is formed along the matrix surface, the so-called Helmholtz-Stern double layer, which depends on the type, structure and electrical charge of the immobilization matrix and the enzyme. Under these circumstances the substrate meets diffusive resistance before each enzyme to react and although excluding the case of porous matrices, however, it is almost unattainable to produce any rate equation without introducing several reasonable assumptions.

At first, we should assume that immobilized enzyme follows H-M-M kinetics with the same substrate before immobilization. Secondly, we should take into account the Fick's laws for diffusion (Laidler & Meiser 1982) and the fact that the concentration of the substrate, which reaches and reacts with the immobilized enzyme is actually unknown; then we could form equation (17) and under steady-state condition, where $\left(\dfrac{\partial[S]}{\partial t}\right)_t = 0$, its equivalent equation (18), assuming also that $[S]_t$ depends only from x, the thickness of the double layer.

$$\left(\frac{\partial[S]}{\partial t}\right)_t = \left(\frac{\partial[S]}{\partial t}\right)_{dif} + \left(\frac{\partial[S]}{\partial t}\right)_E = \frac{\partial}{\partial x}\left(D\frac{\partial[S]}{\partial x}\right) - \frac{k_{cat}[E]_t[S]_S}{[S]_S + K_m} \tag{17}$$

$$\frac{\partial}{\partial x}\left(D\frac{\partial [S]}{\partial x}\right) = \frac{k_{cat}\,[E]_t\,[S]_S}{[S]_S + K_m} \quad \text{or} \quad \frac{d^2[S]_l}{dx^2} = \frac{k_{cat}\,[E]_t\,[S]_S}{D\{[S]_S + K_m\}} \tag{18}$$

The indices *t*, *dif*, *E*, *s*, and *l*, in equations (17) and (18) stand for *total*, *Diffusion*, *Enzymatic*, *surface* (of the immobilized enzyme molecules), and *layer*, respectively; *D* is the experimentally estimated diffusion coefficient, which has been assumed equal for all three dimensions, and *[S]* is the concentration of the substrate in solution. The differential equation (18) has not a general solution, and it could be solved only under specific conditions ($[S]_s << K_m$ or $[S]_s >> K_m$); in addition, the introduced simplifications do not lead to a handy solution of the problem (Thomas et al. 1972, Bunting & Laidler 1972). The key for a relatively simple solution seems to be based on the assumption that each element j of the enzymatic reaction (substrates, products, etc) is distributed between the surface of the immobilized enzyme (*s*) and the solution (*sn*) according to a distribution coefficient $P^{[j]}_{s,\,sn} = \frac{[j]_s}{[j]_{sn}}$; however, we should assume an equilibrium between phases according to relation $[j]_s \rightleftarrows [j]_{sn}$ (Heirwegh et al. 1988, 1989). By using common assumptions and reasonable rearrangements, equation (19) is produced (Aiba et al. 1973).

$$v = \frac{k_{cat}^{app}\,[E]_t\,[S]}{[S] + K_m^{app}} \tag{19}$$

To understand what k_{cat}^{app} and k_m^{app} represent, let us take into account the rate of flux per unit surface of substrate, from solution to the surface of the immobilized enzyme through the double layer due to a homogeneous diffusion $J_{df} = -D\frac{d[S]}{dx} \approx \frac{D\{[S] - [S]_S\}}{\Delta x}$. However, it is not uncommon to deal with charged surfaces of immobilization and/or charged reactants; hence another flux $J_{dl} = -\frac{DF\Psi_H\,[S]}{RT}\,\frac{\Delta\Psi_s}{\Delta x}$ should be accounted, where Ψ_H is the electrochemical valence of charged reactants, Ψ_S is the electrostatic valence of immobilization matrix, and F = Ne (N is the Avogadro's number and e the electron charge). As it has been assumed that immobilized enzyme follows H-M-M kinetics with substrate, equation (19a) is obtained, where $V_{s_{unit}}$ and α are the reaction rate per unit surface of immobilized enzyme, and the surface of unit volume, respectively; K_{cat_s} and K_{m_s} are the well-known parameters for the reaction at the surface of the immobilized enzyme. Then, equation (19b) could be written, whereas under steady-state conditions equation (19c) is obtained.

$$v_s = v_{s_{unit}}\,\alpha = -\frac{d[S]_s}{dt} = \frac{k_{cat_s}\,[E]_t\,[S]_s}{[S]_s + K_{ms}} \tag{19a}$$

$$v_{s_{unit}} = \frac{\frac{k_{cat_s}\,[E]_t}{\alpha}\,[S]_s}{[S]_s + K_{ms}} \tag{19b}$$

$$\nu_{s_{unit}} = J_{df} + J_{dl} = \frac{\frac{k_{cat_s}[E]_t}{\alpha}[S]_s}{[S]_s + K_{ms}} = \frac{D\{[S]-[S]_s\}}{\Delta x} - \frac{DF\Psi_H[S]}{RT}\frac{\Delta\Psi_s}{\Delta x} \tag{19c}$$

By eliminating $[S]_s$ from equation (19c), the quadratic equation (19d) is formed, whose an approximate real solution is $\nu_{s_{unit}} = \frac{A}{B}$, where $A = \frac{k_{cat_s}[E]_t}{\Delta x\,\alpha} D[S]\left\{1 - \frac{F\Psi_H[S]\Delta\Psi_s}{RT}\right\}$, and $B = \frac{D}{\Delta x}\left\{K_{m_s} + [S] + \frac{\Delta x\, k_{cat_s}[E]_t}{D\,\alpha} - \frac{F\Psi_H[S]\Delta\Psi_s}{RT}\right\}$ (Aiba et al. 1973). Subsequently, the rate equation (19e) is obtained, by means of which the features both $K_{cat}^{app} = \frac{k_{cat_s}}{\alpha}$ and $K_m^{app} = \left(K_{m_s} + \frac{\Delta x}{D}\frac{k_{cat_s}[E]_t}{\alpha}\right)\left(\frac{RT}{RT - F\Psi_H\Delta\Psi_s}\right)$ of equation (19) are illuminated.

$$(\nu_{s_{unit}})^2 - B(\nu_{s_{unit}}) + A = 0 \tag{19d}$$

$$\nu_{s_{unit}} = \frac{\frac{k_{cat_s}[E]_t}{\alpha}[S]}{[S] + \left(K_{m_s} + \frac{\Delta x}{D}\frac{k_{cat_s}[E]_t}{\alpha}\right)\left(\frac{RT}{RT - F\Psi_H\Delta\Psi_s}\right)} \tag{19e}$$

2.2 Alternative formulations of enzyme kinetics: *fractal* and *virial* approaches

The rate of any reaction depends on the number of contacts between the different kinds of molecules and in isotropic systems is proportional to the product of concentrations of reactants. When the reactive species belong to macromolecular systems the evaluation of the average number of their contacts should take into account all different conformations of these macromolecules. This latter is rarely taken into account in enzymatic reactions, which are handled as homogeneous ones (Mattiasson & Mosbach 1975). Thus, a variety of equations have been produced in order to describe different behaviour in enzyme kinetics, where the steady-state differential equations may have analytical solutions only under limiting conditions, or may have not any. Furthermore, the non-linear fitting of these multiparametric equations to experimental data is not strictly objective depending on the weighting criteria and the employed program and/or algorithm; besides, the encountered kinetic parameters, are surprisingly complicated functions of the individual rate constants (López-Quintela & Casado 1989, Lymperopoulos et al. 1998). Attempts to counterbalance this situation led a number of authors to present alternative mathematical formulations of the enzyme kinetic equations.

Historically, first López-Quintela & Casado (1989) presented a revision of the methodology in enzyme kinetics, by introducing the fractal dimension D in the H-M-M equation. These authors adopted the concept of Mandelbrot (1983) and by applying the QSSA in the reaction Scheme I, they obtained the general rate equation (20), where V_{max}^{eff} and K_m^{eff} are the effective individual H-M-M parameters. Equation (24) offers an appreciable economy in numerical treatment of enzyme kinetic experimental data, compared with the conventional methods of searching for numerous multiparametric equations, and allows an overall view of the complexity of the reaction path of enzyme catalysis. When $D = 1$ equation (24) takes on the form of equation (6), and V_{max}^{eff}, and K_m^{eff} receive their ordinary meaning. Much later other authors, based on the approach introduced by López-Quintela & Casado developed suitable fractal enzyme kinetic theories providing novel means to achieve important features of biochemical pathway design (Savageau 1995, 1998).

$$v = \frac{V_{max}^{eff}\,[S]^{2-D}}{[S] + K_m^{eff}} \tag{20}$$

However, other attempts led to an alternative conception for a general enzyme kinetic equation, based on a virial expansion of the H-M-M equation (Lymperopoulos et al. 1998). Equation (3) can be written as, $\left(+\frac{d[ES]}{dt}\right) = k_1\, C(E,S)$, where $C(E,S)$ is the average number of contacts between the two reactants. In this complex macromolecular system the dependence of $C(E,S)$ i.e. the number and kind of contacts of S on E is more complicated, and leads also to a complicated dependence on [S]. Therefore, the number of these contacts can be written as: $C(E,S) = [E][S](1 + A_2[S] + A_3[S]^2 + A_4[S]^3 + \ldots)$, where A_2, A_3, etc. are the equivalent virial coefficients, which can be estimated by means of molecular models of statistical thermodynamics by taking into account all possible configurations of the macromolecular system. These coefficients can be positive or negative expressing positive or negative contributions to $C(E,S)$ from pairs, triples etc. of S in the neighborhood of E. Then, under steady-state conditions the rate equation (21) is generated, which can fit and explain a variety of experimental observations and/or mechanisms.

$$v = \frac{V_{max}\,[S](1 + A_2[S] + A_3[S]^2 + \ldots.)}{K_m + [S](1 + A_2[S] + A_3[S]^2 + \ldots.)} \tag{21}$$

2.3 Reversible enzyme inhibitors

Enzymes and substrates are not usually the only reactants of enzyme-catalyzed reactions. Other reactants generally called *ligands* (including substrates) participate in enzymatic reactions and affect their rate. Ligands, which reduce reversibly the rate of enzymatic reactions, and they act bound to enzyme molecules, are called *reversible enzyme inhibitors* (NC-IUB 1983); other ligands, *enzyme activators*, increase the rate of enzymatic reactions. In this part we deal only with reversibly inhibited enzymatic reactions, comprising single substrate and inhibitor molecules, as it is depicted in Scheme IV.

Scheme IV

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (a)$$

$$E + I \overset{K_i}{\rightleftharpoons} EI \qquad (b)$$

$$ES + I \overset{K_i'}{\rightleftharpoons} ESI \qquad (c)$$

In Scheme IV, the parameters K_i and K_i' hold the meaning of K_m, and express qualitatively similar relations. As we worked out previously in expressing the H-M-M equation, we should now state other assumptions, including the QSSA, in order to formulate the relation of velocity for inhibited enzymatic reactions. Thus, firstly the mass-balance equation should be stated as $[E]_t = [E] + [ES] + [EI] + [ESI]$; in addition, the velocity-dependence equation $v = k_2[ES]$, as well as the relations $[E]_t << [S]$ and $[E]_t < [I]$, should be valid, where [I] represents the concentration of the inhibitor. In cases of multi-reaction systems, as in Scheme IV, alternatively to QSSA we could assume that, shortly after the beginning of the reaction, an equilibrium is established; accordingly, the relations, $K_m = \frac{[E][S]}{[ES]}, K_i = \frac{[E][I]}{[EI]}$, and $K_i' = \frac{[ES][I]}{[ESI]}$ could be written, and equation (22) is produced, known as the mixed inhibition equation. Equation (22) takes on the form of equation (22a) for competitive inhibition, if $\{K_i' \rightarrow \infty\}$ i.e. equivalent to [ESI] = 0, and reaction (c) in Scheme IV, should be neglected. Conversely, if $\{K_i \rightarrow \infty\}$ i.e. equivalent to [EI] = 0, reaction (b) in Scheme IV should be neglected; then equation (22) takes on the form of equation (22b) for uncompetitive inhibition. However, in special cases where $\{K_i = K_i'\}$, equation (22) takes on the form of equation (22c), the so-called non-competitive inhibition.

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_i'}\right) + K_m\left(1 + \frac{[I]}{K_i}\right)} \qquad (22)$$

$$v = \frac{V_{max}[S]}{[S] + K_m\left(1 + \frac{[I]}{K_i}\right)} \qquad (22a)$$

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_i'}\right) + K_m} \qquad (22b)$$

$$v = \frac{V_{max}[S]}{([S] + K_m)\left(1 + \frac{[I]}{K_i}\right)} \qquad (22c)$$

As it can be easily noticed equations (22) to (22c) are more awkward forms than the H-M-M equation, having more parameters to be estimated. Therefore, it is recommended to use special tests to diagnose the kind of inhibition, and then to use the proper equation (Cornish-Bowden 1974). The most commonly used diagnostic test is based on the comparison of plots obtained by two specific linear transformations of equation (22) (Cornish-Bowden 1974), which are expressed by equations (23 - Dixon plot) and (24 - Cornish-Bowden plot). Both equations (23) and (24) represent straight lines in $\left(\frac{1}{v}, [I]\right)$, and $\left(\frac{[S]}{v}, [I]\right)$

space, respectively, as it is depicted in Fig. 4. The application of the diagnostic test is simple. The experimenter should perform a series of measurements, by varying the concentration of inhibitor ([I]) for three to four different, however, constant concentrations of substrate ([S]), and to plot the results as it is shown in Fig. 4. Then, it is easy to deduce the kind of inhibition, and to fit the experimental data properly. Furthermore, it should be emphasized that by using only one equation either (18) or (19), it is impossible to deduce on the kind of inhibition.

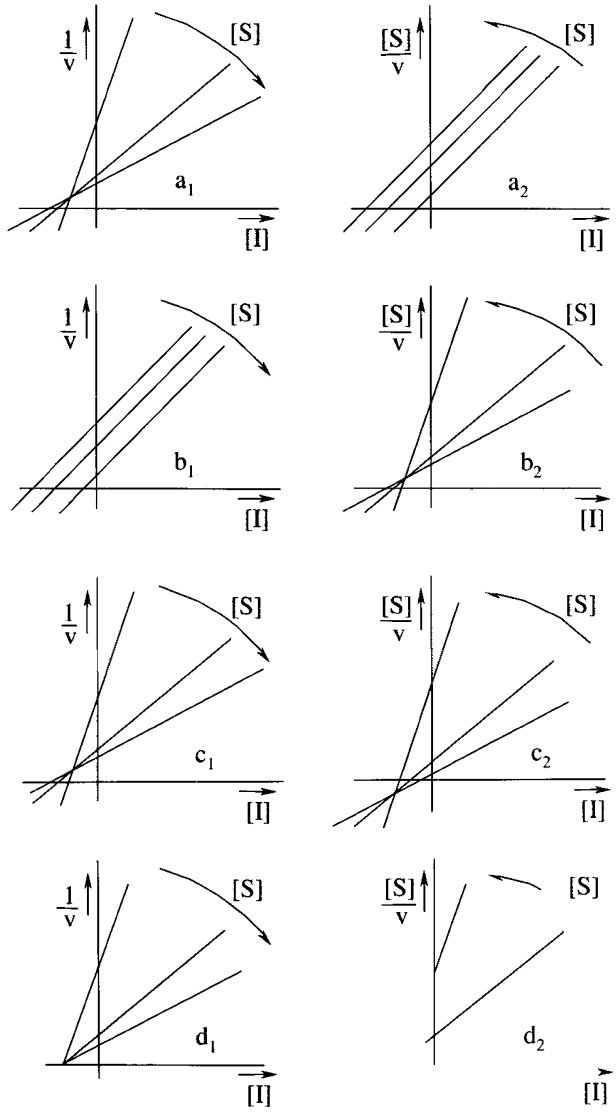


Figure 4: Dixon plots (a_1, b_1, c_1, d_1), and Cornish-Bowden plots (a_2, b_2, c_2, d_2), as a diagnostic tool of the kind of inhibition, which correspond to equations (23) and (24), for competitive, uncompetitive, mixed, and non-competitive inhibition, respectively.

$$\frac{1}{v} = \frac{1}{V_{max}}\left(\frac{K_m}{[S]} + 1\right) + \frac{1}{V_{max}}\left(\frac{K_m}{[S]K_i} + \frac{1}{K_i'}\right)[I] \tag{23}$$

$$\frac{[S]}{v} = \frac{1}{V_{max}}(K_m + [S]) + \frac{1}{V_{max}}\left(\frac{K_m}{K_i} + \frac{[S]}{K_i'}\right)[I] \tag{24}$$

In Fig. 4, a_1, b_1, c_1, d_1 sub-diagrams represent plots of equation (22) for competitive, uncompetitive, mixed, and non-competitive inhibition, respectively, whereas, a_2, b_2, c_2, d_2 sub-diagrams represent the corresponding plots of equation (23). The coordinates of intersection points in a_1, b_2, c_1, c_2, and d_1 or d_2 correspond to $(1/V_{max}, -K_i)$, $(K_m/V_{max}, -K_i)$, $([1 - K_i/K_i']/V_{max}, -K_i)$, $(K_m[1 - K_i'/K_i]/V_{max}, -K_i)$, and to $-K_i = K_i'$, respectively.

Recently, an important work has been published on the diagnosis of the kind of inhibition, which is based on: (a) the dependence of the degree of inhibition, (b) the inhibitor concentration, and (c), the substrate concentration present in the reaction (Antunes et al. 2003). As it is well known the degree of inhibition is a ratio between reaction rates; in this way, kinetic data could be normalized by the rate of uninhibited reaction, whereas the error associated with the kinetic measurements decreases and fewer measurements are necessary to achieve diagnosis. This latter can be easily deduced from Fig. 5. Additionally, in this new process the diagnosis of the kind of inhibition is facilitated by stepwise graphical and/or non-linear fitting procedures, where only two rival models are evaluated in each step; the choice of the best model is based on the application of suitable information statistic theories.

2.4 Substrate inhibition

It is not uncommon to observe a decrease in the rate of an enzymatic reaction, as the concentration of the substrate is increased. In the absence of inhibitors, this is due to special binding effects of substrate onto enzyme, which in a straightforward analogy to competitive inhibition, and by considering similar assumptions and methodologies, is expressed by Scheme V and equation (25).

Scheme V

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (a)$$

$$ES + S \overset{K_{SS}}{\rightleftharpoons} SES \qquad (b)$$

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_{SS}}} \tag{25}$$

3. MATHEMATICAL MODELS IN ENZYME KINETICS

Modeling of enzymatic reactions is not a simple task; a variety of prerequisites steps should be taken into account as for example experimental determination and mathematical formulation of the initial reaction

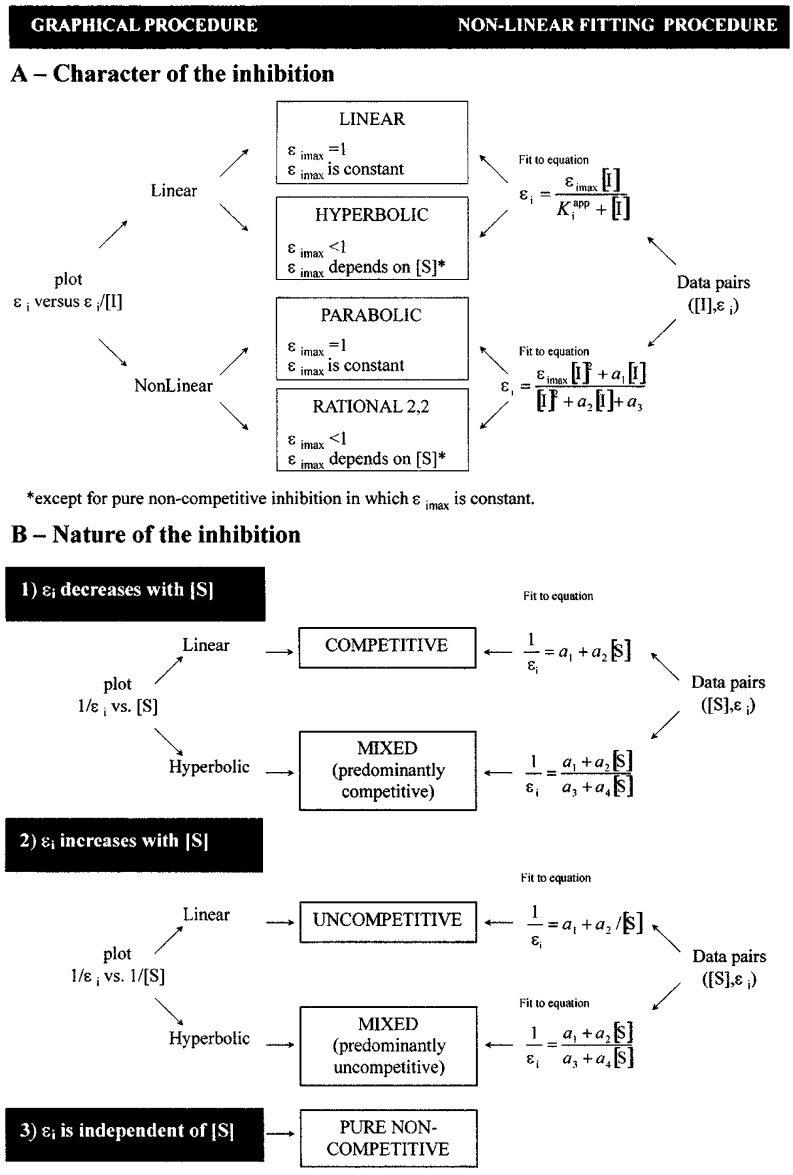


Figure 5: Strategy used to diagnose the type of inhibition (with permission from Antunes et al. 2003). The diagnosis of inhibition is divided in a two-step approach where the character (A) and the nature (B) of the inhibition are identified. Either a graphical (left part of the figure) or a non-linear fitting (right part of the figure) procedure is possible, however it is suggested the combination of both procedures. In the graphical procedure, only the two indicated plots are needed to completely diagnose the inhibition, while in the non-linear one, this is achieved by two sequential fittings of the two pairs of shown equations to the data. Note that diagnoses of the character (obtained with constant [S]), and of the nature of inhibition (obtained with constant [I]) are independent.

rate, parameter identification, mathematical formulation of an overall reaction rate, experimental verification of the model prediction, and calculation and prediction on the basis of the developed model. However, of great importance gets the information about a plausible structure for the transition state of the enzymatic reaction under consideration, which could be based on the search in the literature, in the choice of the appropriate experimental methodology, in theoretical calculations and/or computer simulations (Kędzierski & Sokalski 2001). Below are given some examples demonstrating the benefits of enzyme kinetics in modeling of enzymatic reactions and systems.

3.1 EXAMPLES DEMONSTRATING THE MODELING OF THE ENZYMATIC REACTIONS

3.1.1 Enzymatic dipeptide synthesis (Vasic-Racki et al. 2003)

Although advanced methods were developed for the synthesis of short-chain peptides, which acquire of increased interest, however, their chemical and optical purity is influenced by side reactions and racemisation effects, and it should be minimized during synthesis. These requirements use to be satisfied by kinetically controlled enzyme-catalyzed peptide synthesis.

In Scheme VI an acyl-enzyme A transfers the acyl group either to a second amino acid B (a) or to water W (b), and dipeptide product P or the amino acid Q is obtained, respectively; therefore, the reaction conditions should be controlled, to minimize the secondary hydrolysis (c) (Fischer 1994, Fischer et al. 1994). Thus, enzymes and substrates should be as pure as possible and free from substances affecting the enzymatic reaction, whereas the influence of the composition, pH-value and temperature of the reaction medium and the substrate concentration have to be optimized in order to enzymatic peptide synthesis to proceed under suitable conditions. In the examined case, kinetic equations were fitted data from experimental measurements of the forward and reverse reaction at different substrate and/or product concentrations using non-linear least-squares regression techniques and optimization algorithms (Marquardt 1963, Ratkowski 1983). Firstly, the ping-pong mechanism, which generally leads to rather unwieldy equations (26) to (26c) comprising too many parameters for practical application, and secondly, the Michaelis-Menten double substrate equation (27) to (27c) considering a competitive substrate and/or product inhibition, have been used. Both models were validated (Fischer 1994) with enzymatic dipeptide (Tyr-Ser) synthesis catalyzed with α-chymotrypsine, by comparing the calculated and the experimental concentration-time curves; it has been found a better agreement between the predicted concentration-time curves and the observed experimental data, according to equations (27) to (27c).

$$-\frac{d[A]}{dt} = \frac{\dfrac{V_{m,AB}[A][B]}{K_{m,B}} + \dfrac{V_{m,AI}[A][I]}{K_{m,I}}}{denominator} \quad (26) \qquad \frac{d[P]}{dt} = \frac{\dfrac{V_{m,AB}[A][B]}{K_{m,B}} - \dfrac{V_{m,PI}K_{m,AI}[I][P]}{K_{m,I}K_{m,P}K_{m,BQ}}}{denominator} \quad (26a)$$

$$-\frac{d[B]}{dt} = \frac{\dfrac{V_{m,AB}[A][B]}{K_{m,B}} - \dfrac{V_{m,PI}K_{m,AI}[I][P]}{K_{m,I}K_{m,P}K_{m,BQ}}}{denominator} \quad (26b) \qquad \frac{d[Q]}{dt} = \frac{\dfrac{V_{m,AI}[A][I]}{K_{m,B}} - \dfrac{V_{m,PI}K_{m,AI}[I][P]}{K_{m,I}K_{m,P}K_{m,BQ}}}{denominator} \quad (26c)$$

where $$denominator = [A] + \frac{K_{m,AI}[B]}{K_{m,B}} + \frac{K_{m,AB}[B][P]}{K_{m,B}K_{i,P}} + \frac{K_{m,AI}[I]}{K_{m,I}} + \frac{[A][B]}{K_{m,B}} + \frac{[A][I]}{K_{m,I}} + \frac{K_{m,AI}[I][P]}{K_{m,I}K_{m,Q}K_{m,PQ}}$$

and $\frac{d[A]}{dt} = -R_1[E]_t - R_2[E]_t$ (27) $\frac{d[B]}{dt} = -R_1[E]_t + R_2[E]_t + R_3[E]_t$ (27a)

$\frac{d[C]}{dt} = R_1[E]_t - R_3[E]_t$ (27b) $\frac{d[Q]}{dt} = R_2[E]_t + R_3[E]_t$ (27c)

where, $R_1 = \frac{V_{max,A}[A]}{K_{mA}\left(1+\frac{[P]}{K_{iP}}\right)+[A]} \frac{[B]}{K_{mB}+[B]}$, $R_2 = \frac{V_{max,A}[A]}{K_{mA_h}+[A]}$ and $R_3 = \frac{V_{max,P}[P]}{K_{mP}+[P]}$

Scheme VI

Phth-Tyr-OR + SerOR → Phth-Tyr-Ser-OR (a)
A **B** **P**

Phth-Tyr-OR + H_2O → Phth-Tyr-OH + SerOR (b)
A **W** **Q** **B**

Phth-Tyr-Ser-OR + H_2O → Phth-Tyr-OH + SerOR (c)
P **W** **Q** **B**

R = Et- or NH_2-

3.1.2 Computational analysis of substrate binding by phosphorylase kinase and protein kinase A (Brinkworth et al. 2002)

The large number of protein kinases in eukaryotes makes impractical to determine their specificity by considering also that they exhibit a variety of substrate specificities. By considering three-dimensional structures of several protein kinases with bound substrates and nucleotides (Knighton et al. 1991, Kobe et al. 1999, Brown et al. 1999), a common fold has been shown, made of two lobes forming a cleft inside of which the active site is located, where nucleotides and peptide substrates bind in close proximity. In an attempt to distinguish probable substrates from non-substrates, the binding enthalpies of modeled enzyme-substrate complexes were analyzed by computational techniques and were correlated to experimental enzyme kinetics measurements. Consequently, a number of models of phosphorylase kinase and cAMP-dependent protein kinase with a series of known peptide substrates and non-substrates were generated, and in addition, the crystal structures of their complexes with a substrate peptide have been determined (Madhusudan et al. 1994, Lowe et al. 1997) from appropriate experimental data. Finally, it has been found that V_{max} values were affected, however, there was little effect on K_m values, and no obvious correlation appears to exist between the calculated enthalpies and V_{max}/K_m values. Good substrates showed better binding enthalpies than poor substrates and particularly non-substrates. Nevertheless, this simple method can distinguish good substrates from inadequate ones, and non-substrates.

3.1.3 Investigation of the catalytic mechanism of papain, a cysteine proteinase (Theodorou et al. 2001)

The proton inventory method has been applied during the hydrolysis by papain of four peptide substrates X-F-L-Y, where X={Suc, Pht}, and Y={pNA, Nmec, ONPh}; pNA, Nmec, ONPh, Suc, and Pht stand for p-nitroanilide, 7-amino-4-methylcoumaryl, p-nitrophenyl, Succinyl, and Phthalyl, respectively. This method comprises kinetic studies of solvent isotope effects in mixtures of H_2O and D_2O (Schowen & Schowen 1982). The reaction parameters are expressed as functions, of deuterium atom fraction n in the solvent, according to equation (28) (Szawelski & Wharton 1981); k_0 is the reaction parameter in H_2O, and ϕ_i^T and ϕ_j^G are the isotopic fractionation factors of i^{th} transition state, and j^{th} ground state protons, respectively, which reveal the effect of solvent from a reactant to a transition state (Harmony et al. 1975).

$$k_n = k_0 \frac{\prod_{i=1}^{\mu}(1-n+n\phi_i^T)}{\prod_{j=1}^{\nu}(1-n+n\phi_j^G)} \tag{28}$$

Accordingly, the hydrolytic mechanism of papain, which could be simplified by Scheme VII, has been elucidated; additionally, the rate constants k_1, k_{-1} and k_2 were estimated, and it was found that $k_{cat}/K_m = k_1$ (Theodorou et al. 2001). These results along with the magnitudes and shapes of proton inventories of the kinetic parameters and rate constants, expressed by equations (29) to (32), argue (a) for the formation of a tetrahedral adduct during the reaction governed by the k_1 parameter, and (b) that both acylation and deacylation proceed through similar concerted reaction pathways, whereas the movement of one proton accompanies a nucleophilic attack, according to Scheme VIII (Theodorou et al. 2001), where A, B, and C, denote the enzyme, the so-called Michaelis complex, and the acyl-enzyme, respectively.

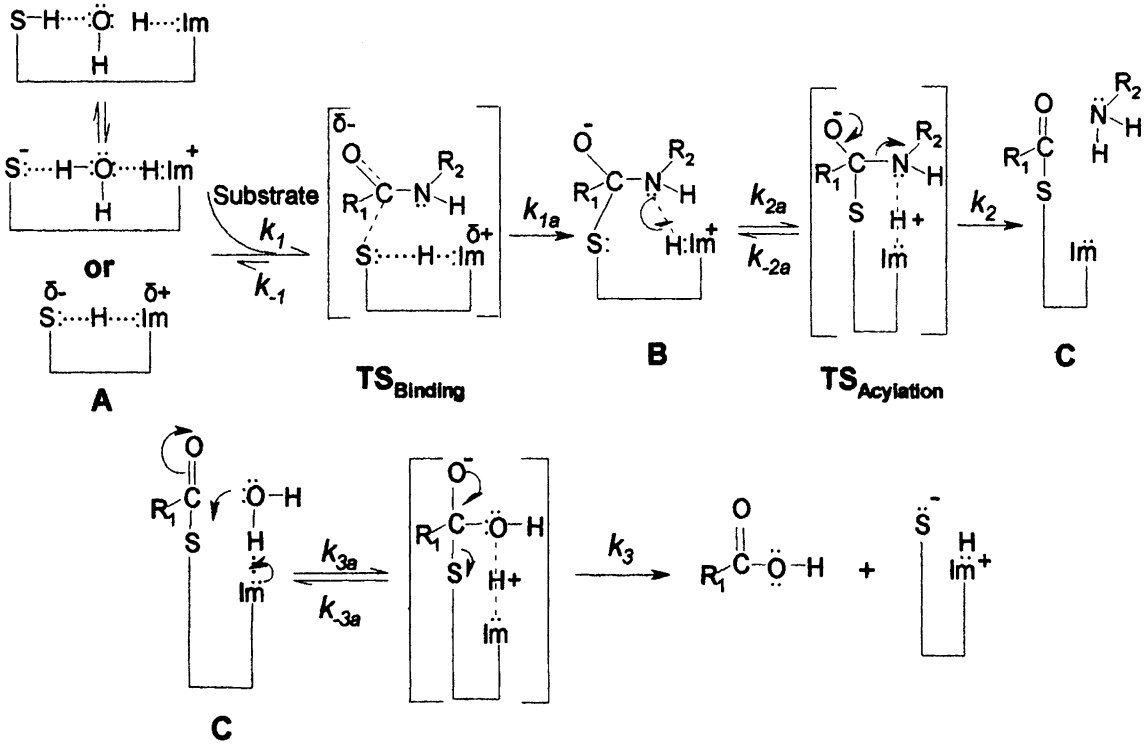
Scheme VII

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{E\text{-acyl}} \xrightarrow[+\ H_2O]{k_3} E + P_2$$

$$k_n = k_0 \frac{1-n+n\phi^T}{1-n+n\phi^G} \quad (29) \qquad K_n = K_0 \frac{1-n+n\varphi^P}{1-n+n\varphi^G} \quad (30) \qquad k_n = k_0(1-n+n\phi^T) \tag{31}$$

$$\frac{(k_2)_n}{(k_{cat}/K_m)_n} = \frac{1-n+n\phi^{G,k_1}}{1-n+nC_1}\left[\frac{k_2}{(k_{cat}/K_m)} + K_R\left(\frac{1-n+nC_2}{1-n+n\phi^{T,k_1}} - 1\right)\right] \tag{32}$$

Scheme VIII



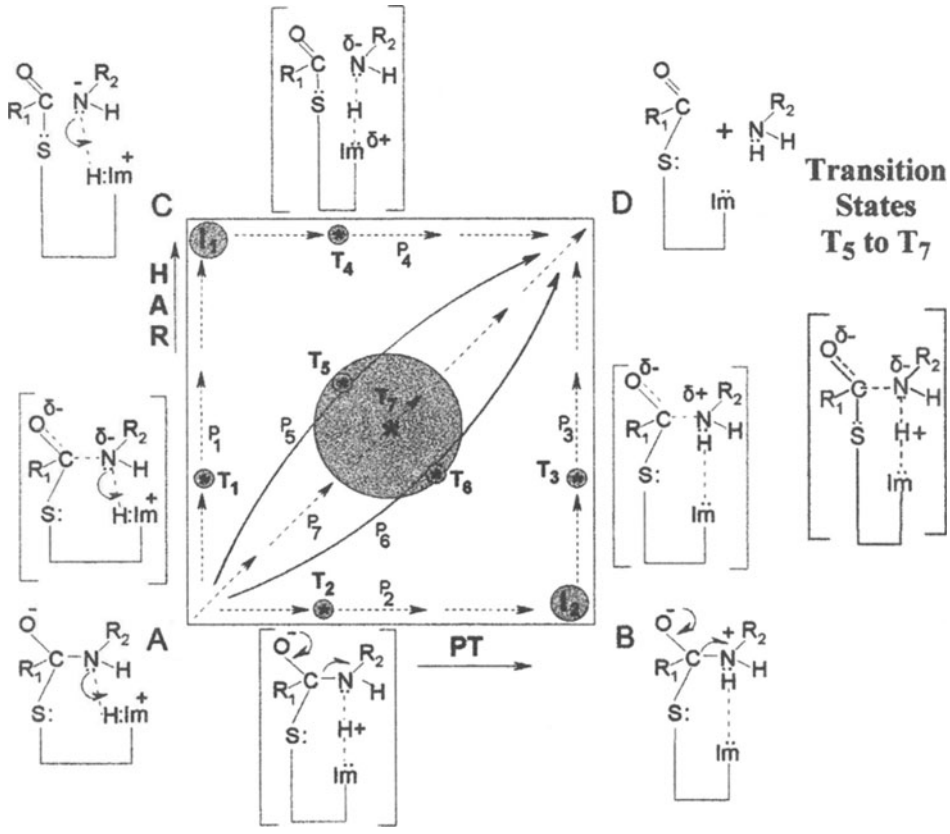
However, previously a hybrid quantum-mechanical/molecular-mechanical (QM/MM) approach was used where the effective Hamiltonian was written as: $\hat{H}_{eff} = \hat{H}_0 + \hat{H}_{QM/MM}$, and $\hat{H}_0$ and $\hat{H}_{QM/MM}$, are the Hamiltonian of the QM fragment, and the interaction between the QM and MM fragments, respectively, according to equation (33) (Harrison et al. 1997). In this work, the amide hydrolysis was elucidated through the stabilization of thiolate-imidazolium ion-pair of papain by means of the codes *Gaussian94* (Pearlman et al. 1992) and *AMBER* (Frisch et al. 1995) for the QM, and MM calculations, respectively. In equation (33) R and M label QM and MM centers, respectively, whereas its first term accounts for the effect of the formal charges of the MM fragment (q_M) on the QM part and may readily be incorporated into standard QM codes; the final two terms are the remaining Coulombic and Lennard-Jones interaction requisites between the QM and MM fragments (Harrison et al. 1997).

$$\hat{H}_{QM/MM} = -\sum_{iM}\frac{q_M}{r_{im}} + \sum_{aM}\frac{z_a\, q_M}{R_{aM}} + \sum_{aM}\left\{\frac{A_{aM}}{R_{aM}^{12}} - \frac{B_{aM}}{R_{aM}^{6}}\right\} \tag{33}$$

Within the limitations of the AM1 method (Nemukhin et al. 2003) the transition state of this hydrolytic reaction was identified and found that it is a concerted process without the involvement of a tetrahedral intermediate. Besides, a mutation of the active site residue Asn-175 to Ala-175 showed a marked effect on the ion-pair stability. Another QM/MM study of the active site of papain and its complex with N-methyl-acetamide (Han et al. 1999) provided similar results as previously (Harrison et al. 1997).
In conclusion, the results of both these theoretical studies (Harrison et al. 1997, Han et al. 1999) are in

complete agreement to those found experimentally (Theodorou et al. 2001) by applying the kinetic methodology of proton inventory, thanks to which the nature of a tetrahedral adduct was assigned to the Michaelis complex, and the transition state of the papain acylation ($TS_{Acylation}$) has been described in details, as it is depicted in Scheme IX; moreover rate constants as k_1, k_{-1} and k_2, were firstly estimated.

Scheme IX



3.1.4 Diagnostic tests on the mode of ligand binding to proteins [Papamichael et al. 2002]

Biochemical analyses of glucose uptake of the *Zymomonas mobilis* derivative CU1Rif2 of strain ATCC 10988 showed biphasic responses in Eadie-Hofstee plots (Douka et al. 1999). On the other hand, it has been accepted that *Zymomonas mobilis* transports glucose by means of a low-affinity, high-velocity glucose-facilitated diffusion system based on GLF protein (Parker et al. 1995 & 1997, DiMarco & Romano 1987), whereas other authors have suggested one or more similar protein molecules as glucose transporters of *Saccharomyces cerevisiae* (Walsh et al. 1994). All series of collected data of glucose uptake of *Zymomonas mobilis* derivative CU1Rif2 of strain ATCC 10988, failed to be fitted by one-kinetic-component equation (analogous to H-M-M), and were given biphasic responses in Eadie-Hofstee

plots. However, both the two-kinetic-components equations (34) and (35) were fitted glucose uptake data equally likely, and therefore one cannot distinguish whether one glucose transporter having two binding sites [equation (34)] exists or two transporters exist, each of them having one binding site [equation (35)]. It should be noticed that equation (35) is the same equation (15). To resolve this uncertainty two suitable diagnostic tests were developed (Papamichael et al. 2002), based on the alternative formulations of enzyme kinetics by *fractal* and *virial* approaches, which are described by equations (24) and (25) above.

$$v_{(uptake)} = \frac{V_{max_1} + \dfrac{V_{max_2}[S]}{K_{m_2}}}{1 + \dfrac{K_{m1}}{[S]} + \dfrac{[S]}{K_{m2}}} \qquad (34)$$

$$v_{(uptake)} = \frac{V_{max_1}[S]}{K_{m1} + [S]} + \frac{V_{max_2}[S]}{K_{m2} + [S]} \qquad (35)$$

In this way, appropriate computer simulations were performed by using suitable software, and experience (Papamichael & Evmiridis 1988, Evmiridis & Papamichael 1991, Box 1971, Cornish-Bowden 1974, Ratkowski 1983). The fractal dimension, which corresponds to equation (34) was estimated as D = 0.75 ± 0.16, whereas that of equation (35) as D = 0.50 ± 0.09. Likewise, it was found that an equation similar to (25) having two virial coefficients, the first of which was estimated as negative and the second one as positive, could replace equations (34) and (35); the absolute value of the first virial coefficient was estimated as 30 ± 0.9 times larger than the second one, in case of equation (34), whereas as 22952 ± 100 times larger than the second one, in case of equation (35). Finally, by fitting all available collected data of glucose uptake of *Zymomonas mobilis* derivative CU1Rif2 of strain ATCC 10988 by means of equation (24) a fractal dimension D = 0.73 ± 0.22 was estimated, whereas by fitting the same data by means of a two virial coefficients equation, a negative first virial coefficient was estimated whose absolute value founds as 76 ± 12 times larger than that of the second positive one. In conclusion, the system under investigation seems more likely to comprise only one proteinaceous factor, which functions as glucose transporter having two binding sites, and where the binding of one glucose molecule enhances the binding of a second one.

4. CONCLUSIONS

The first steps of enzyme kinetics, i.e. the theories developed by Henri, Michaelis, Menten, Hill and other pioneers were based on rather simple chemical and mathematical concepts, and assumptions. In this regard, the modeling of enzymatic reactions was poor in fitting the experimental data, and estimating appropriate rate constants and kinetic parameters; in most cases a vague description of the mechanism under consideration was obtained. Nowadays, with the aim of more powerful means (e.g. computers, and computer assisted analytical instruments), of new theories and applications (e.g. nonlinear fitting algorithms, non parametric statistics, QM/MM and fractal approaches, enzymes equipped by mutagenically transformed active site residues, etc), enzyme kinetics became a powerful tool of studying complex bio-catalyzed reactions offering robust estimation of rate constants and kinetic parameters, elucidation of composite mechanisms, and development of appropriate mathematical models; however, it should not be considered as panacea, and occasionally enzyme kineticists use to confirm their results by complementary methods and means.

This chapter has presented all conventional theories of enzyme kinetics applied in cases of single-enzyme - single-substrate reactions, including the kinetics of immobilized enzymes, and alternative formulations using fractal and/or virial approaches. This also includes several not uncommon effects, which could influence enzymatic reactions as the activity of reversible enzyme inhibitors and the substrate inhibition. Examples and applications concerning the mathematical modeling of specific enzymatic reactions by means of the tool of enzyme kinetics have also been provided.

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Thermodynamics of Enzyme-Catalyzed Reactions

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1. INTRODUCTION

Chemical thermodynamics is the science that deals with transformations of matter and energy. As such, it has found wide scientific and industrial applications in treating chemical reactions and processes. The important practical side of this discipline is that it serves as a primary tool for process design, optimization, and decision-making. Examples of chemical production where thermodynamics play a major role are the production of synthetic diamonds from graphite and the production of aviation fuel from hydrocarbons. In the much younger bioprocessing industry, examples of thermodynamic applications are seen in the production of food and pharmaceutical products such as high-fructose corn syrup and semi-synthetic penicillins. Indeed, many, if not most, bioprocess applications involve enzyme catalysis.

In this chapter, we shall review the principles needed to treat the thermodynamics of both simple and complex reactions. A full discussion of this topic is not possible in this Chapter and the reader is referred to a recently published book on this subject (Alberty 2003). We shall discuss industrial applications of thermodynamics, including enzyme-catalyzed reactions that use non-aqueous media, as well as some physiological applications. We shall give an overview of the experimental methods used to measure thermodynamic data as well as the use of thermochemical cycles and networks that can be used both to check the accuracy of and to greatly expand the amount of useful property values. We shall discuss estimation methods that can be used to provide approximate property values in the absence of experimental data. Finally, recognizing that the desired macroscopic property values can, in principle, be obtained from first principles by use of quantum chemistry, there will be a brief discussion of this application.

2. GENERAL PRINCIPLES

2.1. Thermodynamics of reactions involving specific chemical species

Chemical thermodynamics provides the general principles that allow one to treat the transformations of matter and energy associated with chemical reactions. For purposes of our discussion a chemical reaction is a transformation that involves specific species. An example of such a reaction is

$$ATP^{4-}(aq) + H_2O(l) \; f \quad ADP^{3-}(aq) + HPO_4^{2-}(aq) + H^+(aq), \tag{1}$$

where ATP is the abbreviation for adenosine 5'-triphosphate and ADP is adenosine 5'-diphosphate. This is a useful example because $ATP^{4-}(aq)$, $ADP^{3-}(aq)$, and $HPO_4^{2-}(aq)$ all bind $H^+(aq)$ and metal ions such as $Mg^{2+}(aq)$ and $Ca^{2+}(aq)$. Later, this will be shown to result in a very complicated behavior of the apparent equilibrium constant and thus makes this reaction an excellent model for illustrative purposes. Additionally, the hydrolysis of ATP is a primary energy source for living systems. The designators for the states of the reactants have also been given: (aq) for aqueous and (l) for liquid. Since the empirical formulas of ATP^{4-} and ADP^{3-} are, respectively, $C_{10}H_{12}N_5O_{13}P_3^{4-}$ and $C_{10}H_{12}N_5O_{10}P_2^{3-}$, the fundamental requirements that the number of atoms and that the charges balance is met. The thermodynamic functions that are of primary interest to our discussion are:

$\Delta_r G_m^\circ$ – the standard molar Gibbs energy change,

$\Delta_r H_m^\circ$ – the standard molar enthalpy change,

$\Delta_r S_m^\circ$ – the standard molar entropy change, and

$\Delta_r C_{P,\,m}^\circ$ – the standard molar heat-capacity change at constant pressure P.

The notation used here follows that recommended by the IUPAC (Mills 1993). The symbolism is important: Δ implies a change; the subscript "r" designates a reaction, the subscript "m" implies a molar quantity and the "°" specifies that the change in the thermodynamic function pertains to the standard state. While the choice of the standard state is arbitrary, the conventions adopted by the National Bureau of Standards (now the National Institute of Standards and Technology) (Wagman et al. 1982) are commonly used. For aqueous reactants the standard state for the solute is the hypothetical ideal solution at unit molality; the standard state of the solvent is the pure solvent. The standard pressure P° = 0.1 MPa. These formal statements are now clarified. In the hypothetical ideal solution at unit molality, the activity of the solute $a_2 = m_2/m^\circ$, where m_2 is the molality of the solute and m° is the standard molality (m° = 1 mol·kg^{-1}). This is equivalent to the real solution where $(a_2/m_2) \rightarrow 1$ as $\Sigma m_i \rightarrow 0$ (i.e. infinite dilution). For the solvent standard state, the activity of the solvent $a_1 \rightarrow 1$ as $x_1 \rightarrow 1$; here x is the mole fraction and the subscript "1" denotes the solvent. The difference between the partial molar Gibbs energy of a solute G_2 and the standard partial molar Gibbs energy of that solute G_2° is

$$G_2 - G_2^\circ = RT \ln a_2 = RT \ln m_2\gamma_2, \tag{2}$$

where γ_2 is the activity coefficient of the solute, R is the gas constant (8.314472 J·K^{-1}·mol^{-1}), and T is the thermodynamic temperature. The corresponding difference for the solvent is

$$G_1 - G_1^\circ = RT \ln a_1. \tag{3}$$

Thus, the properties of the solutes and of the solvent in their respective standard states are the same as those as the real solution in the limit of infinite dilution. However, one must appreciate that, formally, the standard state is not infinite dilution.

The natural variables for a chemical reaction such as reaction (1) above are the temperature T, the pressure P, and the amounts of the species n_i. One can think of the natural variables as those independent variables that make a difference in the description of a system or of a process. Thus, the fundamental equation for the Gibbs energy G for a reaction {e.g. reaction (1)} is given by

$$dG = -SdT + VdP + \Sigma\mu_i dn_i \,, \tag{4}$$

where μ_i and n_i are, respectively, the chemical potential and the amount (moles) of species i. From the fundamental equation one can obtain the relationship

$$\Delta_r G_m = \Delta_r G^\circ_m + RT \ln Q, \tag{5}$$

where Q is the reaction quotient. For reaction (1)

$$Q = m\{ADP^{3-}(aq)\}\cdot m\{HPO_4^{2-}(aq)\}\cdot m\{H^+(aq)\}/\{m\{ATP^{4-}(aq)\}\cdot(m^\circ)^2\}. \tag{6}$$

By convention, the activity of water has been omitted from eq. (6). Also, the quantity $m^\circ = 1$ mol·kg^{-1} has been used to make Q dimensionless and to avoid the impossibility of taking the logarithm of "mol·kg^{-1}". The absolute values of the stoichiometric numbers ν_i in reaction (1) are all equal to unity. If this is not the case, it is necessary to raise the molalities in eq. (6) to the powers of their respective stoichiometric numbers, which are positive for the products and negative for the reactants. At equilibrium, the Gibbs energy of the system is a minimum and $\Delta_r G = 0$. This leads to the important relationship

$$\Delta_r G^\circ_m = -RT \ln K, \tag{7}$$

where K is the equilibrium constant (i.e. the value of the reaction quotient at equilibrium). If one knows the equilibrium constant of a reaction and the initial molalities (calculations can also be performed using concentrations) of the species, one can calculate the extent of reaction and the molalities of the various species at equilibrium. Thus, the knowledge of the equilibrium constant for a given reaction provides useful information. The temperature dependence of the equilibrium constant can be calculated by using

$$R\ln K = -\{\Delta_r G^\circ_m (T_{ref})/T_{ref}\} + \Delta_r H^\circ_m (T_{ref})\cdot\{(1/T_{ref}) - (1/T)\} + \Delta_r C^\circ_{p,m}\{(T_{ref}/T) - 1 + \ln(T/T_{ref})\}. \tag{8}$$

Here, T_{ref} is a reference temperature (298.15 K is most commonly used); $\Delta_r G^\circ_m(T_{ref})$ and $\Delta_r H^\circ_m(T_{ref})$ are $\Delta_r G^\circ_m$ and $\Delta_r H^\circ_m$, respectively, evaluated at T_{ref}. The above equation is a variant on the van't Hoff equation

$$\Delta_r H^\circ_m = RT^2(\partial \ln K/\partial T)_P \,. \tag{9}$$

In many cases one does not know $\Delta_r C^\circ_{p,m}$. However, neglecting this quantity generally causes only a small error unless T is far removed from T_{ref}. The variation of $\Delta_r G^\circ_m$ with pressure is given by

$$(\partial \Delta_r G^\circ_m / \partial P)_T = \Delta_r V^\circ_m \tag{10}$$

where $\Delta_r V^\circ_m$ is the molar volume change for the reaction. For the vast majority of chemical reactions carried out in aqueous solutions, one finds only a negligible effect on $\Delta_r G^\circ_m$ (and on K) until very high pressures are reached.

It is important to recognize that, for chemical reactions, K does not depend on pH. In fact, $m\{H^+(aq)\} = 1$ under standard state conditions and, in this case, one can consider the pH to be zero. In reaction (1) it is also seen that there is one hydrogen atom produced as a consequence of the reaction. Thus, the change in binding of hydrogen atoms $\Delta_r N(H^+) = -1$ for this reaction.

The above treatment was for a single chemical reaction. If one has several chemical reactions that are occurring, for example in a reactor, one can write equations for these various reactions. If one knows the values of the equilibrium constants for these several reactions, the initial molalities, and the conservation equations, one can again solve the pertinent set of equilibrium equations to obtain the molalities of the various species at equilibrium. For more than a few reactions, this can turn into a very difficult calculation if done by hand. However, numerical methods have been developed (Smith and Missen 1982; Krambeck 1991) that allow for easy solution of these equations with a computer.

The above discussion on equilibrium constants assumed ideal behaviour of the species in solution, i.e. that the activity coefficients γ_i were equal to unity. Since $\gamma_i \rightarrow 1$ only in the limit of infinite dilution, one must make adjustments for the non-ideal behaviour of the solute species when dealing with real solutions. This can be done rigorously if one has information on the activity coefficients of the species in the actual solution (Pitzer 1991). In general, and particularly for biochemical species, this is not always possible. However, a reasonable approach is to use the extended Debye-Hückel equation

$$\ln \gamma_i = -A_m z_i^2 I^{1/2}/(1 + BI^{1/2}), \tag{11}$$

where A_m is the Debye-Hückel constant ($A_m = 1.1758$ kg$^{1/2}$·mol$^{-1/2}$ at 298.15 K), z_i is the signed charge of species i, I is the ionic strength $\{I = (1/2)\Sigma m_i z_i^2\}$, and B is an "ion-size" parameter which has often been taken equal to 1.6 kg$^{1/2}$·mol$^{-1/2}$ (Alberty et al. 1994). Use of tabulated (Clarke & Glew 1980) values of A_m permits calculation of values of γ_i as a function of temperature and ionic strength. This, in turn, permits the adjustment of equilibrium constants at $I = 0$ to higher values of I. How this can be done is shown for the generalized reaction

$$\Sigma \nu_i R_i = 0 \tag{12}$$

where R_i is reactant i (species i in the case of a chemical reaction involving specific species) and ν_i is its stoichiometric number. Note that reactants on the left-hand side of reaction (12) have negative values of ν_i. The equilibrium constant K for this reaction is

$$K(I = 0) = \Pi\{a(R_i)\}^{\nu i} = \Pi\{m(R_i)^{\nu i}\}\cdot(\Pi\gamma_i^{\nu i}), \tag{13}$$

where a_i is the activity of R_i. Since K in eq. (13) was written in terms of activities, it is valid at $I = 0$ and has been designated as such. Eq. (13) can be rewritten

$$K(I = 0) = K(I)\cdot\Gamma, \tag{14}$$

where $\Gamma = \Pi\gamma_i\nu_i$. Thus, if one knows the value of the equilibrium constant at $I = 0$, one can use the activity coefficient product Γ to calculate $K(I)$, the value of K at ionic strength I; the inverse calculation of $K(I = 0)$ from $K(I)$ is also possible. Thus, an equilibrium constant for a chemical reaction is, in general, a function of the temperature, the pressure, and the ionic strength. All three of these quantities, the reaction to which the equilibrium constant pertains, the definition of the equilibrium constant, and the standard state should also be given to avoid any possible confusion about the equilibrium constant.

The above discussion shows how a knowledge of $K(I = 0)$ and $\Delta_r H_m^\circ$ at a reference temperature (e.g. $T = 298.15$ K) (and perhaps a value of $\Delta_r C_{P,m}^\circ$) together with a reasonable activity coefficient model can be used to calculate K at the desired ionic strength and temperature. This calculated value of K, together with a knowledge of the initial molalities of the species in a reaction, permits the calculation of the extent of reaction and the molalities of the various species at equilibrium. This is the essential information needed for predicting the feasibility of a reaction and for process optimization. Since reasonable temperature control is needed for the proper operation of a reaction and a catalyst, a knowledge of $\Delta_r H_m^\circ$ also plays a role in process design. Thus, if $|\Delta_r H_m^\circ|$ is large, the heat liberated or absorbed can cause major temperature changes in the reactor. This can be remedied by the use of heating for an endothermic reaction or cooling for an exothermic reaction.

It is possible to develop large collections and databases of equilibrium constants for chemical reactions. However, an alternative and more general approach is to tabulate values of standard molar Gibbs energies of formation $\Delta_f G_m^\circ$ and standard molar enthalpies of formation $\Delta_f H_m^\circ$. Thus, for the generalized reaction (12), one can calculate values of $\Delta_r G_m^\circ$ and $\Delta_r H_m^\circ$, respectively, by using the relationships

$$\Delta_r G_m^\circ = \Sigma\nu_i \Delta_f G_m^\circ \text{ (i)}, \tag{15}$$

$$\Delta_r H_m^\circ = \Sigma\nu_i \Delta_f H_m^\circ \text{ (i)}, \tag{16}$$

where $\Delta_f G_m^\circ$(i) and $\Delta_f H_m^\circ$(i) are, respectively, the standard molar Gibbs energies of formation and standard molar enthalpies of formation of species i. For inorganic substances and for organic substances containing no more than two carbon atoms, extensive tables of $\Delta_f G_m^\circ$ and $\Delta_f H_m^\circ$ have been published by the NBS (Wagman et al. 1982). These tables also contain values of the standard molar entropies S_m° and standard molar heat capacities $C_{P,m}^\circ$ as well as an excellent discussion on the construction of these thermochemical tables and on the use of these quantities in performing thermodynamic calculations.

2.2. Thermodynamics of reactions involving sums of chemical species – Biochemical reactions

Reaction (1) involves specific chemical species. However, it is well known that the biochemical reactant ATP is a mixture of different species. Thus, the total molality of the various ATP species is given by

$$m(\text{ATP})_{\text{total}} = m(\text{ATP}^{4-}) + m(\text{HATP}^{3-}) + m(\text{H}_2\text{ATP}^{2-}) + m(\text{H}_3\text{ATP}^{-}) + m(\text{MgATP}^{2-})$$
$$+ m(\text{MgHATP}^{-}) + m(\text{MgH}_2\text{ATP}) + m(\text{Mg}_2\text{ATP}). \tag{17}$$

If other metal ions (e.g. Ca^{2+}) are present, eq. (17) would have to include additional terms for the corresponding Ca^{2+}– ATP species. Similar considerations apply when one considers reactants such as

ADP and phosphate. Thus, the hydrolysis of ATP to ADP and phosphate is generally written as

$$\text{ATP} + \text{H}_2\text{O} = \text{ADP} + \text{phosphate}, \tag{18}$$

where it is understood that each biochemical reactant is a mixture of species. A group such as ATP, ADP, or phosphate that differ only in the number of H^+ or Mg^{2+} or Ca^{2+} atoms bound to a central ionic species is called a pseudoisomer group (Alberty 2003) when the pH and pMg are constrained. The apparent equilibrium constant K' for reaction (16) is

$$K' = m(\text{ADP}) \cdot m(\text{phosphate}) / \{m(\text{ATP}) \cdot m^\circ\}. \tag{19}$$

In eq. (19) it is understood that one is dealing with total molalities or sums of species. Also, the designation "(aq)" has not been used in the above equations since it is implicit that we are dealing with reactants in an aqueous solution. By convention, the molality of water has also been omitted in the expression for K'.

Reactions such as (18) will be referred to as (overall) biochemical reactions as distinct from chemical reactions that involve specific chemical species. It is an observed fact that, for such reactions, K' depends not only on temperature and ionic strength, but also on pH and pMg. Here, pMg is defined analogously to pH as $-\log_{10}\{m(Mg^{2+})/m^\circ\}$. Biochemical reactions do not balance hydrogens if the pH is constrained or magnesium if pMg is constrained. Therefore, a biochemical reaction does not balance charge (Alberty et al. 1994). Clearly, the other atoms in the reaction must balance. It is important to distinguish clearly between these two different kinds of reactions and equilibrium constants. Unfortunately, this is not always done in the literature and one sometimes sees hybrid reactions such as

$$\text{ATP} + \text{H}_2\text{O} \; f \quad \text{ADP} + \text{phosphate} + \text{H}^+ . \tag{20}$$

The hybrid reaction (20) is neither a chemical nor a biochemical reaction. It is incorrect. Additionally, the above reaction shows one hydrogen atom being released as a consequence of the reaction. This will occur only for pH > 8. In general the number of hydrogen atoms released or absorbed in a biochemical reaction is not an integer.

Thermodynamic formalisms and methods (Alberty 1968; Alberty 1969; Goldberg & Tewari 1991; Alberty 1991) have been developed to treat (overall) biochemical reactions in terms of the individual chemical reactions that make up the system of reactions. An essential feature of these methods is that a system of non-linear equilibrium equations must be solved. This can be done relatively easily by hand if only a few equations are involved. However, this is impractical for more complex systems and several approaches have been developed. Presently, the most general approach is to use numerical codes, which perform a minimization of the Gibbs energy of the system, by using the Newton-Raphson method (Smith & Missen 1982, Krambeck 1991). The data needed in these codes are the equilibrium constants of the pertinent individual chemical reactions that make up the overall biochemical reaction and the initial molalities of the species. If one wishes to perform calculations at temperatures other than the reference temperature T_{ref} one also needs values of $\Delta_r H^\circ_m$ (and possibly $\Delta_r C^\circ_{P,m}$) for all of the pertinent reactions. The array of data needed for calculations on the thermodynamics of ATP hydrolysis, reaction (18), is given in Table 1. The results of some calculations performed by using this data and codes written in Mathematica (Akers & Goldberg 2001) are shown in Figure 1. This package of codes written in Mathematica (Wolfram 1996) allows the user to specify

Table 1. Thermodynamic quantities for the chemical reactions pertinent to the overall hydrolysis reaction (ATP + H_2O *f* ADP + phosphate) in aqueous solutions [Alberty and Goldberg 1992]. The equilibrium constant *K* and the standard molar enthalpy of reactions $\Delta_r H^\circ_m$ pertain to *T* = 298.15 K and *I* = 0. The standard state is the hypothetical ideal solution of unit molality

Reaction	*K*	$\Delta_r H^\circ_m$(kJ·mol^{-1})
$ATP^{4-} + H_2O$ *f* $ADP^{3-} + HPO_4^{2-} + H^+$	0.295	-20.5
H_2ATP^{2-} *f* $H^+ + HATP^{3-}$	$2.09 \cdot 10^{-5}$	15
$HATP^{3-}$ *f* $H^+ + ATP^{4-}$	$2.51 \cdot 10^{-8}$	-6.3
$MgHATP^-$ *f* $Mg^{2+} + HATP^{3-}$	$2.34 \cdot 10^{-4}$	-16.9
$MgATP^{2-}$ *f* $Mg^{2+} + ATP^{4-}$	$6.61 \cdot 10^{-7}$	-22.9
Mg_2ATP *f* $Mg^{2+} + MgATP^{2-}$	$2.04 \cdot 10^{-3}$	-10.8
H_2ADP^- *f* $H^+ + HADP^{2-}$	$4.37 \cdot 10^{-5}$	17.6
$HADP^{2-}$ *f* $H^+ + ADP^{3-}$	$6.61 \cdot 10^{-8}$	-5.6
$MgHADP$ *f* $Mg^{2+} + HADP^{2-}$	$3.16 \cdot 10^{-3}$	-12.5
$MgADP^-$ *f* $Mg^{2+} + ADP^{3-}$	$2.24 \cdot 10^{-5}$	-19
$H_2PO_4^-$ *f* $H^+ + HPO_4^{2-}$	$6.03 \cdot 10^{-8}$	3.6
$MgHPO_4$ *f* $Mg^{2+} + HPO_4^{2-}$	$1.95 \cdot 10^{-3}$	-12.2

conveniently, using a chemical notation, an overall biochemical reaction in terms of the system of chemical reactions from which it is formed as well as the necessary thermodynamic properties (*K*, p*K*, $\Delta_r H^\circ_m$, and $\Delta_r C^\circ_{P,m}$) for the individual chemical reactions. The computer then solves the chemical equilibrium equations to yield the molalities of the species and also calculates *K'* as a function of *T*, pH, pMg, and *I*. Plots of *K'* as a function of these quantities can be obtained in two and three dimensions. Additional capabilities include the calculation of $\Delta_r N(X)$ (X = H^+, Mg^{2+}, etc.) and $\Delta_r H'^\circ_m$, the standard molar transformed enthalpy accompanying a biochemical reaction at constant *T*, *P*, pH, pMg, and *I* (see below), and the treatment of experimentally determined values of *K'* and calorimetrically determined enthalpies of reaction. Systems of biochemical reactions can also be treated with these programs to obtain equilibrium compositions. Specifically, the same programs are used, but with different input.

It is important to appreciate that while thermodynamics predicts that reaction (18) will proceed essentially to completion, the actual reaction may take a very long time to reach equilibrium in the absence of an effective catalyst – i.e. an enzyme such as an ATPase. It must also be recognized that the catalyst operates by lowering the activation energy and thereby increasing the rate of reaction. The important point is that the position of equilibrium is unchanged by the catalyst. This is illustrated in Figure 2.

The formal basis of the apparent equilibrium constant has been shown by Alberty (Alberty 1992) to rest on the Legendre transform

$$G' = G - n_c(H)\mu(H^+) - n_c(Mg)\mu(Mg^{2+}), \tag{20}$$

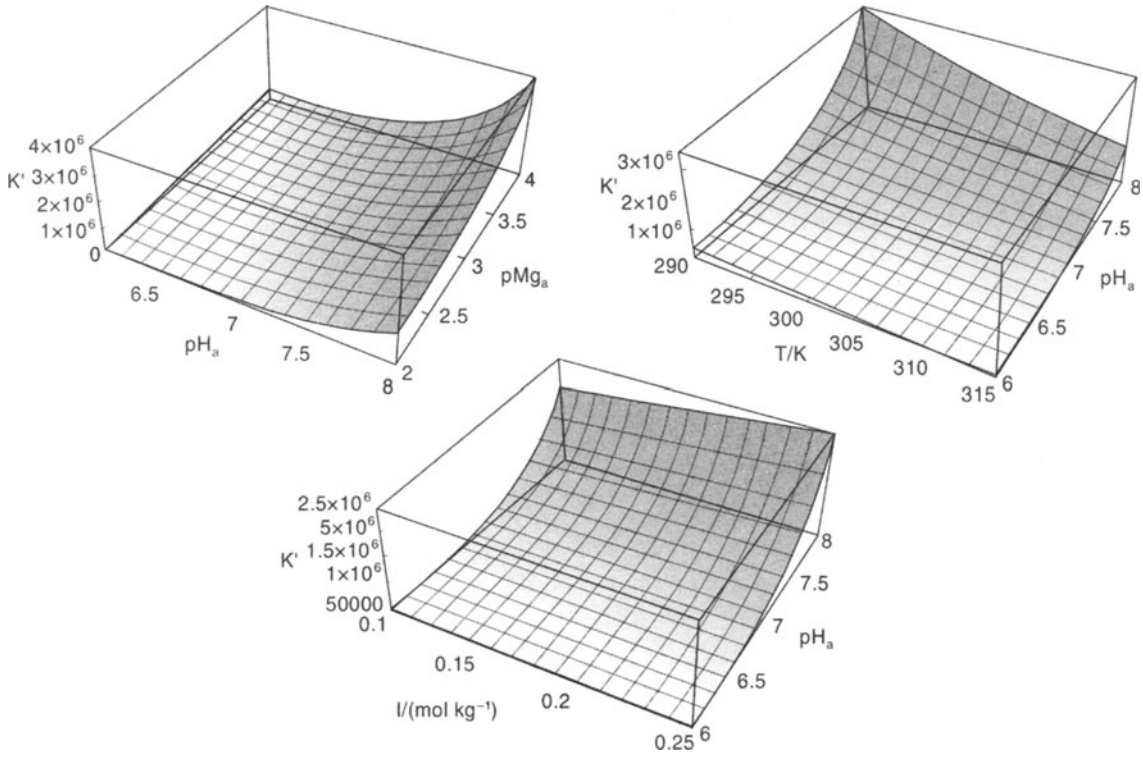

Fig. 1. The apparent equilibrium constant *K'* for the hydrolysis reaction (ATP + H_2O = ADP + phosphate) as a function of temperature *T*, pH, pMg, and ionic strength *I*. Since it is not possible to represent a five dimensional surface in two dimensions, we have shown three 3-D projections where appropriate constraints have been applied in calculating each surface. These constraints are: *T* = 298.15 K and *I* = 0.25 M (top figure); *I* = 0.25 M and pMg = 3.0 (middle figure); and *T* = 298.15 K and pMg = 3.0 (bottom figure).

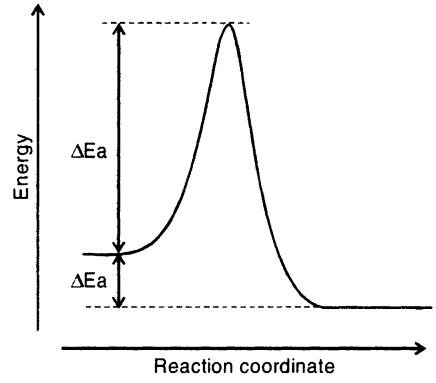

Fig. 2. The energy *E* of a reacting system as a function of the reaction coordinate. ΔE is the change in energy for the reaction and ΔE_a is the activation energy. The role of a catalyst is to lower ΔE_a and thus accelerate the rate of reaction.

where n_c(H) and n_c(Mg) are, respectively, the amounts of the components hydrogen and magnesium in the system; $\mu(H^+)$ and $\mu(Mg^{2+})$ are the chemical potentials of H^+(aq) and Mg^{2+}(aq) under the conditions of the system. If other species (e.g. Ca^{2+} or, in general, species X) are pertinent to the description of system, eq. (2) can be extended by the inclusion of additional terms of the form $n(X)\mu(X)$. A Legendre transform is a mathematical device (Alberty 1994; Alberty 2003) that can be used to define functions that have the desired set of natural independent variables and that are appropriate for treating the system of interest. For example, the Gibbs energy G is the thermodynamic function that is appropriate for treating a chemical reaction at constant T and P. It is defined by using the Legendre transform

$$G = H - TS. \tag{21}$$

This leads to the fundamental relationship {eq. (4)} where T, P, and n_i are the natural variables. As mentioned above, the Gibbs energy G provides the criterion for chemical equilibrium for a reaction involving chemical species at constant T, P, and I. However, the Legendre transform {eq. (20)} that defines the transformed Gibbs energy G' is appropriate for a biochemical reaction occurring at constant T, P, pH, pMg, and I and thus provides the criterion of equilibrium for an overall biochemical reaction under these set of conditions. The use of this Legendre transform leads to several useful relations:

$$\Delta_r G'_m = \Delta_r G'^\circ_m + RT \ln Q', \tag{22}$$

$$\Delta_r G'^\circ_m = -RT\ln K' = \Delta_r H'^\circ_m - T\Delta_r S'^\circ_m, \tag{23}$$

$$\Delta_r H'^\circ_m = RT^2(\partial \ln K'/\partial T)_{P,pH,pMg,I}, \tag{24}$$

$$\Delta_r N(H^+) = -(\partial \log_{10} K'/\partial pH)_{T,P,pMg,I}, \tag{25}$$

$$\Delta_r N(Mg^{2+}) = -(\partial \log_{10} K'/\partial pMg)_{T,P,pH,I}. \tag{26}$$

Here $\Delta_r G'^\circ_m$, $\Delta_r H'^\circ_m$, and $\Delta_r S'^\circ_m$ are, respectively, the changes in the standard molar transformed Gibbs energy, enthalpy, and entropy accompanying a biochemical reaction at constant T, P, pH, pMg, and I. Q' is the apparent reaction quotient which is defined analogously to the reaction quotient for a chemical reaction {see eq. (6)}, i.e. total molalities are used to define Q'. Eqs. (22), (23), and (24) are similar in form to the equations {see, respectively, eqs. (5), (7) and (9) above} for reactions involving specific chemical species. However, eqs. (25) and (26) yield new information, namely, the changes in binding of H^+ and Mg^{2+} that accompany biochemical reactions. Since enzymes often operate optimally over a narrow range of pH and pMg, this information is also useful for the design and operation of bioreactors. Also, at constant T and P, $\Delta_r G'$ is the upper limit to the non-PV work that can appear in the surroundings. It is critical to appreciate that the apparent and transformed quantities K', $\Delta_r G'_m$, $\Delta_r G'^\circ_m$, $\Delta_r H'^\circ_m$, $\Delta_r S'^\circ_m$, Q', and $\Delta_r N(X)$ are functions of temperature, pX, and ionic strength. These dependencies can be both complex and dramatic (Alberty 1968; Alberty 1969; Rose 1968; Tewari & Goldberg 1988; Tewari et al. 1991). In some cases, the direction of reaction can be completely shifted by changes in the conditions of reaction.

Another useful approach for handling these calculations is based on the use of standard molar transformed Gibbs energies $\Delta_f G'^\circ_m$ and enthalpies $\Delta_f H'^\circ_m$ of formation of biochemical reactants. Values of these

properties can be calculated from the standard molar formation properties of chemical species by using the principles of isomer group thermodynamics (Alberty 2003). The working formulas are:

$$\Delta_f G'^\circ_m(\text{reactant}) = -RT\ln\Sigma\exp\{-\Delta_f G'^\circ_m(\text{species i})/(RT)\}, \quad (27)$$

$$\Delta_f H'^\circ_m(\text{reactant}) = \Sigma r_i \cdot \Delta_f H'^\circ_m(\text{species i}), \quad (28)$$

$$r_i = \exp\{[\Delta_f G'^\circ_m(\text{reactant}) - \Delta_f G'^\circ_m(\text{species i})]/(RT)\}, \quad (29)$$

$$\Delta_r G'^\circ_m = -RT\ln K' = \Sigma \nu'_i \Delta_f G'^\circ_m(\text{i}). \quad (30)$$

Here, r_i is the equilibrium mole fraction of species i in the pseudoisomer group and the ν'_i are the apparent stoichiometric numbers of the reactants in the overall biochemical reaction. In eq. (29) , species i is one of the species in the isomer group that defines the biochemical reactant. A detailed example of this type of calculation has been given for the ATP hydrolysis reaction {reaction (18)} and for several related reactions involving the ATP series (Alberty & Goldberg 1992). As expected, the values of K' obtained by using this approach and the approach based on solving the chemical equilibrium equations are identical. An important point is that standard molar Gibbs free energies and enthalpies of formation provide an extremely convenient way to store the essential thermodynamic data for species. A substantial body of such data has been assembled by Alberty (Alberty 2001) in the form of a Mathematica package, which allows the user to calculate values of $\Delta_f G'^\circ_m$ and of $\Delta_f H'^\circ_m$ for, respectively, 131 and for 69 biochemical reactants. The package also allows the user to perform several additional and useful calculations: $\Delta_r G'^\circ_m$, K', and $\Delta_r N(H^+)$ as a function of pH, T, and I; $\Delta_r H'^\circ_m$ as a function of pH and I; and the calculation of equilibrium compositions of a single biochemical reaction or a system of biochemical reactions at specified T, pH, and I.

It is important to recognize that the thermodynamic formalisms described above can be applied to systems of essentially unlimited complexity and that they are not limited to enzyme-catalyzed reactions. However, to use these formalisms for practical calculations one needs either equilibrium constants for the reactions of interest or standard molar Gibbs free energies of formation of the pertinent species. Thus, reliable results (data) are required for performing practical calculations. A substantial body of experimental results for enzyme-catalyzed reactions has accumulated over many years and has been systematized in review articles and on the web (Goldberg 1999). Essentially all of the equilibrium data has been obtained by using a variety of analytical methods with chromatography and enzymatic assays (often coupled with spectrophotometry) being the most commonly used methods. Calorimetry has proven to be an important tool for the determination of molar enthalpies of reaction. These methods are reviewed below (Section 5).

3. INDUSTRIAL APPLICATIONS

The fundamental principles of chemical thermodynamics needed for the practical treatment of both chemical and biochemical reactions have been reviewed above. The information that thermodynamics can provide for industrial applications involving enzyme-catalyzed reactions is:

- The ability to calculate the position of equilibrium. This determines the feasibility, the product yield(s), and can be used to determine the optimal operating conditions for a process.

- The heat released or absorbed by the reaction. This information can be used to optimize energy efficiency.
- The change(s) in binding associated with a biochemical reaction. This information is important for the maintenance of pH and/or pMg.

Having this information in hand is invaluable not only for the design of a bioreactor but also for the industrial decision making process that should be used prior to the expenditure of large amounts of capital on a given project. As an example, it makes little sense to engineer either a process or a catalyst (i.e. an enzyme) if the desired product yield cannot be achieved. If these and the other necessary engineering calculations are not performed early on, substantial resources may be wasted.

Fortunately, the thermodynamics of several of the major industrially prominent enzyme-catalyzed reactions have been studied (Tewari 1990). These reactions include the conversions of penicillin G to 6-amino-penicillanic acid using the enzyme penicillin acylase; starch to glucose using amylases; glucose to fructose using glucose (xylose) isomerase (Tewari & Goldberg 1984; Tewari & Goldberg 1985); cellulose to glucose using cellulase; fumaric acid and ammonia to L-aspartic acid using L-aspartase; transcinnamic acid and ammonia to L-phenylalanine using L-phenylalanine ammonia-lyase; L-histidine to urocanic acid and ammonia using L-histidine ammonia-lyase; lactose to glucose and galactose using lactase; and the reactions catalyzed by amino-acylases and proteases. Additionally, there are extensive tabulations of reaction property values available for enzyme-catalyzed reactions in published reviews and on the web (Goldberg 1999). Considering the enormous potential of enzymology for carrying out useful reactions, it is clear that there will be many useful applications that will be forthcoming in future years for which additional thermodynamic investigations will prove useful.

Enzyme catalysis also works in non-aqueous media such as organic solvents. This has a particular utility in carrying out reactions where the reactants have a very low solubility in water. It should be noted that there is almost always a small amount of water present in any reacting system and the actual catalysis may indeed be occurring in the very small aqueous phase present in the system. Nevertheless, the reactants and products are partitioned between the aqueous and non-aqueous phases and, if one has taken care to demonstrate that equilibrium has been achieved, one still has a valid determination of the equilibrium constant pertinent to the reaction in the non-aqueous phase. In the case of ester hydrolysis reactions, the position of equilibrium can be shifted significantly in the favour of the ester if the reaction is carried out in a non-aqueous solvent. The information in the literature on hydrolysis reactions in non-aqueous solvents is suggestive of the rule that the values of the equilibrium constants for hydrolysis reactions in different solvents are comparable if the reaction refers to neutral species and the concentration of water is included in the formulation of the equilibrium constant (Tewari et al. 1995). There is a very recent thermodynamic investigation (Tewari et al. 2003) of the lipase-catalyzed transesterification of benzyl alcohol and butyl acetate, which was carried out using supercritical carbon dioxide. This solvent has the very nice feature that it is easily removed from the final reaction mixture simply by releasing the system to the atmosphere. Thus, it is now possible to design the synthesis of a compound via enzyme catalysis in supercritical carbon dioxide followed by the supercritical fluid extraction for the separation of the reaction products. This completely avoids any problems associated with the presence of an organic solvent. In the near future, biocatalysis in supercritical carbon dioxide could become the preferable route for synthesis and separation of drug intermediates.

4. PHYSIOLOGICAL APPLICATIONS

Enzyme-catalyzed reactions are an essential and important part of the chemical machinery whereby cells and living systems are able to carry out the necessary transformations of metabolites to accomplish useful work and to construct the building blocks of life. Since thermodynamics provides the foundation for any quantitative discussion of this chemical machinery, equilibrium and calorimetric data have been widely used in metabolic calculations (Minakami & Yoshikawa 1966; Thauer et al. 1977; Veech et al. 1979; Battley 1987; Alberty 1992; Kashiwaya et al. 1994). In this regard, it is particularly helpful to have measurements of concentrations of the biochemical reactants (metabolites) under *in vivo* conditions. With this information, one can obtain the value of the apparent reaction quotient Q' for a given reaction. This value can then be compared with the value of the apparent equilibrium constant K' determined from *in vitro* measurements performed at or near the presumed physiological conditions. Since the ratio K'/Q' is equal to $\exp(-\Delta_r G'_m/RT)$ {see eq. (22)}, both K'/Q' and $\Delta_r G'_m$ are quantitative measures of the departure of a given reaction from equilibrium. As mentioned above, $\Delta_r G'_m$ is also the upper limit to the chemical energy available from a given reaction or series of reactions. Clearly, kinetic considerations are also of importance in understanding the operation of a given metabolic pathway. An illustration of this comes from a recent study (Kashiwaya 1994) in which both thermodynamic and kinetic data were used with metabolic control analysis (Fell 1997) to quantitatively assess the effects of different physiological states on the metabolic flux in glycolysis and glycogen metabolism in working perfused rat hearts. In a related study (Masuda et al. 1990), the gradients of the major inorganic ions across the plasma membrane of a perfused rat heart were also determined. The Gibbs energy changes involved in the formation of these gradients are generally established by enzyme-catalyzed reactions. Thus, these Gibbs energy changes can be evaluated by the usual thermodynamic methods.

5. EXPERIMENTAL METHODS

Equilibrium measurements on enzyme-catalyzed reactions have been performed using a wide variety of techniques. Historically, the principal tool has been spectrophotometry, which has often been carried out in conjunction with enzymatic assay(s). Here, the well-known spectral absorbance of reduced β-nicotinamide-adenine dinucleotide (NADH) at the wavelength $\lambda = 340$ nm has proven to be a very useful tool. However, the advent of modern chromatographic methods offers a real advantage in that one can frequently carry out both the separation of the reactants as well as the desired analyses for the amounts of the substances present in solution. Both high-performance liquid chromatography (HPLC) and gas chromatography (GC) are well-established methods, although the GC method usually requires the additional step of the preparation of a derivative. Additionally, modern chromatography has a major advantage in that a wide variety of commercially available columns and developed methods are available. These established methods can also be modified by appropriate variations in the method of operation (e.g. mobile phase, flow-rate, gradient, etc.). Essentially any type of detector can be attached to a chromatograph. Thus, spectrophotometric, fluorescence, refractive index, electrical and thermal conductivity, pulsed-amperometric, electron capture, flame ionization, and radioactivity detectors are currently in wide use. The chromatograph can also be coupled with a mass spectrometer. This gives additional analytical capability as well as a more positive identification of the substance(s) that are analyzed. Other methods such as NMR, capillary-zone electrophoresis, and chemical analysis have been, and will continue to be, useful tools for specific applications.

A problem common to all equilibrium measurements is the establishment that the reaction(s) of interest is at equilibrium. This can be a very serious problem for enzyme-catalyzed reactions because of possible loss of enzymatic activity due to product inhibition and denaturation. Thus, it is important to demonstrate that equilibrium has been established by showing agreement between results obtained by approaching the position of equilibrium from different directions. Also, unless the method is capable of distinguishing between pseudoisomer species, it is the apparent equilibrium constant K' that is determined experimentally.

Calorimetry provides a direct measurement of the enthalpy change accompanying a biochemical reaction. Microcalorimetry (Wadsö 1970; Steckler et al. 1986) also allow one to work with relatively small amounts of substances - a frequent concern in biochemistry. While the measurement of the enthalpy change can be a straight-forward matter, one must also deal with the chemical part of the thermochemical investigation. Specifically, "it is necessary to establish that the actual reaction or process that occurs is the specified one, and second, to measure with the necessary accuracy the extent of the given reaction or process that occurs in each experiment for which the heat energy has been evaluated" (Rossini 1956). Here, the aforementioned analytic methods can be used both to measure the extent of reaction and to check for possible side reactions. Additionally, in calorimetric investigations of biochemical reactions carried out in buffered solutions (the usual case), it is important to know $\Delta_r N(H^+)$ the change in binding of H^+. A correction for the enthalpy of protonation of the buffer must then be applied (Alberty & Goldberg 1993) to the calorimetrically determined molar enthalpy of reaction $\Delta_r H_m(\text{cal})$ in order to obtain $\Delta_r H'^\circ_m$. Clearly, additional corrections are needed if other ions such as Mg^{2+} or Ca^{2+} also interact with the buffer. $\Delta_r N(X)$ can be obtained from the dependence of K' on pX {see eqs. (25) and (26)}, by measuring $\Delta_r H_m(\text{cal})$ in two different buffers that have sufficiently different values of enthalpies of binding with the ligand X, or by using equilibrium modeling calculations.

Calorimetry often yields more accurate and precise values of $\Delta_r H^\circ_m$ and $\Delta_r H'^\circ_m$ than can be obtained from values of equilibrium constants determined at several temperatures {see eqs. (9) and (24)}. In any case, if one has agreement of values of $\Delta_r H^\circ_m$ and $\Delta_r H'^\circ_m$ that have been obtained by using these two different approaches, one has additional confidence in the correctness of the results.

6. THERMOCHEMICAL CYCLES AND TABLES

An important principle that greatly expands the power of chemical thermodynamics is the fact that the Gibbs energy G, the enthalpy H, and the entropy S are state functions. Thus, the values of these properties are independent of pathway. This permits one not only to compare property values obtained by two different methods or pathways, but also to calculate property values of substances and reactions that have not been measured directly. As an example, a thermochemical cycle was used to establish the consistency of the series of reactions shown in Table 2 (Larson et al. 1993). Reactions (31) to (34) form a thermochemical cycle which requires that

$$\Delta_r H^\circ_m(31) + \Delta_r H^\circ_m(32) - \Delta_r H^\circ_m(33) - \Delta_r H^\circ_m(34) = 0. \qquad (35)$$

When the values of $\Delta_r H^\circ_m$ given in Table 2 are introduced into the left-hand side of the above equation, the calculated value is (0.6 ± 1.0) $kJ \cdot mol^{-1}$ and is in agreement with the prediction that the sum equal zero. A second example involves the determination of the apparent equilibrium constant for reaction (18), the hydrolysis reaction of ATP to (ADP + phosphate). Since the hydrolysis of ATP is essentially complete under normal conditions, a direct equilibrium study does not appear to be possible with existing

methods. However, it was possible (Guynn & Veech 1973) to measure apparent equilibrium constants for the following three reactions

$$\text{acetate(aq)} + \text{ATP(aq)} = \text{acetyl phosphate(aq)} + \text{ADP(aq)}, \quad (36)$$

Table 2. Standard molar enthalpies of reaction $\Delta_r H_m^\circ$ for four chemical reactions in aqueous solution at $T = 298.15$ K and $I = 0$ (Larson et al. 1993). AMP and IMP are, respectively, abbreviations for adenosine 5'-monophosphate and inosine 5'-monophosphate. The standard state is the hypothetical ideal solution of unit molality

Reaction		$\Delta_r H_m^\circ$/(kJ·mol^{-1})
$AMP^{2-}(aq) + H_2O(l) + H^+(aq)$ *f* $H\text{·}IMP^{2-}(aq) + NH_4^+(aq)$	(31)	-(49.6 ± 0.5)
$H\text{·}IMP^{2-}(aq) + H_2O(l)$ *f* $H\text{·Inosine(aq)} + HPO_4^{2-}(aq)$	(32)	1.7 ± 0.2
$\text{Adenosine(aq)} + H_2O(l) + H^+(aq)$ *f* $H\text{·Inosine(aq)} + NH_4^+(aq)$	(33)	-(49.4 ± 0.7)
$AMP^{2-}(aq) + H_2O(l)$ *f* $\text{adenosine(aq)} + HPO_4^{2-}(aq)$	(34)	0.9 ± 0.4

$$\text{acetyl phosphate(aq)} + \text{CoA(aq)} \; f \; \text{acetyl-CoA(aq)} + \text{phosphate(aq)}, \quad (37)$$

$$\text{acetyl-CoA(aq)} + H_2O(l) \; f \; \text{CoA(aq)} + \text{acetate(aq)}. \quad (38)$$

The sum of reactions (36), (37), and (38) is reaction (18). Thus, by having (reliable) values of K' for the above three reactions under the same set of conditions (T, I, pH, and pMg), one can calculate the values of K' and $\Delta_r G_m'^\circ$ under these conditions. Alternatively, one can use the values of K' for the above three reactions to calculate values of K and $\Delta_r G_m^\circ$ for chemical reference reactions that correspond to the respective (overall) biochemical reactions (36), (37), and (38). These values can then be used to calculate values of K and $\Delta_r G_m^\circ$ for reaction (1), the hydrolysis reaction of $ATP^{4-}(aq)$.

The use of thermochemical cycles can be generalized in terms of thermodynamic networks that lead to values of the standard molar formation properties $\Delta_f G_m^\circ$, $\Delta_f H_m^\circ$, and of the standard molar entropies S_m° for the species. These networks also include and use values of standard molar enthalpies of formation of the condensed phases, standard molar enthalpies of solution and saturation molalities (solubilities), pKs and standard molar enthalpies of binding of ions such as $H^+(aq)$ and $Mg^{2+}(aq)$. One of the earliest tabulations of formation properties for biochemical substances appeared as an Appendix in the monograph of Krebs and Kornberg (Krebs & Kornberg 1957). More extensive tables were produced later by Wilhoit (Wilhoit 1969). More recent activities in this area include the tables on the metabolites in the Krebs cycle (Miller & Smith-Magowan 1990) and the tables on the monosaccharides and their monophosphates (Goldberg & Tewari 1989). All of the aforementioned tables contain values of standard molar Gibbs energies of formation $\Delta_f G_m^\circ$. Values of standard molar enthalpies of formation and standard partial molar entropies are also given in two of these tables (Wilhoit 1969; Goldberg & Tewari 1989). The computer package developed by Alberty (Alberty 2001) also contains values of $\Delta_r G_m^\circ$ and of $\Delta_r H_m^\circ$ – some of which are from previously published tables as well as some newly calculated values. In spite of these efforts, the calculation and tabulation of formation properties is rather limited and is significantly less than what could potentially exist given the full extent of the data in the literature (Goldberg 1999).

7. ESTIMATION METHODS AND QUANTUM CHEMICAL CALCULATIONS

In the absence of a needed property value, it may sometimes be possible to make progress by estimating a value for that property. This approach has proven to be most useful for the estimation of values of S_m°. The general principle in making such estimates is that of looking for property values for substances or reactions that have molecular similarities to the substance or reaction for which the property value is needed. This approach has been generalized in terms of the Benson method (Benson & Buss 1958; Benson et al. 1969) in which specific molecular group contributions are assigned and then summed to obtain the estimated property value. Extensive tables of parameters that use this method are available for organic compounds in the condensed and gaseous phases (Domalski & Hearing 1993). However, aqueous solutions remain the media of most general interest for enzyme-catalyzed reactions. While there has been some development (Cabani et al. 1981; Domalski 1998) of the Benson approach for organic solutes in water, the database is not nearly as extensive as that currently available for organic compounds in the condensed and gaseous phases. This is unfortunate, as there are simply too many reactions and substances for which one would like to have thermodynamic property values.

There have been very significant advances in quantum chemistry in the last several years. In one study (Kast et al. 1997), quantum chemistry was used to calculate values of $\Delta_r H_m^\circ$ and $\Delta_r S_m^\circ$ for the conversion of chorismate^{2-}(aq) to prephenate^{2-}(aq). The calculated value of $\Delta_r H_m^\circ$ for this reaction differed by only 9 kJ·mol^{-1} from the measured value $\Delta_r H_m^\circ = -(55.4 \pm 2.3)$ kJ·mol^{-1}. This agreement between the measured and the calculated value may have been due to a fortuitous cancellation in the calculations of the solvation effects. In any case, it was concluded that the quantum mechanical method used was capable of adequately describing the increased solvent-solute interaction of prephenate^{2-}(aq) relative to chorismate^{2-}(aq). Clearly, very significant progress in treating solvation effects is needed for quantum chemistry to be competitive with carefully done equilibrium and calorimetric measurements on enzyme-catalyzed reactions.

The foregoing discussion leads to a need to state the fact that the development of both quantum chemistry and the Benson and other estimation methods to biochemical thermodynamics are dependent on the availability of accurate thermodynamic data for both "key" and representative biochemical reactions. Specifically, the current Benson parameters (Domalski & Hearing 1993) are essentially based upon enthalpies of combustion and third law entropies for condensed or gaseous phases. However, to use these data in a thermochemical pathway that leads to the formation properties of the aqueous species, one must also have the molar enthalpy of solution, the saturation molality m_{sat}, the number of water molecules of hydration of the condensed phase, and a value of the activity coefficient of the solute at the molality $m = m_{sat}$. The difficulty of obtaining all of this data with sufficient accuracy suggests that thermodynamic data {K' and $\Delta_r H_m$(cal)} on enzyme-catalyzed reactions will most likely provide the bulk of the data for the thermodynamic pathways needed for the development of tables of thermodynamic data for organic and biochemical species in water. It is these tables of thermodynamic data that can most profitably be used for the development of estimation methods and for testing quantum chemical calculations. As pointed out above, these thermodynamic tables are useful not only for reproducing the experimental results upon which they are based and for checking the consistency of these results, but also for the calculation of thermodynamic quantities that have not been directly measured. Therefore, the more extensive these tables are, the more valuable they become.

8. CONCLUSIONS AND PERSPECTIVES

Thermodynamics provides a formalism that allows one to predict the position of equilibrium, extent of reaction, and energy yield for both chemical and biochemical reactions. Consequently, thermodynamics can be used to optimize product yields and the conditions of reaction for biochemical reactions. This chapter has provided a brief and general outline of the thermodynamic principles that underlie the calculations needed for these applications. Most importantly, the use of the thermodynamic formalisms requires the availability of accurate and reliable property values for the substances and reactions of interest. Fortunately, a substantial body of thermodynamic data has been collected and evaluated both in the form of reaction properties and, in some cases, formation properties. In the absence of a given piece of data, one can attempt to make an estimate for the value of the property by using property values for structurally similar substances. In any case, there is still a real need for many additional equilibrium and calorimetric measurements on key enzyme-catalyzed reactions. Also, the utility of the existing body of thermodynamic data can be substantially enhanced by taking advantage of the fact that the thermodynamic functions (properties) G, H, and S are state functions and independent of path. Thus, thermochemical cycle calculations can be used to establish tables of standard molar formation properties and standard molar entropies just as has been done for inorganic substances. Such tables would serve to very significantly enhance the utility of the existing body of information.

Acknowledgements

The authors thank Professor Robert A. Alberty (Massachusetts Institute of Technology) for helpful discussions.

Glossary

Symbol	*Name*	*Unit*
a	activity	dimensionless
A_m	Debye-Hückel constant	$kg^{1/2} \cdot mol^{-1/2}$
B	"Ion-size" parameter	$kg^{1/2} \cdot mol^{-1/2}$
C_P	heat capacity	$J \cdot K^{-1}$
$\Delta_r C^\circ_{P,m}$	change in the standard molar heat capacity accompanying a reaction at constant T, P, and I	$J \cdot K^{-1} \cdot mol^{-1}$
E	energy	$J \cdot mol^{-1}$
ΔE_a	activation energy	$J \cdot mol^{-1}$
G	Gibbs energy	kJ
G'	transformed Gibbs energy	kJ
$\Delta_r G^\circ_m$	change in the standard molar Gibbs energy accompanying a reaction at constant T, P, and I	$kJ \cdot mol^{-1}$
$\Delta_r G'^\circ_m$	change in the standard molar transformed Gibbs energy accompanying a biochemical reaction at constant T, P, pH, pMg, and I	$kJ \cdot mol^{-1}$
H	enthalpy	kJ
$\Delta_r H^\circ_m$	change in the standard molar enthalpy accompanying a reaction at constant T, P, and I	$kJ \cdot mol^{-1}$
$\Delta_r H'^\circ_m$	change in the standard molar transformed enthalpy accompanying a biochemical reaction at constant T, P, pH, pMg, and I	$kJ \cdot mol^{-1}$
$\Delta_r H_m$(cal)	calorimetrically determined molar enthalpy of reaction that includes the enthalpies of reaction of H^+ and Mg^{2+} (consumed or produced) with any buffer in the solution	$kJ \cdot mol^{-1}$
I	ionic strength	$mol \cdot dm^{-3}$
K	equilibrium constant	dimensionless
K'	apparent equilibrium constant	dimensionless
m	molality	$mol \cdot kg^{-1}$
m°	standard molality (m° = 1 $mol \cdot kg^{-1}$)	$mol \cdot kg^{-1}$
n	amount of substance	mol
$\Delta_r N$(X)	change in binding in X in a biochemical reaction	dimensionless
P	pressure	Pa
pH	$-\log_{10}\{m(H^+)/m^\circ\}$[a]	dimensionless
pK	$-\log_{10} K$	dimensionless
pMg	$-\log_{10}\{m(X)/m^\circ\}$[a]	dimensionless

Q	reaction quotient	dimensionless
Q'	apparent reaction quotient	dimensionless
r	mole fraction at equilibrium within a specified class of species	dimensionless
R	gas constant (8.314472 $J \cdot K^{-1} \cdot mol^{-1}$)	$J \cdot K^{-1} \cdot mol^{-1}$
S	entropy	$J \cdot K^{-1}$
$\Delta_r S_m^\circ$	change in the standard molar entropy accompanying a reaction at constant T, P, and I	$kJ \cdot mol^{-1}$
$\Delta_r S'^\circ_m$	change in the standard molar transformed entropy accompanying a biochemical reaction at constant T, P, pH, pMg, and I	$kJ \cdot mol^{-1}$
T	temperature	K
V	volume	dm^3
$\Delta_r V_m^\circ$	change in the standard molar volume accompanying a reaction at constant T, P, and I	$dm^3 \cdot mol^{-1}$
x	mole fraction	dimensionless
z	signed charge	dimensionless
γ	activity coefficient	dimensionless
Γ	product of activity coefficients	dimensionless
λ	wavelength	m
μ	chemical potential	dimensionless
ν	stoichiometric number of a species	dimensionless
ν'	apparent stoichiometric number of a reactant	dimensionless
(aq)	denotes an aqueous substance	----------
c(subscript)	denotes a component	----------
f(subscript)	denotes a formation reaction	----------
i	denotes a species	----------
(l)	denotes a liquid	----------
m(subscript)	denotes a molar quantity	----------
R_i	denotes reactant i	----------
r(subscript)	denotes a reaction	----------
$^\circ$	denotes a standard quantity	----------
Δ	denotes a change	----------

[a]A definition more closely related to the measurement of this quantity uses the activity of the species rather than its molality.

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Regeneration of Cofactors for Enzyme Biocatalysis

Ryan D. Woodyer, Tyler W. Johannes and Huimin Zhao

1. INTRODUCTION

Industrial biocatalytic processes continue to become increasingly common as interest in the area multiplies (Faber 2000, Koeller & Wong 2001, Schmid et al. 2001, Schoemaker et al. 2003). This interest is driven by recent advances in genomics and genetic engineering coupled with the need for more selective, safer, and cleaner reactions. Enzymes as catalysts meet many of the needs of industrial processes. However, most of the biocatalysts in current use are limited to cofactor-independent enzymes such as hydrolases, which perform relatively simple chemistry (Faber 2000). In contrast, cofactor-dependent enzymes, such as oxidoreductases and transferases, can perform more complex chemistry. Many of these reactions are synthetically useful. For example, nicotinamide adenine dinucleotide/(NAD)dependent oxidoreductases catalyze the asymmetric reduction of carbonyl groups to alcohols and amines (Stewart 2001, Li et al. 2002) and acetyl-Coenzyme A-dependent (AcCoA) synthetases catalyze asymmetric carbon-carbon bond formation (Patel et al. 1986, Ouyang et al. 1990, Jossek & Steinbuchel 1998, Satoh et al. 2003). However, because these enzymes use cofactors, they have seen little use in large-scale applications.

Cofactors such as NAD and AcCoA are low molecular weight compounds that are essential for many enzymatic reactions. Some cofactors such as adenosylcobalamin, pyridoxal phosphate, biotin, and flavins are tightly bound to the enzymes and are self-regenerating in most cases. However, other cofactors such as pyridine dinucleotides (NAD(P)(H)) and nucleoside triphosphates (NTPs) act more like cosubstrates. They are loosely bound (K_D ~ μM-mM) and act as functional group transfer agents, and therefore are consumed in stoichiometric amounts (Chenault et al. 1988). The limited use of enzyme-cofactor reactions in industry stems from the high cost of cofactors, which ranges from $30/mmol for NAD, to several orders of magnitude more expensive for cofactors such as AcCoA and 3-phosphoadenosine-5'-phosphosulfate (PAPS). This high cost prohibits stoichiometric addition of cofactors, so they must be regenerated *in situ* for any large-scale reaction. Cofactor regeneration can also drive the reaction to completion, simplify product isolation, and allow the removal of inhibitory cofactor by-products, further reducing the cost of synthesis (Chenault et al. 1988). Various methodologies of cofactor regeneration have been developed to allow the use of catalytic amounts of cofactors (Chenault & Whitesides 1987, Chenault et al. 1988, Wong & Whitesides 1994, Koeller & Wong 2001, van der Donk & Zhao 2003, Zhao & van der Donk 2003) and many are described in this chapter.

In order for cofactor regeneration to be economical and practical, several requirements must be met. First and foremost, the total turnover number (TTN) or the total number of moles of product formed per mole of cofactor during the course of a complete reaction must be high (Chenault & Whitesides

1987, Chenault et al. 1988). TTNs of 10^3 to 10^5 are often large enough to make a regenerative process economically viable. However, this depends greatly on the cost of the cofactor in comparison with the value of the product being produced. If the value of the product is high, this number may be lower. Conversely, if the cofactor is more costly, this number may need to be higher. The TTN of regeneration reflects several important factors such as concentration and degradation of the cofactor over time, regioselectivity of regeneration, rate of catalysis, and time of reaction. Additionally, the availability of regenerative enzymes or reagents and their stability under process conditions is an important concern. The equipment must also be readily available, inexpensive, and easy to operate. The regeneration step should be favorable both kinetically and thermodynamically. Reagents or by-products of the reaction should not interfere with product isolation or the other components of the system (i.e. regeneration cosubstrate or product should not interfere with synthetic enzymes) unless they can be easily removed (e.g. by ultrafiltration in a continuous flow reaction).

In order to meet all of the criteria listed above, enzymatic reactions are usually the choice for cofactor regeneration. The primary factor is selectivity, which must be 99.99993% correct in order to reach a TTN of 10^6 (Chenault & Whitesides 1987, Chenault et al. 1988). There are very few examples where a catalyst other than an enzyme is this efficient. Chemical and electrochemical strategies often lack this high selectivity and are frequently incompatible with the other components of enzymatic reactions. However, in some cases chemical regeneration is as efficient as or better than the best enzymatic process and some examples are presented below as in the regeneration of AcCoA. As listed in Table 1, methods of regeneration have been described for oxidoreductions with NAD(P)(H), phosphoryl transfer reactions with nucleoside di- and triphosphates (NDP, NTP), glycosylations with sugar nucleotides, sulfuryl transfer reactions with PAPS, and acyl transfer reactions with AcCoA. Many of these have been successfully implemented in large-scale synthesis. Herein we discuss what we believe to be the current best, most widely used, and most interesting regeneration systems for these cofactors. Not discussed are methods that are of little utility or cofactors such as S-adenosyl methionine (SAM), which cannot currently be regenerated *in situ*.

2. NAD(P)(H) REGENERATION

The nicotinamide cofactors NAD(P)(H) play a pivotal role in numerous biochemical oxidation and reduction reactions. Generally, NAD(H) functions in dehydrogenase (DH)-catalyzed reactions involved in biodegradation reactions whereas NADP(H) functions in DH-catalyzed reactions involved in biosynthesis. Both cofactors are involved in a broad range of synthetically useful redox reactions.

Several enzymatic methods have been developed for the regeneration of NADH, such as reduction with formate/formate dehydrogenase (FDH), glucose/glucose dehydrogenase (GDH), glucose/glucose-6-phosphate dehydrogenase (G6PDH), and isopropanol/*Pseudomonas* alcohol dehydrogenase (ADH). The most widely used method for NADH regeneration uses formate dehydrogenase (FDH) isolated from *Candida boidinii* to convert formate into carbon dioxide (Wichmann et al. 1981). Degussa currently produces L-*tert*-leucine on an industrial scale using FDH to regenerate NADH (Bommarius et al. 1998). Continuous large-scale enzymatic regeneration systems typically use ultrafiltration (UF) membrane reactors in which the enzymes and cofactors are retained within the reactor. The cofactor NADH is usually covalently coupled to a large macromolecule such as polyethylene glycol (PEG) to increase its effective size such that it is larger than the molecular weight cut-off of the UF membrane. Various other reactor

Table 1. Common cofactors required for biotransformation and their representative *in situ* regeneration methods (Table adapted from Zhao & van der Donk 2003).

Cofactor	Reaction type	Representative regeneration method	Reference
NAD^+	Removal of hydrogen	Glutamate dehydrogenase with α-ketoglutarate	(Lee & Whitesides 1986)
NADH	Addition of hydrogen	Formate dehydrogenase with formate	(Wichmann et al. 1981)
$NADP^+$	Removal of hydrogen	Glutamate dehydrogenase with α-ketoglutarate	(Lee & Whitesides 1986)
NADPH	Addition of hydrogen	Glucose dehydrogenase with glucose	(Wong et al. 1985)
ATP	Phosphoryl transfer	Acetate kinase with acetyl phosphate	(Crans & Whitesides 1983)
Sugar nucleotides	Glycosyl transfer	Bacterial coupling	(Koizumi et al. 1998)
CoA	Acyl transfer	Phosphotransacetylase with acyl phosphate	(Billhardt et al. 1989)
PAPS	Sulfuryl transfer	Aryl sulfotransferase IV with *p*-nitrophenyl sulfate	(Burkart et al. 1999)
SAM	Methyl transfer	No demonstrated method	(Koeller & Wong 2001)
Flavins	Oxygenation	Self-regeneration*	(Faber 2000)
Pyridoxal phosphate	Transamination	Self-regeneration	(Faber 2000)
Biotin	Carboxylation	Self-regeneration	(Faber 2000)
Metal porphyrin complexes	Peroxidation, oxygenation	Self-regeneration	(Faber 2000)

*: Many flavin-dependent mono- or di-oxygenases require additional NAD(P)H as an indirect reducing agent.

configurations have been reviewed elsewhere (Devaux-Basseguy et al. 1997, Leonida 2001). Advantages of the FDH regeneration system include the use of formate as an inexpensive, stable, innocuous substrate and the production of CO_2, which can be easily removed from the reaction. Disadvantages of FDH are its initial expense, low specific activity (~ 6 U mg^{-1}) (Slusarczyk et al. 2000), and general sensitivity to organic solvents.

NADP-dependent enzymes are far less common than their NAD-dependent counterparts (Faber 2000). Two general regenerative approaches have been used for NADP-dependent enzymes. The first strategy focuses on regenerating NADPH using G6PDH, ADH from *Thermoanaerobium brockii* or an engineered FDH that can accept $NADP^+$ instead of its natural substrate NAD^+ (Tishkov et al. 1999). G6PDH

catalyzes the oxidation of glucose-6-phosphate to 6-phosphoglucolactone, which spontaneously hydrolyzes to the corresponding gluconate. The enzyme from *Leuconostoc mesenteroides* accepts both NAD^+ and $NADP^+$ whereas yeast-G6PDH accepts only $NADP^+$. Although the specific activity of G6PDH is quite high compared to FDH, a major disadvantage of this method is the high cost of glucose-6-phosphate. The current best method for NADPH regeneration is based on the engineered FDH isolated from *Pseudomonas* sp. 101 (mut-Pse FDH), which is available from Jülich Fine Chemicals (Jülich, Germany). In the second general approach, the production enzyme is engineered so that it can utilize NAD^+. This approach eliminates the need for more costly $NADP^+$ cofactor and allows the use of the more stable NAD^+ cofactor.

A particularly promising new method for NAD(P)H regeneration uses a newly discovered enzyme, phosphite dehydrogenase (PTDH) (Costas et al. 2001, Vrtis et al. 2001) (Fig 1). This enzyme catalyzes the nearly irreversible oxidation of phosphite to phosphate. Although the wild type PTDH has a preference for NAD^+ over $NADP^+$ by about 100-fold, a mutant PTDH was created by rational design with relaxed specificity toward both nicotinamide cofactors (Woodyer et al. 2003). The mutant PTDH has a similar specific activity toward NAD^+ as FDH, and a 33-fold higher catalytic efficiency toward $NADP^+$ ($k_{cat}/K_{M,NADP}$) than mut-Pse FDH with a comparable turnover number. Thus, the mutant PTDH can efficiently regenerate both cofactors. This phosphite/PTDH system has features similar to the formate/FDH system, including inexpensive substrate, easily removable byproduct, and both substrate and byproduct are innocuous to enzymes.

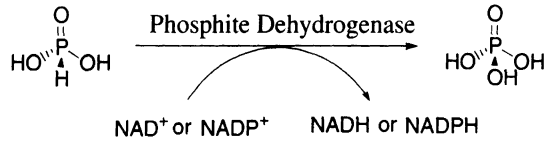

Figure 1. Enzymatic regeneration of reduced nicotinamide cofactors using the phosphite/phosphite dehydrogenase method.

3. NAD(P)$^+$ REGENERATION

Although NAD(P)H regeneration has been heavily investigated, methods of recycling NAD(P)$^+$ *in situ* remain far fewer and less developed. Motivation to develop a practical regeneration scheme for NAD(P)$^+$ stems from the potential to either synthesize ketones, which can be difficult to create chemically or use DHs to resolve racemic mixtures of chiral alcohols and amines. The best general method for NAD(P)$^+$ regeneration has been achieved by using glutamate dehydrogenase (GluDH) to catalyze the oxidation of ammonium α-ketoglutarate to glutamic acid. Disadvantages of GluDH are its moderate specific activity (40 U/mg) and its by-product, glutamate, which can complicate product isolation (Chenault et al. 1988).

4. ATP/NTP REGENERATION

Nucleoside triphosphates (NTPs) are utilized as sources of nucleosides, nucleoside phosphates and phosphate in living organisms. NTPs, particularly adenosine triphosphate (ATP) can be used as cofactors to phosphorylate compounds or enzymes frequently making high-energy bonds. Other NTPs such as uridine triphosphate (UTP) and cytidine triphosphate (CTP) usually form activated intermediates by donating the nucleoside phosphate moiety. For example, CTP activates choline by the formation of

CDP-choline for the synthesis of phospholipids. ATP is one of the cheapest cofactors, costing about $11/mmol, while other NTPs cost around $160/mmol (Sigma catalog 2003). Consequently, ATP is more frequently used as a phosphorylating agent and it has received more attention than other NTPs. The products of NTP catalyzed reactions are usually the corresponding nucleotide diphosphates (NDPs) or monophosphates (NMPs) and regeneration of these to the NTPs would allow recycling.

Several enzymatic and whole-cell based methods have been described for regeneration of ATP from ADP or AMP. Due to the high specificity of these systems, chemical methods are inferior and will not be discussed here. However, it should be noted that production of nucleotide analogs by chemical synthesis has been very successful for treatment of cancer and viral diseases such as HIV (Gulick 2003). There are four enzymes commonly employed in the regeneration of ATP from ADP, including the use of phosphoenolpyruvate (PEP) in a coupled reaction catalyzed by pyruvate kinase (PK), acetylphosphate coupled with acetate kinase (AK), creatine phosphate (CP) coupled with creatine kinase (CK), and polyphosphate (pPi) coupled with polyphosphate kinase (PPK). Since these enzymes have broad substrate specificities, other NTPs such as guanosine 5'-triphosphate (GTP), UTP and CTP can be regenerated by these ATP regeneration methods.

PK catalyzes the phosphoryl transfer from PEP to ADP to produce ATP and pyruvate. This method has been coupled to several enzymatic syntheses at 50-900 mmol scale (Gross et al. 1983, Bolte & Whitesides 1984, Bednarski et al. 1988) and typically the TTNs are on the order of 100. The advantage of this system is that the reaction has a favorable K_{eq} and PEP is very stable. The disadvantages are that PEP is very expensive and the product of reaction (pyruvate) inhibits PK. PEP can be prepared on the mole scale from pyruvate (Hirschbein et al. 1982), cutting down its cost as a substrate. PPK catalyzes the transfer of phosphoryl groups from pPi to ADP to form ATP. pPi is a low cost and high stability linear polymer of orthophosphate, which has high-energy phosphoanhydride linkages (Kornberg 1995, Kuroda & Kornberg 1997, Shiba et al. 2000). PPK/pPi has been used in several ATP regeneration schemes including those where ATP is regenerated from AMP and are discussed in more detail below. The biggest advantage of this system is that polyphosphate is by far the cheapest phosphoryl donor per phosphate of any ATP regeneration system. AK catalyses the hydrolysis of acetylphosphate with transfer of the phosphoryl group to ADP to form ATP and acetate. This method was once the most widely used method, primarily because acetylphosphate (also used for AcCoA regeneration, see below) can be prepared cheaply and easily on the mole scale (Crans & Whitesides 1983). However, due to the very fast hydrolysis of acetylphosphate, with a half-life of less than nine hours in neutral solution (Zhang et al. 2003), this regeneration scheme cannot be used for long-term regeneration. Finally, CK catalyzes the phosphoryl transfer from creatine phosphate to ADP forming creatine and ATP. This reaction occurs with a high K_{eq} because the free energy of hydrolysis of creatine phosphate is higher than that of ATP by about –13 kJ/mol (the same as acetyl phosphate, but smaller than –30 kJ/mol of PEP) (Zhang et al. 2003). This method has been used to regenerate ATP in the synthesis of fructose 1,6-diphosphate (Sakata et al. 1981) and to regenerate ATP, CTP, and UTP in the synthesis of sugar nucleotides (discussed further below) (Zhang et al. 2003) achieving TTNs of around 10. The expense of both the enzyme and substrate for this system are low and the enzyme has broad nucleotide specificity and therefore this regeneration method deserves further attention in the future.

The ATP regeneration methods discussed above are effective for enzyme-catalyzed synthesis. However, an additional method has been developed specifically for cell-free protein synthesis (Kim & Swartz

2001). The methods above are not well adapted to this application because the accumulation of phosphates in the cell-free system will eventually inhibit protein synthesis and uncoupled enzymes in the cell extracts would degrade the phosphoryl regeneration substrates (Kim & Swartz 2001). This novel ATP regeneration approach takes advantage of the enzymes normally present in cell extracts, and rather than using expensive phosphoryl sources such as PEP or creatine, it utilizes glycolytic intermediates such as glucose-6-phosphate. In this study it was discovered that endogenous enzymes convert pyruvate into acetylphosphate upon NAD^+ addition. Acetylphosphate then regenerates ATP by endogenous acetate kinase. Furthermore, glucose-6-phosphate can regenerate ATP in two steps by being first converted into pyruvate and then acetate. The economic efficiency of cell-free protein synthesis will be improved by this system. This improvement results from the lower cost of the substrates used in this system and either comparable or higher synthetic protein yield (for pyruvate/NAD^+ or for glucose-6-phosphate/NAD^+, respectively) than systems using PEP (Kim & Swartz 2001).

There has also been a significant interest in regenerating NTPs from the corresponding NMPs. Many ATP-dependent enzymes produce AMP rather than ADP making an efficient method for regenerating ATP from AMP highly desirable. It has been shown that AMP can be converted to ADP by adenylate kinase (AdK), which converts one ATP and one AMP to two ADPs. This enzyme was used in combination with AK and acetylphosphate to convert AMP into ATP in a bioreactor at 99 % yield for six days (Kondo et al. 1984). Drawing upon this principle, Whitesides and coworkers (Kim & Whitesides 1987, Simon et al. 1988) created a NMP regeneration system based on AdK and PK with PEP as the phosphoryl donor. This system was further used to regenerate CTP from CMP for production of CMP-*N*-acetylneuraminic acid (CMP-NeuAc) by CMP-NeuAc synthetase from NeuAc and CTP in batch reactions on the gram scale (Simon et al. 1988). A novel enzymatic method using pPi as a phosphoryl source has also been used for the regeneration of CTP from CMP (Ishige et al. 2001). In this scheme, the collective activity of PPK and CMP kinase from *E. coli* converts CMP into CDP, while PPK further phosphorylates CDP to CTP. This regeneration system was coupled with CMP-NeuAc synthetase to synthesize CMP-NeuAc. CMP-NeuAc can also be produced by a recombinant *E. coli* strain containing an acetylphosphate/acetate kinase-based ATP regeneration system (Lee et al. 2002). More details about these systems will be discussed in the sugar nucleotide section.

An important advance in ATP regeneration from AMP is the use of inorganic pPi and the enzyme polyphosphate-AMP phosphotransferase (PAP) (Resnick & Zehnder 2000, Kameda et al. 2001). Using pPi with PAP and AdK, Resnick and Zehnder (Resnick & Zehnder 2000) designed an ATP regeneration system in which AMP is converted to ADP by PAP. AdK then converts two ADPs into one AMP and one ATP. This regenerative system was successfully applied to the synthesis of glucose-6-phosphate with hexokinase, which produces ADP, thus the only AMP created comes from AdK. The advantage of this over previous approaches lies in the use of relatively inexpensive AMP and poly(P) as the substrates. The drawbacks of this system are the low TTN of 3–4 and the need for a cloned PAP gene. A similar process was developed in which PAP and a recombinant PPK from *E. coli* were used together to regenerate ATP from AMP and pPi (Kameda et al. 2001). This method was coupled with acetyl-CoA synthase to synthesize AcCoA (described in AcCoA section) and inorganic pyrophosphatase (PPase) helped to alleviate the inhibitory effect of pyrophosphate and to provide an additional driving force in this method (Fig 2). This approach has several advantages, including a TTN of 40 for ATP, low cost of the phosphate donor pPi, and loose substrate specificity that allows regeneration of guanosine triphosphate

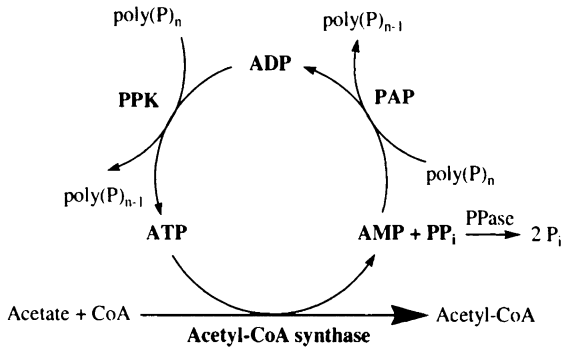

Figure 2. The PAP-PPK ATP regeneration system from AMP coupled with the acetyl-CoA synthetic reaction adapted from (Kameda, Shiba et al. 2001).

(GTP) from GMP using the same system. Unfortunately, PAP and PPK are both specific for their particular nucleotide substrate (NMP and NDP, respectively) (Ishige & Noguchi 2000). Consequently, NTP regeneration from NMP requires two enzymes at this point.

The regeneration of ATP and other NTPs from their corresponding NDPs is now a routine process using a selection of several enzymes. The most widely used enzyme is PPK with pPi as a substrate. This method uses low cost substrate and when coupled with PAP, regeneration of NMPs is also possible. An interesting endeavor would be to engineer a PPK that could phosphorylate both NMPs and NDPs, thus allowing a one-enzyme conversion to NTPs. For cell-free protein synthesis, a novel ATP regeneration method using glycolytic intermediates was developed for cost-effective synthesis. One of the remaining challenges for ATP regeneration is the production of immobilized derivatives with high activity. Unfortunately, most immobilization methods for nucleotides cause a significant loss in biological activity.

5. SUGAR NUCLEOTIDE REGENERATION

There is an increasing interest in glycobiotechnology as glycoconjugates and polysaccharides are being explored as potential pharmaceuticals (Thematic Issue 2001, Zhang et al. 2001, Nahalka et al. 2003). This research has resulted from discovery of their roles in transmembrane signaling, molecular recognition (especially in bacterial and viral infections), tumor development, and many other physiological and pathological pathways. Of the many known potential sugar-based drugs, only a few have been used in pharmaceutical industry due to the complexity of their chemical synthesis and other limiting factors like the high cost of individual oligosaccharides. In order to overcome the complexity of carbohydrates and their synthesis, biosynthetic pathways are usually used for their synthesis. There are many methods developed for oligosaccharide synthesis. However, the Leloir-glycosyltransferase mediated method, with its high regioselectivity and stereoselectivity, is acknowledged as the most useful approach for large-scale preparation (Bulter & Elling 1999, Thematic Issue 2001, Nahalka et al. 2003). The down side to this methodology is that glycosyltransferases require a sugar nucleotide as donor substrate. In order to make the process economically feasible, the sugar nucleotide must be regenerated *in situ* by recycling the nucleotide moiety. There are many possible sugar nucleotides in different organisms that could act as donors for glycosyltransferases in the biosynthesis of oligosaccharides. However, the

primary research interest to this point has been the nine sugar nucleotides that function as donor substrates in mammalian systems, including CMP-NeuAc, UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), UDP-glucuronic acid (UDP-GlcUA), GDP-mannose (GDP-Man), GDP-fucose (GDP-Fuc), UDP-*N*-acetylglucosamine (UDP-GlcNAc), UDP-a-D-xylose (UDP-Xyl) and UDP-*N*-acetylgalactosamine (UDP-GalNAc) (Ichikawa et al. 1994, Bulter & Elling 1999, Liu et al. 2002). A detailed review of the enzymatic synthesis of each of these sugar nucleotides has been recently published (Bulter & Elling 1999).

Regeneration of UDP-Gal, UDP-Glc, UDP-GlcNAc, CMP-NeuAc and GDP-Fuc for preparative functions has been the focal point of several new approaches. Bacterial coupling may be the most notable approach. In this technique, combinations of different strains are used together to produce the final product from whole cells. For instance, *Corynebacterium ammoniagenes* can produce UTP from orotic acid by producing UMP, which is converted inside the cell to UDP and then UTP. This strain has been coupled with recombinant *E. coli* strains expressing the enzymes required for sugar nucleotide generation (Fujio & Maruyama 1997; Fujio et al. 1997). Since all of the regenerative and synthetic catalysis occurs inside the cells, no enzyme purification has to be preformed. However, the cells often have to be permeabilized. Using this approach, sugar nucleotides including UDP-Gal (44 g l^{-1}), CMP-NeuAc (17 g l^{-1}), UDP-GlcNAc (7.4 g l^{-1}) and GDP-Fuc (18.4 g l^{-1}) have been successfully produced on a large-scale (Koizumi et al. 1998, Endo et al. 1999, 2000, Koizumi et al. 2000, Tabata et al. 2000). This method has also been shown to be an effective method for making CDP-choline (11 g l^{-1}), which is an important intermediate in phospholipid biosynthesis and is given as a treatment for brain injuries (Fujio & Maruyama 1997, Fujio et al. 1997). As shown in Figure 3, this method was used to obtain CMP-NeuAc by the combination of *C. ammoniagenes* and two *E. coli* strains; one expressing CTP synthetase (converting UTP to CTP)

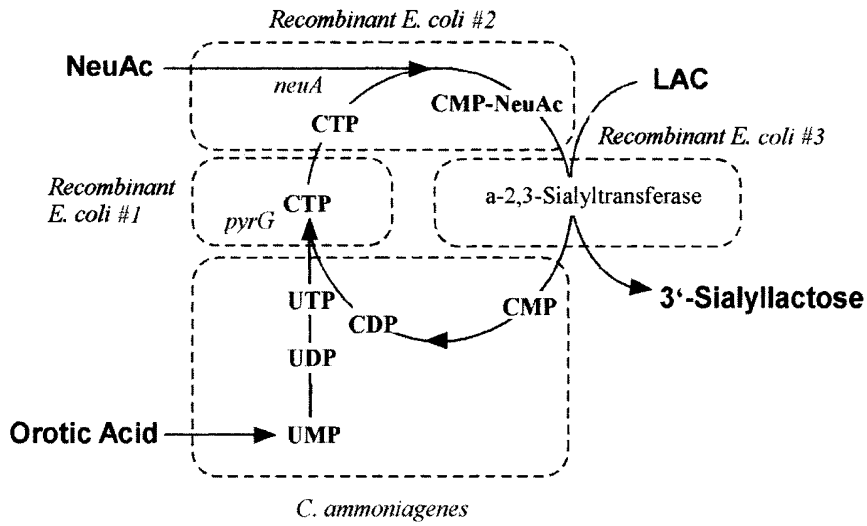

Figure 3. A CMP-NeuAc regeneration system by bacterial coupling. The system consisted of *C. ammoniagenes* that converts orotic acid into UTP and two recombinant *E. coli* strains expressing the CTP synthetase gene (*pyrG*) from *E. coli* K12 and the CMP-NeuAc synthetase gene (*neuA*) from *E. coli* K1, respectively. Further coupling of a recombinant *E. coli* strain over-expressing the α-(2, 3)-sialyltransferase gene from *N. gonorrhoeae* led to the large scale production of 3'-sialyllactose using NeuAc, orotic acid and lactose (LAC) as substrates (adapted from Endo et al. 2000).

and the other expressing CMP-NeuAc synthase (converting NeuAc and CTP to CMP-NeuAc) (Endo et al. 2000). Coupling of these CMP-NeuAc or GDP-Fuc regeneration systems with other recombinant *E. coli* strain(s) over-expressing the desired glycosyltransferase(s) allowed the large-scale production of important oligosaccharides including globotriose, 3'-sialyllactose and Lewis X (Endo et al. 2000, Koizumi et al. 2000) as well as the sialyl-Tn epitope of the tumor-associated carbohydrate antigen (Endo et al. 2001) and NeuAc (Tabata et al. 2002).

Bacterial coupling is one of the most efficient methods for sugar nucleotide regeneration, especially when one considers that the enzymes do not have to be purchased or purified and the starting materials are often inexpensive. However, there are a few drawbacks to the method as well, including the need to permeabilize certain strains and the slow transport of intermediates in other strains. Additionally, many bacteria have different optimal growth conditions and thus compromises sometimes have to be made for fermentation of several strains at once. One possible solution to these problems is a new approach using a single recombinant bacterial strain or "superbug". This method has been recently demonstrated (Chen et al. 2001, Chen et al. 2002, Lee et al. 2002) where one strain contains all the necessary enzymes for sugar nucleotide regeneration and oligosaccharide synthesis. CMP-NeuAc regeneration (Lee et al. 2002) was achieved by cloning and expressing CMP kinase, sialic acid aldolase and CMP-NeuAc synthetase in a single *E. coli* strain. In this system several biomedically important trisaccharides were produced by simply choosing different galactosyltransferases. Once these recombinant strains were created they were used for large-scale production of Galα1,4Lac (globotriose) and Galα1,3Lac (α-Gal epitope) while regenerating UDP-Gal in a two-step process. The engineered strains were first fermented and then harvested, permeabilized, and used as whole-cell biocatalysts for oligosaccharide synthesis. Similarly, the α-Gal epitope was produced when a biosynthetic pathway was created from five genes from the Galα1,3Lac producing pathway and transferred into an *E. coli* strain (Chen et al. 2002).

When weighed against the whole-cell based approaches, enzymatic methods can offer more flexibility for sugar nucleotide regeneration and oligosaccharide synthesis in some cases. Additionally, using immobilized enzymes allows greater productivity and a simplified process for product isolation when compared to the whole cell methods. A cost-effective enzymatic UDP-Gal regeneration method was developed by Wang and coworkers (Liu et al. 2002, Nahalka et al. 2003) in which seven enzymes involved in the biosynthesis of UDP-Gal were immobilized onto nickel agarose beads ("superbeads"). First, the genes were cloned, expressed, and purified individually as 6His-tag fusions. Then the corresponding enzymes were immobilized by their 6His-tags to the nickel containing beads (Chen et al. 2001) by simply mixing them in the desired amounts. Incubating equal amounts of UMP and galactose with catalytic quantities of ATP and glucose-1-phosphate, resulting in the formation UDP-Gal with a 50 % yield based on UMP. Later, this methodology was used for the preparation of several oligosaccharides including globotriose and the α-Gal epitope (Nahalka et al. 2003). Ishige and coworkers (2001) described a new efficient enzymatic method for CMP-NeuAc regeneration based on purified enzymes. In this method, the gene encoding a novel CMP-NeuAc synthetase was cloned and the corresponding enzyme was coupled with the pPi/PPK enzymatic CTP regeneration system discussed in the previous section. Zhang and coworkers also described preparation of CMP-NeuAC by CMP-NeuAc synthetase, where they used AdK and CK in the presence of CP to regenerate the CTP to form CMP-NeuAc, which was used to synthesize 3'-sialyllactose. This same regeneration system was used to regenerate UTP from UDP to make UDP-gal, which was used to make α-Gal epitope with the cloned galactosyltransferase (Zhang et al. 2003).

As mentioned above, there are nine sugar nucleotides donors involved in mammalian glycobiology. However, there are other sugar nucleotides that have gained some interest. For example, the dTDP- and UDP-activated deoxyhexoses utilized in plant and bacteria secondary metabolism (Liu & Thorson 1994) have recently received a lot of attention. They have potential applications in modifying the structures of antibiotics that are decorated with carbohydrates (Walsh et al. 2003) possibly allowing the creation of antibiotic analogs. Many of the glycosyltransferase genes involved have been cloned and expressed heterologously. Additionally, the chemoenzymatic syntheses of various pyrimidine diphosphosugars with sugar nucleotide regeneration have been reported (Stein et al. 1995, Amann et al. 2001, Jiang et al. 2001). Although there are only a few examples where sugar nucleotides have actually been regenerated *in situ*, there are many newly developed sugar nucleotide production methods. Of those methods, the whole-cell approaches, using bacterial coupling or a single strain with several genetically engineered genes, represent the most cost-effective and efficient routes. These methods have been successfully used to create sugar nucleotides and pharmaceutically important oligosaccharides. Purified enzyme and bead chemistry should not be dismissed either as it also has been proven effective at regenerating sugar nucleotides *in situ* where other methods have not. These methods will continue to improve as genomic and proteomic researches bring about the discovery of more bacterial glycosyltransferases. The development of more efficient and versatile protein expression systems for these glycosyltransferases will allow a large number of glycosyltransferases to become available, making the synthesis of very diverse carbohydrates possible.

6. PAPS REGENERATION

Sulfated carbohydrates and glycopeptides play an important role in specific cell signaling and recognition events of both normal and disease processes such as chronic inflammation, cancer metastasis, and hormone regulation (Bowman & Bertozzi 1999). The cofactor PAPS (3'-phosphoadenosine 5'-phosphosulfate) is the universal sulfate donor in organisms. Sulfotransferases catalyze the transfer a sulfuryl group (SO_3) from PAPS to an acceptor molecule.

Traditional regeneration schemes for PAPS involve multi-enzyme systems. These regeneration schemes have typically focused on the *in vivo* metabolic machinery of PAPS. Key enzymes identified in the *in vivo* synthesis of PAPS were ATP sulfurylase, adenosine-5'-phosphosulfate (APS) kinase, 3'-nucleotidase, myokinase and pyruvate kinase. However, a recent study has shown that recombinant rat liver aryl sulfotransferase IV can be used to sulfonate 3'-phosphoadenosine-5'-phosphate from simple available chemicals (Burkart et al. 1999). In this cycle (Fig 4), a high concentration of *p*-nitrophenyl sulfate drives the *in situ* regeneration of PAPS. Coupling this regeneration reaction to the enzymatic sulfation of *N, N',N", N"'*-diacetylchitobiose resulted in an 95% yield of *N, N',N", N"'*-diacetylchitobiose 6-sulfate (Burkart et al. 2000). Several other sulfated oligosaccharides and glycopeptides have been produced by recycling the sulphotransferase cofactor PAPS.

The large-scale production of enzymatically sulfated products has been hampered by the high cost and instability of PAPS ($27,902/mmol (Sigma catalog 2003); $t_{1/2}$ = 20 h at pH 8.0). Another limiting factor to large-scale applications has been the product inhibitory effect of 3'-phosphoadenosine-5'-phosphate. Even micromolar concentrations of 3'-phosphoadenosine-5'-phosphate in the reaction mixture can result in product inhibition of sulfotransferases. A practical regeneration system would remove 3'-phosphoadenosine-5'-phosphate from preparative reactions and increase the stability of PAPS. While

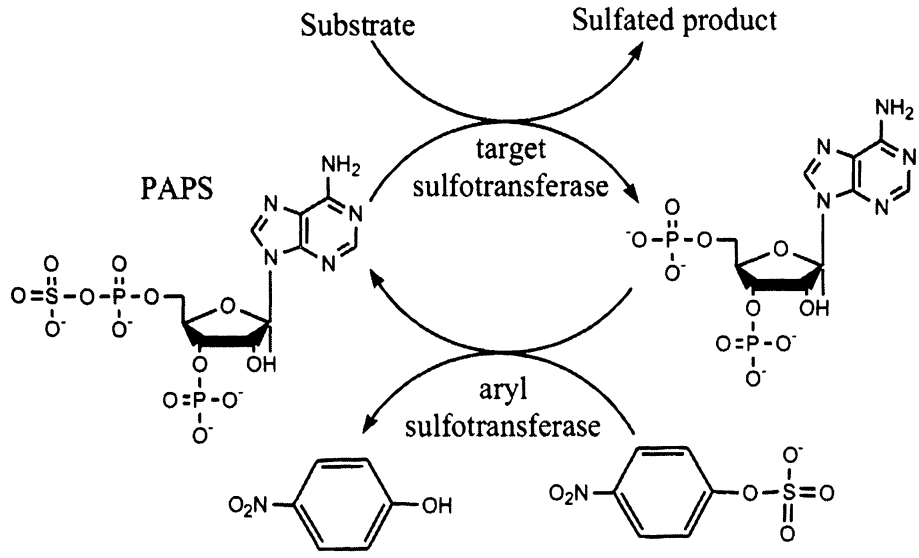

Figure 4. A novel PAPS regeneration method consisting of a recombinant aryl sulfotransferase and *p*-nitrophenyl sulfate (Reproduced from Zhao & van der Donk 2003).

small-scale synthesis of PAPS can produce research quantities of PAPS, a multi-gram method of synthesis has yet to be demonstrated.

7. ACETYL-COENZYME A REGENERATION

As mentioned above most enzymes used in industry are hydrolytic enzymes and there has not been any large effort toward carbon-carbon bond formation in biocatalysis. This is in part due to the small number of commercially available enzymes that catalyze these reactions. However, the larger problem is that cofactors are often required in these reactions. In fatty acid, cholesterol/steroid, and polyketide biosynthetic pathways, there is a requirement for acetyl-coenzyme A (AcCoA). One of the primary pharmaceutical targets for lowering cholesterol is hydroxymethylglutaryl-CoA reductase, which catalyses the first committed step of cholesterol biosynthesis (Grundy 1988). It has been estimated that about 4% of all known enzymatic reactions require coenzyme A (CoA) in some form as a substrate (Martin et al. 1992). CoA functions as an acyl carrier, activating acyl groups as thioesters toward nucleophilic substitutions and Claisen or aldol condensations at the α-carbon, while serving as a good leaving group. CoA is a very expensive cofactor at nearly $1400/mmol and the thioester forms of the coenzyme are even more expensive (Sigma catalog 2003), making regenerative processes necessary.

There are several enzymatic and chemical methods that have been employed for the regeneration of AcCoA from CoA. The chemical methods include reacting CoA with acyl chlorides (Seubert 1960), acid anhydrides (Simon & Shemin 1953), N-hydroxysuccinimide esters (Al-Arif & Blecher 1969), or thioacetic acid (Wilson 1952). Chemical acylations of CoA in general are unattractive because they experience non-specific acylation and cannot be preformed *in situ* due to the use of organic solvents, making batch extraction and acylation of CoA necessary for each turnover. However, there are a few notable exceptions in which the chemical regeneration of AcCoA is very attractive. Ouyang and Walt showed that S-acylthiocholine iodide could be used to transfer acyl groups to CoA in aqueous buffer

with high efficiency (Ouyang & Walt 1991). With these inexpensive and commercially available reagents, they were able to charge CoA with acetyl, proponyl, butyryl, and benzoyl groups. The reaction to form AcCoA appeared to occur with a relatively high K_{eq} as the reaction proceeded to apparent completion when monitored by HPLC. They then examined the possibility of regeneration for the conversion of oxaloacetate to citrate by AcCoA-dependant citrate synthase (CS). By adding S-acetylthiocholine iodide, oxaloacetate and catalytic amounts of CoA and CS, they were able to obtain citrate with a TTN for AcCoA of 1160. They tested this method on a second system for the conversion of carnitine to L-acetylcarnitine catalyzed by the AcCoA-dependant carnitine acetyltransferase and were able to obtain a TTN of 340. The same group also showed that acid anhydrides could regenerate AcCoA effectively in a two-phase system. In the organic phase, acetic anhydride was used to acylate the phase transfer catalyst dimethyl aminopyridine, which would then transfer to the aqueous phase and acylate CoA. This system was used in conjunction with CS to produce citrate. However, it required a lot of fine-tuning to optimize the TTN, which ranged from 17 to 964. Although it would also allow diverse and unnatural acyl groups transferred, additional challenges such as enzyme stability in trace organic solvent and acid anhydride water stability would have to be solved.

Among the enzymatic regeneration schemes, the same enzymes are typically used for recycling AcCoA. These have included, but are not limited to, phosphotransacetylase (PTA), carnitine acetyltransferase (CAT), and AcCoA synthetase (ACS). PTA transfers the acetyl group of acetylphosphate to CoA to form AcCoA with a high K_{eq} of ~150 (Bergmeyer et al. 1963). This enzyme has been coupled with production of citrate by CS on a small scale (0.3 mmol) and a large scale (5 mmol) with TTNs of 11,800 and 560, respectively (Patel et al. 1986). In this same study, PTA regeneration of AcCoA was coupled with CAT in the resolution of DL-carnitine to L-acetylcarnitine with a TTN ranging from 400 to 2,500. Additional benefits of PTA include its ability to catalyze the transfer of some unnatural and short chain alkanoyl groups to CoA from the corresponding phosphates (Billhardt et al. 1989). Some problems with PTA regeneration include the loss of enzymatic activity in solution (Billhardt et al. 1989), and the hydrolysis of acyl phosphates in neutral solutions (Patel et al. 1986).

ACS catalyzes the coupling of a broad range of carboxylic acids to CoA in the presence of ATP (Patel & Walt 1987). ACS has been used to regenerate AcCoA for the production of citrate catalyzed by CS with a TTN of 1000 for a mmol scale reaction (Patel et al. 1986). The obvious disadvantage of this system is that ATP is consumed by ACS and must be regenerated, used in whole cells, or added in stoichiometric amounts. The effectiveness of ACS for regenerating AcCoA has been shown by two groups, both for the production of poly 3-hydroxybutyric acid (P3HB). In the first system, AcCoA regeneration was coupled to propionyl-coenzyme A transferase and 3HB to make 3HB-CoA, which was then coupled to polyhydroxyalkanoic acid (PHA) synthase to produce P3HB (Jossek & Steinbuchel 1998). A TTN of 60 for AcCoA by ACS in a small-scale reaction was achieved. The second system was based on three enzymes from the P3HB biosynthetic pathway in *Ralstonia eutropha* (Satoh et al. 2003). In this biosynthetic pathway (Fig 5), two regenerated AcCoAs are condensed by β-ketothiolase to form acetoacetyl-CoA, which are then converted to 3HB-CoA by NADPH-dependent acetoacetyl reductase (phaB) and further polymerized by PHA synthase (phaC) as in the first system. When these three enzymes were coupled to ACS regeneration of AcCoA, a TTN of 26 was obtained when additionally utilizing the NADPH recycling method by glucose dehydrogenase (GDH) (described in nicotinamide regeneration section).

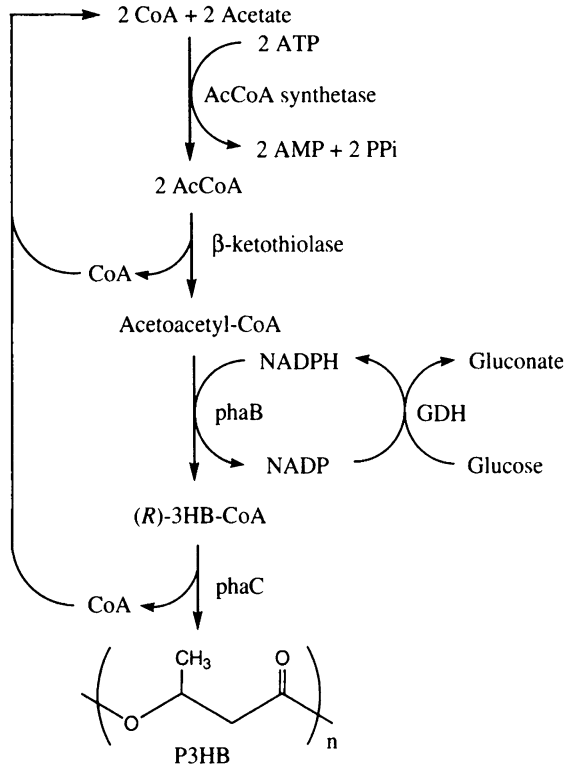

Figure 5. A novel AcCoA regeneration method based on the three enzymes β-ketothiolase (phaA), phaB and phaC from the P3HB biosynthetic pathway in *Ralstonia eutropha*.

Additional systems described for acyl-CoA regeneration include using CAT in the reverse reaction (converting acyl carnitine to carnitine while producing AcCoA from CoA). This system was used successfully for citrate production (a TTN of 690) when coupled to CS (Billhardt et al. 1989). This system accepts other acyl derivatives of L-carnitine, but they are difficult to prepare and the TTN is lower than several other systems described above. Regeneration of succinyl-CoA was demonstrated by α-ketoglutarate dehydrogenase (Hunter & Ferreira 1995), which catalyzes the reaction between NAD, α-ketoglutarate, and CoA to produce succinyl-CoA, NADH, and CO_2. The synthetic utility of this system has yet to be demonstrated and it would require the regeneration of NAD from NADH. In general, high TTNs have not been obtained for AcCoA or other acyl-CoA derivatives. Using S-acylthiocholine iodide chemical regeneration is thus by far the best non-enzymatic method, but large-scale use requires the preparation of large amounts of S-acylthiocholine iodide. Since AcCoA is expensive and must be turned over many times to make a process economically feasible, it is likely that the enzymatic and whole cell methods will prevail. Acyl carnitine/CAT and acyl phosphate/PTA are the most straightforward enzymatic systems for AcCoA regeneration. However, the acyl substrates are difficult to prepare and are unstable. ACS does not require substrate synthesis or use unstable substrates, but does require ATP. Since ATP regeneration has been well studied (described in section 4), ACS may provide an interesting alternative to CAT or PTA. In the end, there is no clear advantage of one system over another among these AcCoA regeneration methods.

8. CONCLUSIONS

Although substantial progress has been made in the past two decades, further improvements on existing regeneration methods for nicotinamide cofactors, ATP/NTP, sugar nucleotides, PAPS, and AcCoA need to be accomplished to make their use more widespread in industrial biocatalysis. Advances in metabolic engineering and protein engineering will be increasingly important in making cofactors more applicable in large-scale operations. Future work should also focus on cofactors for which there is not a practical regeneration method such as *S*-adenosyl methionine (SAM). The development of inexpensive and efficient *in situ* regeneration methods for the different cofactors should greatly expand their use in the pharmaceutical and chemical industries in the coming years.

Abbreviations:

AcCoA	acetyl-Coenzyme A
AdK	adenylate kinase
ADH	alcohol dehydrogenase
ADP	adenosine 5'-diphosphate
AK	acetate kinase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CK	creatine kinase
CoA	Coenzyme A
CP	creatine phosphate
CMP-NeuAc	cytidine 5'-monophosphate *N*-acetylneuraminic acid
CS	citrate synthase
CTP	cytidine 5'-triphosphate
dTTP	deoxy thymidine triphosphate
DH	dehydrogenase
FDH	formate dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase
GDH	glucose dehydrogenase
GDP-Fuc	GDP-fucose
GTP	guanosine 5'-triphosphate
HIV	human immunodeficiency virus
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NTP	nucleoside triphosphate
NDP	nucleoside diphosphate
NMP	nucleoside monophosphate
PAP	polyphosphate:AMP phosphotransferase
PAPS	3-phosphoadenosine-5'-phosphosulfate
PEP	phosphoenolpyruvate
pPase	pyrophosphatase
PPK	polyphosphate kinase
pPi	poly phosphate
TTN	total turnover number
UDP-Gal,	UDP-galactose
UDP-GlcNAc	UDP-*N*-acetylglucosamine
UF	ultrafiltration
UTP	uridine 5'-triphosphate

Acknowledgments

We thank Biotechnology Research and Development Consortium (BRDC) for supporting our work on developing a phosphite dehydrogenase-based nicotinamide cofactor regeneration system for reductive biocatalysis.

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Biocatalysis in Organic Media using Enzymes

Adrie J.J. Straathof

Although water is the default solvent for enzymatic reactions, organic media can be used as an alternative. This requires the proper selection of organic solvent type, water activity and enzyme formulation. The application of organic media for enzymatic reactions is particularly interesting for reversing hydrolytic reactions or for preventing hydrolytic side reactions that compete with a desired synthetic reaction. Besides, substrate solubility and product recovery can be improved by using organic media. This has led to a significant number of industrial applications. Immobilized lipases dominate these applications.

1. INTRODUCTION

In nature, water is the predominant solvent. Although some enzymes are associated with membrane structures containing mainly hydrophobic lipids, it is not surprising that studies of enzymes traditionally have been carried out mainly in aqueous media. Despite the high selectivities and rapid catalysis performed by enzymes under ambient reaction conditions, synthetic chemists used to be reluctant to employ enzymes as catalysts. The reason was that aqueous media were supposed to be required for enzyme activity, whereas many organic compounds are poorly water-soluble and many traditional organic reagents decompose in water. Carrying out reactions in an aqueous-organic mixture would be a solution to overcome the solubility problem. This was only partly successful due to the limited stability of enzymes in these systems. Hence, other approaches were sought.

When it was found that enzymes can retain and, in some cases, improve their high specificity in nearly anhydrous solvents (Zaks & Klibanov 1984), the scope of enzymatic catalysis as a tool in synthetic organic chemistry was widened dramatically. In the absence of water, hydrolytic enzymes now could be used for synthetic reactions, and some organic reagents that are unstable in aqueous solution now could be used in highly selective enzyme-catalyzed synthesis reactions.

1.1. Solvent systems

The addition of a solvent is not a prerequisite for enzymatic reaction. For example, a liquid ester may be hydrolyzed upon mixing with only the amount of water needed for the hydrolysis, in the presence of a lipase. Even the presence of a liquid phase is not a prerequisite for enzymatic reaction. Solid peptides have been shown to be formed in solventless mixtures of their solid precursors with protease powder (Gill & Vulfson 1994). However, the use of solvents or other fluids will usually improve the conversion rate and simplify the conversion process.

Fluids for enzymatic catalysis may be categorized as:

1. Aqueous solvents
2. Organic solvents
3. Ionic solvents, i.e. salts that are liquid at room temperature such as 1-ethyl-3-methylimidazolium tetrafluoroborate (Van Rantwijk et al. 2003)
4. Supercritical and near-supercritical fluids (Hartmann et al. 2000)
5. Gaseous fluids (Lamare & Legoy 1993)

Often mixtures of these five fluids are used, in particular:

- Water: water-miscible solvent mixtures (monophasic aqueous-organic or aqueous-ionic liquids)
- Water: water-immiscible solvent mixtures (biphasic aqueous-organic or aqueous-ionic, including emulsion systems with reverse micelles and systems where the two phases are on either side of a membrane)

More categories would be required to indicate that frequently not all components in the reaction system are dissolved. Enzymes are often suspended as solids in the reaction medium. Substrates and products may be partly suspended as solids as well, dispersed as gases (O_2 for glucose oxidase, for example), or emulsified as non-miscible liquids.

2. ENZYME INACTIVATION IN ORGANIC SOLVENTS

Enzymes that are in their native conformation and that are added to organic media may lose their intrinsic activity because of

- Denaturation, i.e. unfolding of the protein chain
- Dehydration, i.e. essential water molecules are removed from the microenvironment of the enzyme by dissolution in the organic solvent, but unfolding does not occur
- Inhibition, i.e. organic solvent molecules reversibly bind to the enzyme molecule, for example at the active site
- Chemical modifications, i.e. the organic solvent causes reactions that change the primary structure of the enzyme

The two latter reasons are relatively rare and the associated problems can usually be circumvented by changing the solvent type. The two former reasons will be treated here.

Denaturation is a very general phenomenon that will also occur to some extent in aqueous solution, especially at higher temperatures. Therefore, understanding of the effects of temperature on enzyme structure may also help to understand the effects of different solvents on enzyme structure (Cowan 1997). In general, enzymes are thought to occur in their native structure due to a large amount of hydrophobic interactions between amino acid residues in the interior of the folded structure. Hydrophilic and charged residues may support the stability, by forming hydrogen bridges and charge-charge interactions, respectively. The latter two interactions occur in particular at the enzyme surface, and may be stabilized by structural water molecules.

Comparison of structures of enzymes from organisms living at different temperatures has shown that the 3-dimensional folding of the protein chains of hyperthermophilic enzymes is generally more compact than for corresponding mesophilic enzymes that share most of the sequence. The hyperthermophilic enzymes contain many charged amino acid residues at positions where the corresponding mesophilic enzymes contain hydrophilic amino acid residues. Presumably this leads to a relatively large number of salt bridges in the native protein chain structure of hyperthermophilic enzymes, at the expense of the weaker hydrogen bridges. Therefore, hyperthermophilic enzymes will have a relatively rigid structure, and high temperatures are required for unfolding. For enzymatic activity, however, there should be a certain degree of conformational flexibility in the protein structure, facilitating entry of the substrates into the active site and attainment of the transition state structure. Mesophilic enzymes will have the required flexibility at much lower temperatures than their hyperthermophilic counterparts (Spiller et al. 1999).

This delicate balance that exists between hydrophobic interactions, charge interactions and hydrogen bonds in aqueous solvents can easily be disturbed by an organic solvent. If the organic solvent is relatively polar, it may strip the structural water molecules from the enzyme. Moreover, it may break the hydrogen bonds and the hydrophobic interactions in the native protein structure, allowing rapid unfolding and inactivation of the enzyme. Examples of such polar organic solvents are ethanol, acetone and dimethyl sulfoxide. The amount of these water-miscible solvents that can be added without much negative effects on the enzyme varies depending on the enzyme and the solvent, but is typically 30-60% (Khmelnitsky et al. 1991). There are various exceptions of which lipases are a particular case. For instance, porcine pancreatic lipase was shown to be active in 100% pyridine or acetone (Zaks & Klibanov 1985).

When hydrophobic solvents such as toluene or methyl-*tert*-butyl ketone (MTBE) are added in intermediate proportions (10-90 % v/v) to an aqueous enzyme solution, a two-liquid phase system will be obtained. The enzyme may remain relatively stable and active within the aqueous phase. Then, inactivation of the enzyme may be caused mainly by contacts with the aqueous-organic interface, depending on the enzyme and the organic solvent. Unfolding of enzymes at interfaces by exposure of the hydrophobic protein interior to the interface is a common cause for inactivation. Lipases, however, are usually stable at aqueous-organic interfaces due to their particular structure.

If the ratio of water to hydrophobic solvent is decreased, one may reach the situation that enzyme molecules with only a layer of tightly bound structural water molecules are dispersed in the hydrophobic solvent. In the absence of bulk water or hydrogen-bonding organic solvents, unfolding of the protein structure slows down due to reduced conformational flexibility. The reduced flexibility in hydrophobic solvents, however, may not only be a reason for increased stability but also for reduced catalytic activity.

The ratio of water to hydrophobic solvent may be decreased further, for example by addition of strong drying agents to the aforementioned system. At some stage, the enzyme will lose structural water molecules and consequently its catalytic activity, perhaps due to a too low flexibility. This is the "dehydration" phenomenon mentioned at the beginning of this section.

3. WATER ACTIVITY CONTROL

In the absence of a bulk aqueous phase, the total amount of water will be the sum of the amounts water bound to the enzyme, bound to the organic solvent, bound to the immobilization matrix, etc. Different solvents and different immobilization matrices will have a different affinity for water, leaving different

amounts bound to the enzyme at the same overall water concentration. The activity and stability of the enzyme in an organic medium will depend on the amount of water bound to it, and not on the overall concentration of water. Therefore, instead of measuring and controlling the water concentration, it is more convenient to measure and control the thermodynamic water activity (a_w) (Halling 1994). The water activity is defined so that it is 0.0 in a completely dry system and 1.0 in pure water. Dilute aqueous solutions have water activities close to 1. Organic media are found in the whole range of water activities between 0 and 1. When they are water-saturated their water activity will be close to 1, so the same concentration of water will lead to widely different water activities for different organic solvents. There is a good correlation between the water activity and enzyme hydration and thus enzyme activity in organic media. In different solvents, the maximal reaction rate will be observed at widely different water concentrations.

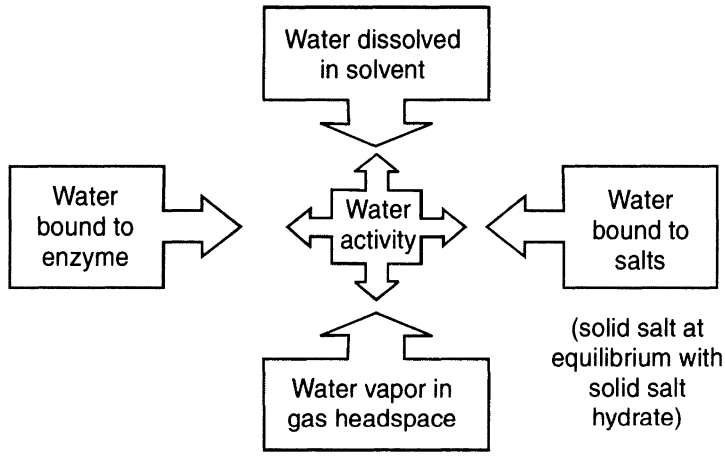

Figure 1. Water activity is the same at all phases at equilibrium.

The thermodynamic activity of a component is the same in all phases at equilibrium (see Fig 1). The water activity in the liquid is most conveniently determined by measuring the water activity in the gas phase with a special sensor, after equilibration. To control water activity at a desired level for an enzymatic reaction, one can equilibrate the reaction mixture via the gas phase with another compartment that contains a saturated salt solution of known water activity. If water is formed or consumed in the reaction, water activity will remain the same, because the water will be taken up or released by the salt solution, provided that the equilibration of the phases is fast enough. One way to achieve this is to pass the saturated salt solution through silicon tubings that are immersed in the reactor (Wehtje et al. 1997).

An alternative method is based on the fact that salt hydrates containing different numbers of water molecules are inter-converted at fixed water activities. Salt hydrates are crystalline compounds like Na_2CO_3.10 H_2O. This compound is at equilibrium with Na_2CO_3.7 H_2O at a water activity of 0.74 at 24°C. The salt hydrates act as a water buffer. As long as both salt hydrates are present the water activity remains at 0.74. The salt hydrates can be added directly to the organic reaction mixture. The pair of salt hydrates should be chosen to give a water activity suitable for the enzymatic conversion (Zacharis et al. 1997).

For developing a process, such salts may have to be avoided. Then it will be more attractive to use a reflux with a water trap (Bloomer et al. 1992) or an organic solvent with such a high capacity for dissolving water that a_w will not shift easily (Slotema et al. 2003). Other techniques are pervaporation or addition of molecular sieves (Wehtje et al. 1997).

4. PREDICTING SOLVENT EFFECTS ON ENZYMATIC REACTIONS

4.1. Solvent effects on enzyme stability

As dehydration inactivates enzymes, a scale that quantifies solvent polarity will be useful to predict enzyme stability in organic solvents. The most popular parameter is the log *P* value, which is defined as the logarithm of the partition coefficient of the solvent between 1-octanol and water. Log *P* values can be determined experimentally or can be calculated using a group contribution method (Rekker 1977). Table 1 gives some values.

Enzymes are usually more stable in water-immiscible solvents with high log P values (>4) (hydrophobic solvents) than in more polar solvents (Laane et al. 1987). For some applications, solvents from the former group do not dissolve the substrates well enough and a compromise taking into account enzyme stability and substrate solubility must be made.

4.2. Solvent effects on equilibrium yields

In aqueous-organic biphasic systems, the equilibrium yield of an enzymatic reaction can be shifted

Table 1. Log *P* values for some organic solvents
http://www.syrres.com/esc_kowdemo.htm

Solvent	Log *P*
dimethyl sulfoxide	−1.4
methanol	−0.8
acetone	−0.2
butanone	0.3
ethyl acetate	0.7
butan-1-ol	0.9
methyl tert-butyl ether	0.9
diethyl ether	0.9
dichloromethane	1.3
toluene	2.7
dibutyl ether	3.2
cyclohexane	3.4
hexane	3.9
octane	5.2
dodecane	6.1
dioctyl phtalate	8.5

dramatically by changing the phase ratio, the initial substrate concentration, the pH and the organic solvent type. An experimental screen of the influence of all these variables will be time-consuming, and, moreover, will require enzyme that remains active at all experimental conditions until equilibrium is reached.

As the influence of the different variables is reasonably well understood, it may be worthwhile to try a quantitative prediction (Martinek & Semenov 1981, Martinek et al. 1981, Semenov et al. 1988). If calculations show that interesting yields might be obtained for particular reaction conditions, the experimental program can be targeted at those conditions. To perform these calculations, the partitioning equilibria, dissociation equilibria and solubilities of the substrates and products have to be measured or calculated. Figure 2 schematically indicates an example of such equilibria for the penicillin G hydrolysis in butyl acetate – water (Diender et al. 2002). In this example, 12 species are present: $PenG^{0}$ (org), $PenG^{0}$ (aq), $PenG^{-}$ (aq), PAA^{0} (org), PAA^{0} (aq), PAA^{-} (aq), APA^{0} (org), APA^{+-} (aq), APA^{+} (aq), APA^{-} (aq), APA^{+-} (s) and H^{+} (aq). The superscripts indicate the charges. It is generally assumed that concentrations of charged species in organic phases are negligible. For a given pH, there are 11 unknown concentrations. These are related by the 8 equilibria shown in Figure 2. The remaining 3 equations required to solve the system are the enzymatic reaction equilibrium, which is usually known in water, and two stoichiometric equations. For either product, the number of moles of substrate added equals the sum of moles of substrate and product at equilibrium, and the numbers of moles can be converted into concentrations.

The system of 11 equations can conveniently be solved in a mathematic solver for different values of phase volume ratio, initial substrate concentration and pH. Figure 2 shows some yields that follow from calculated equilibrium concentrations. For widely different conditions, good yields can be obtained. For varying the organic solvent type, the partition coefficients in the calculations have to be changed, so the influence of the solvent can be screened less easily.

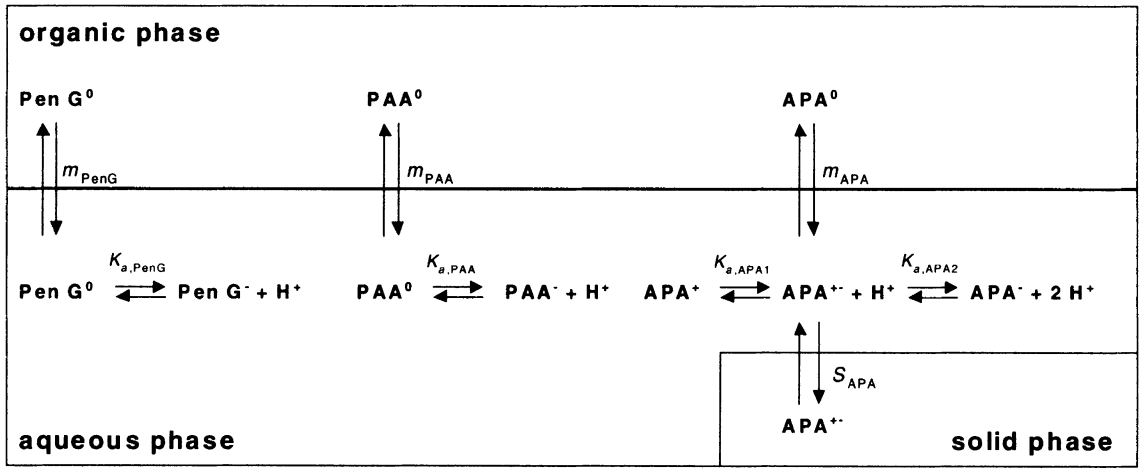

Figure 2. Penicillin G (Pen G) and its enzymatic hydrolysis products 6-aminopenicillanic acid (APA) and phenylacetic acid (PAA) in a aqueous-organic-solid 3-phase system. Equilibrium constants indicated are for acid dissociation (K_a), partitioning (m), and solubility (S).

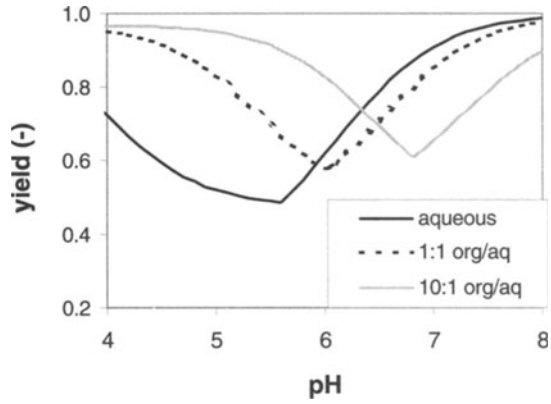

Figure 3. Calculated equilibrium yield for the enzymatic hydrolysis of 0.1 mol Pen G per L mixture at 25 °C in water and in 1:1 and 10:1 (w/w) biphasic mixtures of butyl acetate and water, with optional precipitation of 6-aminopenicillanic acid. (Adapted from Diender et al. 2002)

For a particular organic solvent type, the highest yield of a reverse hydrolysis reaction will be found for infinite ratios of organic solvent phase to aqueous phase, i.e. for a monophasic organic solvent rather than for an aqueous-organic biphasic solvent. In this monophasic organic solvent, the water activity should be as low as the enzyme allows, and pH is undefined. The organic solvent type will be the key variable. The highest equilibrium yield can be expected in the solvent with the highest concentration-based equilibrium constant, K_c. For a conversion of HAc and EtOH to water and ester, $K_c = c_{ester}/(c_{HAc} \cdot c_{EtOH})$, and the ratio of these constants for solvents A and B at the same water activity is:

$$\frac{K_c^A}{K_c^B} = \frac{m_{ester}^{A/B}}{m_{HAc}^{A/B} \cdot m_{EtOH}^{A/B}} = \frac{m_{ester}^{A/aq}\,(m_{HAc}^{A/aq} \cdot m_{EtOH}^{A/aq})}{m_{ester}^{B/aq}\,(m_{HAc}^{B/aq} \cdot m_{EtOH}^{B/aq})}$$

Here $m_{ester}^{A/B}$ is the partition coefficient of ester between solvent A and B. In general these partition coefficients will not be available. Instead, the use of partition coefficients between organic solvent and water, according to the right-hand side of the equation, may lead to useful estimations. However, activity coefficient ratios should be used instead of partition coefficient ratios for a formally correct treatment (Straathof et al. 1992, Halling 1994).

4.3. Solvent effects on reaction rates

In an aqueous-organic biphasic system, the enzyme will usually be in the bulk aqueous phase and may show its normal rate of catalysis, when the mass transfer rate of the substrate between both phases is taken into account (Straathof 2003). Lipases are exceptional in the sense that they may be activated by the interface. In the absence of a bulk aqueous phase, enzymatic reactions usually become slower. Partly this can be attributed to phenomena that have been described in earlier sections, like enzyme denaturation. Another reason is the following.

Suppose that a hydrated enzyme is converting a substrate S, and the enzyme conformation is not affected by the bulk liquid. Then, once the substrate is bound in the active site, the reaction rate will be the same

in any solvent. However, binding of the substrate to the active site may be more difficult than in water if the substrate is solvated better by the solvent than by water. Improved solvation will be reflected by a partition coefficient m_S (between organic solvent and water) that is larger than 1. Organic media are mostly used for substrates that do not dissolve very well in water, so this situation is very common.

Using transition state theory, an increase in the Michaelis constant of S is predicted (Straathof et al. 1992):

$$K_m^{org} = K_m^{aq} \cdot m_s$$

Higher Michaelis constants lead to lower reaction rates at a given substrate concentration. In addition, often lower k_{cat} values are found than in water. This may be due to a reduced flexibility of enzymes in organic media, so that the transition state is reached slower.

4.4. Solvant effects on enantioselectivity

If only the aforementioned phenomena would apply, enzyme enantioselectivity would be the same in all solvents, because the effects of the solvent would be the same for either enantiomer. In some cases, the enantioselectivity was indeed independent of the solvent (Wolff et al. 1997), but usually dependencies are found. This means that the solvent will for example influence the enzyme conformation (Overbeeke et al. 2000) or conformational flexibility (Broos 2002). Methods for reliable predictions of such effects are still in development.

5. CONTROLLING ENZYME FORMULATION IN ORGANIC MEDIA

The microenvironment of the enzyme, and thus the enzymatic reaction, is not only determined by the properties of the bulk solvent but also by the physical state of the enzyme when added to the solvent. A large variety of formulations have been investigated. Different formulations will be preferable for large-scale applications, for laboratory syntheses and for enzymological studies. Diffusion limitation has to be taken into account for most enzyme preparations.

5.1. Lyophilized enzymes

The most straightforward way of using enzymes in organic media is to suspend solid enzyme powder directly in the solvent. For quick results, this method is the obvious first choice, and there are many examples in the literature where the results were positive. Negative results have also attracted the attention of many researchers. Most enzyme powders are prepared by lyophilization (freeze drying) which might reversibly denature the enzyme to some extent. Refolding will be much easier in water than in organic solvent. Often the measured number of active sites is lower in organic solvent than in water (Wangikar et al. 1996). To increase the activity of lyophilized enzymes in dry organic solvents, the lyophilization should be carried out in the presence of lyoprotectants such as sugars or, preferably, inorganic salts (Ru et al. 2000). Also, it is important to carry out the lyophilization starting from a suitable pH for the enzyme. The enzyme keeps its ionization state from the aqueous solution. This has been called the "pH memory" of enzymes in organic media (Zaks & Klibanov 1985). To increase the buffering capacity of the system, buffer salts are often present in the enzyme preparation.

5.2. Solubilized and micelle-bound enzymes

Enzymes can be made soluble in organic media by covalent attachment of polymers. The most common method is to couple polyethylene glycol chains to the amino groups of the enzyme (Koops et al. 1999).

When solubilized enzymes are used, diffusion limitations will be absent, but product isolation and enzyme recovery are usually more difficult than with solid enzyme preparations. However, solubilized enzyme preparations are well suited for many fundamental studies, for example for spectroscopic investigations requiring transparent solutions.

The inactivation of the enzyme that can occur during the derivatization procedure can be avoided by using surfactants to solubilize enzymes (Okahata & Mori 1997). One method starts with mixing aqueous solutions of the surfactant and the enzyme. The enzyme-surfactant complex precipitates and can subsequently be dissolved in organic media.

By mixing water, organic solvent and surfactant, one can obtain microemulsions. Reversed micelles (water droplets surrounded by a surfactant film and dispersed in the bulk organic phase) may be formed. One way to solubilize an enzyme in a reverse micelle is to add a small amount of an aqueous solution of the enzyme to a solution of the surfactant in the organic solvent. After mixing, a transparent solution is formed and the enzyme sometimes expresses high catalytic activity (Creagh et al. 1993). These microemulsions can be considered as aqueous-organic biphasic systems with a very large interfacial area, allowing rapid mass transfer. Their main drawback is that the surfactant will cause problems in the isolation of the reaction product.

5.3.Adsorbed enzymes

Most enzyme immobilization methods used for organic media rely on adsorption of the enzyme on a porous support. The reason for not requiring covalent binding is that enzymes normally have a low tendency to dissolve in organic media. Popular supports are porous polypropylene, polyacrylamide, glass and Celite particles (Barros et al. 1998). Wetting the particles with an aqueous enzyme solution, optionally followed by drying, may be sufficient to obtain active and stable preparations. The most successful enzyme for use in organic media, immobilized *Candida antarctica* lipase B, is sold as an adsorbed enzyme preparation uder the trade name Novozym 435.

5.4.Entrapped enzymes

Encapsulation of enzymes is often more time-consuming than adsorption on supports, but may offer a better protection from organic media. Entrapment has been performed using photo-crosslinkable polymers, and hydrogels such as 2-hydroxyethylmethacrylate (Vulfson et al. 2001). Efficient procedures have also been developed for encapsulation of enzymes in sol-gel materials produced by hydrolysis of mixtures of RSi $(OCH_3)_3$ and $Si(OCH_3)_4$ (Reetz et al. 2003). Sol-gel lipase immobilizates showed excellent activity and stability in organic media.

5.5. Cross-linked enzyme crystals and aggregates

The most pure and concentrated form of an enzyme is its crystal. For some enzymes, crystallization has been developed as a large-scale purification technique. Upon cross-linking with bifunctional reagents such as glutaraldehyde, the crystals may be used as catalysts in organic media. Very high catalytic activity and stability have been reported for such cross-linked enzyme crystals (Margolin & Navia 2002). However, crystallization is slow and needs expensive pre-purification steps for most enzymes.

Enzyme precipitation is much faster and easier than enzyme crystallization. Precipitation may be performed at conditions that minimize protein unfolding. When enzyme precipitates are cross-linked with glutaraldehyde,

enzyme aggregates may be obtained that are active and stable in organic media (Cao et al. 2003).

6. APPLICATIONS

The main reasons to apply enzymes in organic media are

- To reverse enzymatic hydrolytic reactions
- To suppress side-reactions that require water
- To increase the substrate solubility
- To simplify product recovery

For a set of 134 published biotransformation processes (Straathof et al. 2002), about 20% involve the use of organic media (Fig 4). For processes using enzymes this percentage is about the same as for processes using cells. The latter are not treated here. The number of synthetic applications on laboratory scale of enzymes involving organic solvents is very large. Therefore, the subsequent overview only mentions examples that have been developed to industrial scale.

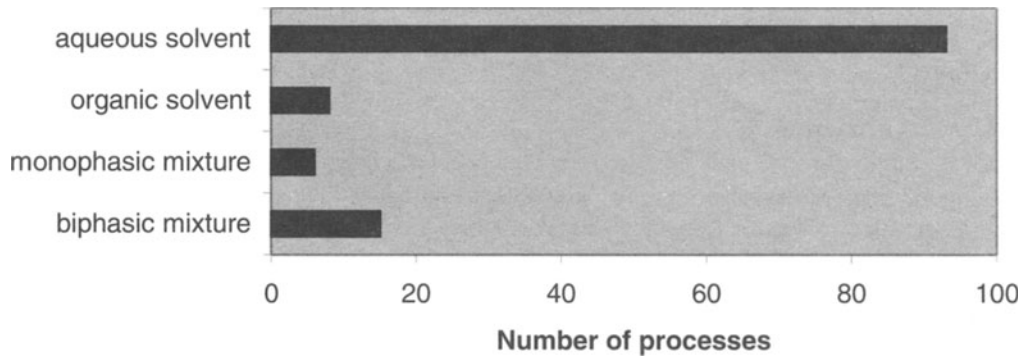

Figure 4. Solvents use in published industrial biotransformations.

6.1. Kinetic resolution of enantiomers

Chiral building blocks have become the most important type of fine-chemicals produced by enzymes (Straathof et al. 2002). A common synthetic route to chiral building blocks is to convert one of the enantiomers of a racemic mixture using an enantioselective catalyst such as an enzyme. For an acceptable yield there are several requirements. The enzyme's enantioselectivity, expressed as the enantiomeric ratio, should be sufficiently high, typically $E > 20$ (Straathof & Jongejan 1997). Besides, the reaction should not stop at an equilibrium if the undesired enantiomer has to be converted completely.

Table 2 gives some industrially important enzymatic kinetic resolutions that are performed using organic media. Kinetic resolution of some chiral alcohols or amines is being performed in anhydrous organic solvents by esterification and amidation, respectively. For full conversion of the undesired enantiomer, activated carboxylic acids have to be used instead of free carboxylic acids. Vinyl acetate is a typical example of a useful acylating agent. A simpler situation occurs when esters are hydrolyzed. At neutral pH, such hydrolysis reactions usually run to completion. However, the substrates typically have a low

Table 2. Some enzymatic kinetic resolutions that have been scaled up to commercial quantities

Racemate	Reagent	Medium	Enzyme	Company	Reference
Amine	Ethyl methoxyacetate	Methyl *tert*-butyl ether	*B. plantarii* lipase	BASF	(Balkenhohl et al. 1997)
Piperidine derivative	Trifluoroethyl isobutyrate	Methyl *tert*-butyl ether	*P. aeruginosa* lipase	Schering-Plough	(Morgan et al. 2000)
2-methoxy-cyclohexanol	Vinyl acetate	Cyclohexane	*C. antarctica* lipase B	Glaxo Wellcome	(Stead et al. 1996)
Glycidyl butyrate	Water	Substrate emulsion with water	Porcine pancreas lipase	DSM and others	(Kloosterman et al. 1988)
Phenylglycidyl ester	Water	Toluene-water	*S. marcescens* lipase	Sepracor, DSM	(Matsumae et al. 1999)
Allothronone acetate	Water	Substrate emulsion with water	*Arthrobacter* lipase	Sumitomo	(Hirohara et al. 1985)
Phe ester	Water	Toluene-water	α-chymotrypsin	Stauffer	(Dahod & Empie 1987)

aqueous solubility, so that using a biphasic aqueous-organic mixture is quite common. The hydrolysis of glycidyl butanoate is a typical example of a kinetic resolution where the organic phase consists of liquid reactant only, without organic solvent addition.

The processes are typically performed in batch mode in a stirred tank. Sepracor, however, has developed the use of kinetic resolution in membrane reactors, with aqueous and organic phase on either side of the membrane and enzyme adsorbed on it.

6.2. Asymmetric synthesis of enantiopure hydroxynitriles

Hydroxynitriles are useful synthetic intermediates, especially when they are in the form of pure *(R)*- or *(S)*-enantiomer. Pure enantiomers can be prepared using enzymatic synthesis in reactions between aldehydes or ketones and hydrogen cyanide (Brussee & Van der Gen 1999). A commercial-scale example is the production of *(S)*-3-phenoxymandelonitrile by DSM (Pöchlauer 1998). The hydroxynitrile lyase from rubber tree (*Hevea brasiliensis*) has been expressed in a microbial host and is being used in a batch stirred tank for this reaction. Other hydroxynitrile lyases can be used in the same way, so the formation of a large range of enantiopure *(R)*- and *(S)*-hydroxynitriles is possible.

The aldehyde and HCN used as reactants can react spontaneously as well, leading to undesired racemic product. To suppress this spontaneous reaction as much as possible, the aldehyde concentration in the aqueous phase that contains the enzyme should be quite low. On the other hand, high aldehyde loads to the reactor and high conversion rates are desirable. Therefore aqueous-organic biphasic liquids

are used. The organic phase serves as a reservoir for aldehyde. A low aqueous phase aldehyde concentration is obtained by using so much enzyme that the rate of transfer of aldehyde from organic phase to aqueous phase becomes limiting (Gerrits et al. 2001, Willeman et al. 2002). The latter mass transfer rate will depend on the interfacial area and therefore on the aqueous/organic phase volume ratio, amongst others.

6.3. Peptide and semisynthetic penicillin synthesis

Activated esters are not only used for esterification and amidation in pure organic solvents but, when the reactants are insoluble in organic solvents, also in monophasic aqueous-organic mixtures. In this case some hydrolysis of the activated ester occurs. This undesired hydrolysis competes with its conversion in the desired synthesis reaction. Secondary hydrolysis of the synthesized product will also occur. By using cosolvent, the hydrolysis reactions are suppressed to some extent. This approach is applied for example by Novo Nordisk for converting pro-insulin to human insulin using trypsin (Mollerup 1999). Similar approaches can be used for other peptides (Gill et al. 1996), and for semisynthetic penicillin antibiotics. If the enzymes are compatible with anhydrous organic solvents, and the substrates sufficiently soluble, water can be left out. However, an opposite trend is to develop enzymes that hardly catalyze the undesired hydrolysis reactions, so that the organic solvent can be left out.

6.4. Glycoside synthesis and esterification

A conceptually related reaction is glycoside synthesis. In water-organic solvent mixtures, enzymatic transglycosylation can be carried out, but in the absence of water the reaction yield may be better because the competing hydrolysis reactions are suppressed (Van Rantwijk et al. 1999). BioEurope has developed a process for converting maltose in butanol to butyl a-glucoside using a transglucosidase (Monsan et al. 1996). Subsequently, an enzyme-catalyzed esterification was carried out. Enzymatic glycoside esterification has been developed to process scale by Novo Nordisk (Björkling et al. 1991). *Candida antarctica* lipase B (*Ca*LB) was used in a reaction medium to which no solvent was added.

6.5. Fats and their derivatives

In addition to the aforementioned esters, a range of esters such as isopropyl palmitate and isopropyl myristate have been produced industrially using immobilized *Ca*LB (Björkling et al. 1991). The reactions are carried out in solvent-free systems containing melted fatty acids. In order to get high yields in the reactions, water is removed continuously. The esters are common components in cosmetics and skin-care products.

Lipases are also used as transferase for exchanging fatty acids in fats. This is of considerable interest to the food industry. The enzymatic production of cocoa butter substitutes is the best known example. Cocoa butter is the fat component of chocolate. It melts at body temperature, but natural cocoa butter is rather expensive. Fats with melting behavior virtually identical to that of natural cocoa butter are now produced industrially starting from cheap palm oil fractions. The catalysts used are lipases which are specific for the 1- and 3-positions in the triglyceride molecules (Björkling et al. 1991). The most common reaction of fats is their hydrolysis. This is commonly done using inorganic bases as catalyst, but in Japan lipases are also applied (Hoq et al. 1984). Aqueous-organic biphasic systems are used, without organic solvent addition, and continuous reactors may be used.

6.6. Effects on safety and environment

From the industrial point of view, it is advantageous to work with minimal amounts of solvents. This minimizes the reactor size and costs, and the product recovery costs. The extreme case is to omit the solvent completely. This is also attractive because organic solvents cause hazards with respect to explosions, health and environment.

It is ironic that in the same time that chemical industry started trying to shift towards water as the solvent, enzymatic catalysis in organic solvents was being developed. Therefore it is very important that for applied studies on enzymatic catalysis only organic solvents are used that are still acceptable for industrial purposes. Table 3 list extraction solvents for natural flavorings recommended by the IOFI Committee of Experts. Carrier solvents, other flavoring substances and some natural materials also can be used as extractions solvents; their limits have not been specified.

In addition to toxicity, fire and explosion hazards will have to be considered. For ionic liquids, investigation of health and safety properties is still in its infancy. Supercritical carbon dioxide is worth mentioning in particular, because it is nontoxic and nonexplosive, and can be removed easily after the enzymatic reaction (Hartmann et al. 2000). As solvent, supercritical carbon dioxide resembles hexane, hence it is water-immiscible and dissolves hydrophobic compounds easily. The main drawback with supercritical reaction media is that high pressures must be used, which requires special reactors and other equipment.

Table 3. Extraction solvents for natural flavorings recommended by the IOFI Committee of Experts as of January 1997, and their maximum concentrations in the flavorings when used for food purposes

Solvent	Max. concentration / p.p.m.
Propane	1
Butane	1
Isobutane	1
Hexane	1
Cyclohexane	1
light petroleum	1
Toluene	1
Methanol	10
butan-1-ol	10
Acetone	2
Butanone	2
diethyl ether	2
dibutyl ether	2
methyl *tert*-butyl ether	2
ethyl acetate	10
Dichloromethane	2

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Biotransformations with Crude Enzymes and Whole Cells

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1. INTRODUCTION

Biotransformation can be defined as a process dealing with the conversion of a compound, often called precursor, into structurally related compound(s) by a biocatalyst in a limited number of enzymatic steps. Such operation is also called microbial transformation or even bioconversion. Straathof et al (2002) thus define biotransformation as "a reaction or a set of simultaneous reactions in which a pre-formed precursor molecule is converted, in contrast to a fermentation process with *de novo* production from a carbon and energy source such as glucose via primary metabolism". The biocatalyst can be whole cells, spores, crude enzymes or purified enzymes.

Several other terms, such as biodegradation or biocatalysis, are also used in this area. They have similar meanings, but are used in more restricted contexts. The term biocatalysis is used similarly to biotransformation, but it has the additional connotation of making a useful compound by an enzymatic system, which is not necessarily purified but can be engineered. For example, the synthesis of acrylamide by microorganisms is often referred to as biocatalysis. The term biodegradation, by contrast, is most typically used when the focus is on taking a compound away, ideally, a process by which a potentially toxic compound is transformed into a nontoxic one or, better, to carbon dioxide. Bioremediation is a more recently coined term, which refers to the application of biodegradation reactions to the practical cleanup of a medium such as soil containing a pollutant (Wackett 2001a,b).

2. HISTORY OF BIOTRANSFORMATIONS

Most of biotechnological productions of food have been practised for hundreds or even thousands of years. For example, Sumerians and Babylonians practised beer brewing before 6000 B.C. references to wine making can be found in the book of genesis, and Ancient Egyptians used yeast for baking bread.

Biotransformations were observed by humans well before they were appreciated as having an underlying microbial cause. Indeed, the knowledge of chemical's production by biotransformation is relatively recent and the first reports in the literature appeared only in the second half of the 19th century. One of the oldest examples of biotransformation processes is vinegar production, which dates back to some 2000 years B.C. but has only been clearly described 200 years ago.

Since the important discoveries of Pasteur and other scientists at the end of 19th century, many chemicals as alcohol or organic acids have been produced by microorganisms or resting cells. Details about history of industrial biotransformations are developed in a recent paper by Vasic-Racki (2000). Rapidly,

scientists were interested by this green technology. This approach, compared to chemical transformations, is generally carried out at ambient temperature, neutral pH and without the need for high pressure and extreme conditions. Moreover their chemo-, regio- and stereoselectivities reported at the end of the 1800's simplify manufacturing processes and make them even more economically attractive. The properties of enzymes became generally understood from kinetic studies conducted in the early 1900's (Michaelis & Menten 1913). Enzyme research has led to the introduction of new processes involving immobilized cells or biocatalysis in organic media. This development of biotechnological processes allowed to produce a variety of very important substances, e. g. penicillin, streptomycin and other antibiotics, steroids or amino acids.

In the 1950's, one of the main scientific achievements has changed the approach of biotransformation. The discovery of double helix structure and the chemical nature of RNA and DNA has led to used molecular biology to develop new biotransformation processes. Applications of this technology to the industrial production of small molecules began in the 1980's. Since this time, a lot of processes such as optimization of enzyme activity by site selective mutagenesis, transfer of the gene into high productivity microorganisms, and overexpression by incorporation of a promoter into a gene are used to improve biotransformations.

Currently, microbiology is entering an important stage with respect to understanding and using the catalytic potential of microorganisms. Microbes have become important tools for chemical synthesis, a trend that will almost surely accelerate. Based on its past, the future of microbial transformation looks attractive.

3. BIOCATALYST SELECTION

3.1. Introduction

The discovery of new biocatalysts can be a chance as for *Penicillium notatum* strain isolated by Fleming, coming from accidental contamination of an agar plate seeded by *Staphylococcus aureus.* But most of the time, important strains selections were carried out by screening methods. Primary screening is designed to detect and isolate microorganisms with potentially interesting commercial applications, eliminating many unwanted microorganisms and or products and allowing detection of the small percentage of potentially interesting isolates. It is a key step in process development, but it is obviously very difficult to propose a rational method of screening for novel enzymes or microorganisms. However, there are three important stages in a general strategy:

- designing the process and deciding the type of enzymatic activity desired;
- deciding which groups of microorganisms have to be selected and screened;
- designing an appropriate, convenient and sensitive assay that will allow as many microorganisms as possible to be screened.

3.2. Use of existing biocatalysts

A well-known way to accomplish a desired biotransformation is the use of existing microorganisms or enzymes on natural and unnatural substrates. In order to select the most appropriate biocatalyst, it is at first necessary to search for informations in the literature. Nowadays, Internet services have revolutionised

the rate at which information can be retrieved, and searches that used to take hours can now be done in seconds. Many of these services have restricted access or require a fee, but some are free and the web enables them to be accessed over national boundaries (Kelly 1998). For example, the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) provides links to genomic, peptide sequences, bibliographic and many other databases. Similar information and links are provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/). Moreover, searches in these databases can be linked so that query results in one database can be used to search another. This is by this way that University of Minesota has structured its website (http://umbbd.ahc.umn.edu/) which is the most interesting and complete database on biocatalysis and biodegradation, since it is directly connected to Kyoto University Ligand Chemical Database (DGBET), Kyoto Encyclopedia of Genes and Genomes (KEGG) and the EMBL Nucleotide Sequence Database (also known as EMBL-Bank).

Information can also be found on electronic supports such as the "Biotransformations" CD-ROM (http://www.accelrys.com) which covers the metabolism of drugs, agrochemicals, food additives, and industrial and environmental chemicals. It included over 40,000 reactions in the first release alone but it is very expensive except for institutional use.

Microbial cultures can be obtained from permanent culture collections, the number of which is continuously growing. For example, Martin and Skerman (1972) list 349 collections in their "World Directory of Culture Collections of Microorganisms, while 476 are now registered at the Culture Collections in the World website (http://wdcm.nig.ac.jp/hpcc.html). An important list is also given on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) website (http://www.dsmz.de/species/abbrev.htm).

3.3. Genetic modification of existing biocatalysts

As soon as an useful organism emerges from a screening programme, it becomes necessary to improve its productivity for the metabolite under evaluation. The initial phases of the biotransformation development will often consist of modifications of the medium and bioconversion conditions used, but the major source of progress will be to improve the performance of organisms by selection and genetic manipulation.

Modern methods for the mutational creation of diversity start with sequencing of the gene of the desirable enzyme. For many bacterial genes, complete sequences are already available in databases. Most enzymes can be expressed in heterologous well characterized hosts like *E. coli*, *B. subtilis*, or yeasts. After establishing a heterologous expression system, various methods may be used for creation of diversity by mutagenesis, directed evolution, or by combinatorial methods. Most popular are random mutagenesis, error prone polymerase chain reaction (epPCR) (Mihara et al 2000), and gene shuffling (Crameri et al 1998). All these techniques are described in more details in this book in the chapter on protein engineering of industrial enzymes written by Kammonen et al.

3.4. Screening for novel biocatalysts

Despite the fact that to date more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the present toolbox is still not sufficient to meet all demands, in particular because they do not withstand industrial reaction conditions. That's why considerable efforts have been devoted to the search for new biocatalysts and new enzymes.

Screening may be achieved by genetic selection. A system must be set up in which the presence of the catalytic activity provides a growth advantage to the bacteria or microorganism hosting it. This approach includes the simple use of the substrate of interest as sole carbon source for growth, as well as a range of other schemes for linking catalytic activity to a survival factor as summarized in Table 1.

Table 1. Isolation procedures for specific types of microorganisms

Conditions	Property or organism selected for
Carbon source	Specific catabolic functions
Nitrogen source	Specific catabolic functions; ability to use specific N
Temperature	Psychrophile, mesophile, or thermophile
pH	Acidophile, alkophile, or growth near neutrality
Heat-shock resistance	Spore and cyst former
Oxygen tension	Anaerobe, microaerophile, or aerophile
Frequency of transfer	Fast- or slow-growing organisms
Metals	Organism needing metals in high concentration
Organic solvents	Organic solvent resistance

Although microorganisms are ubiquitous, the most common sources of industrial microorganisms are soils, lake and river mud. Isolation of microorganisms from the environment is an important way to obtain new enzymes and to improve the productivity of known compounds of industrial interest.

The past few years have seen a great increase in the availability of enzymes from the so-called extremophile organisms that grow under extremes of temperature, pH, pressure, or salt concentration. This increased availability is attributable to some extent to advances in the technologies for cultivating organisms from extreme environments, but more to the ability to move genes from extremophiles into conventional hosts, and, thus, produce enzymes with extremophilic properties under milder, less expensive growth reaction.

Table 2 lists extremophiles by habitat and some applications of their enzymes. Most of the work has been devoted to thermophiles and hyperthermophiles, but other groups have received more attention recently because of their biotechnological potential.

3.5. General procedures for isolation and selection of microorganisms

3.5.1. Selective media – Culture enrichment

The more classical method to isolate new bacteria is direct selection on solid media. A natural microbial source, for example a soil sample, is plated directly or diluted to give a microbial cell concentration such that aliquots either applied directly or sprayed using a variety of procedure. For example, Kleinheinz & Bagley (1997) have developed a rapid and inexpensive method to recover and cultivate microorganisms that are able to grow on petroleum hydrocarbons (PHCs) by placing the growth substrate on a filter pad in the lid of a Petri dish containing a minimal agar medium and microorganisms.

Enrichment culture is also frequently used in order to isolate microorganisms having special growth characteristics. It allows selective cultivation of one or more bacterial strains obtained from a complex

Table 2: Industrial applications of enzymes isolated from extremophiles (adapted from Demirjian et al 2001)

Extremophile	Habitat	Enzymes	Representative applications
Thermophile	High temperature Moderate thermophile (45-65°C) Thermophile (65-85°C) Hyperthermophile (>85°C)	Amylases Xylanases Proteases DNA polymerases	Glucose, fructose for sweeteners Paper bleaching Baking, brewing, detergents Genetic engineering
Psychrophile	Low temperature	Proteases Dehydrogenases Amylases	Cheese maturation, dairy production Biosensors Polymer degradation in detergents
Acidophile	Low pH	Sulfur oxidation Chalcopyrite concentrate	Desulfurization of coal Valuable metals recovery
Alkalophile Halophile	High pH High salt concentration	Cellulases	Polymer degradation in detergents Ion exchange resin regenerant disposal, producing poly (γ-glutamic acid) (PGA) and poly (β-hydroxy butyric acid) (PHB)
Piezophile	High pressure	Whole microorganism	Formation of gels and starch granules
Metalophile	High metal concentration	Whole microorganism	Ore-bioleaching, bioremediation, biomineralization
Radiophile	High radiation levels	Whole microorganism	Bioremediation of radionucleide contaminated sites
Microaerophile	Growth in < 21% O_2		

mixture such as that found for example in most soils. The method typically relies on using a particular organic compound as the sole carbon source or, less frequently, as the nitrogen, sulphur, or phosphorus sources. The choice of medium composition or the conditions used in the enrichment culture favours the growth of desired forms.

Resistance to organic solvents is often an important criterion in the selection of suitable biocatalysts. For example, *Pseudomonas* strains have been isolated with the ability to grow in the presence of toluene and aromatic and aliphatic hydrocarbons and long chain alcohols. Many techniques have been improved to avoid toxic effect of these compounds. The problem of volatile and water-insoluble compounds can be overcome by providing the substrate as a vapor, with the liquid suspended in a glass bulb above the growth medium in an enrichment medium or by flushing a volatile compound in the gas phase. Biphasic systems can also be used with organic solvents having low toxicity ($\log_{10}$ Kow>4) (Fontanille & Larroche 2002).

When a toxic or unnatural compound is used as a substrate, an acclimatation technique can be applied, and usually run long term to isolate microorganisms which are not easily isolated by enrichment culture. An adaptation to a synthetic medium containing a target compound often results in the isolation of microorganisms having a new enzyme. Genetic changes in the microorganisms may be expected (Asano

2002). Microorganisms isolated by these procedures are called 'extrachemophiles', showing characteristics on the ability to transform toxic compounds or synthetic compounds, in contrast to microorganisms living in extreme environments named 'extremophiles'.

3.5.2. High-throughput screening

High-throughput screening methods for catalysis have a key role in the search for new enzymes and biocatalysts. Indeed, today, massive screening of diversity is perceived as the most efficient way to find new enzymes. These methods can be applied for screening libraries generated artificially by randomizing the gene that encodes an existing enzyme or collected directly from the biosphere, which is a rich reservoir for new enzymes. In practice, the diversity being considered exists either in libraries of plasmids or phages expressed by bacteria, or in libraries of microorganisms growing in culture. In these conditions, screening is performed by activity detection compared to classical methods where screening is achieved by genetic selection.

There have been recent advances in catalysis assays applicable for screening biocatalyst libraries in high-throughput format. These include instrumental assays such as high-performance liquid chromatography, mass spectrometry, capillary electrophoresis and IR-thermography, reagent-based assays producing spectroscopic signals (UV/VIS or fluorescence) in response to reaction progress, and assays based on fluorogenic or chromogenic substrates. Some of these methods recently developed are summarized in Table 3.

Table 3: Assays for biocatalysts screening

Method type	Techniques	References
	HPLC	Stahl et al (2000)
	MS	Greenbaum et al (2000)
Instrumental methods	CE	Reetz et al (2000)
	IR-thermography	Holwarth et al (1998)
	RMN	Entzeroth (2003)
	Flow Cytometry	Katsuragi & Tani (2000)
Selective substrate-binding or product-binding proteins		
Sophisticated reagents	QUEST	Firestine et al (2000)
	Cat-ELISA	Tawfik et al (1993)
	Competitive cat-ELISA	McBeath & Hilvert (1994)
Chromometric/fluorometric	TLC	Walher & Reymond (2001a)
	FRET	Olsen et al (2000)
	Product staining	Taylor et al (1999)
Phage display		Marrs et al (1999)

3.5.2.1. Instrumental methods

By monitoring the exact reaction of interest, instrumental approaches such as analysis by HPLC, MS and capillary array electrophoresis (CE) are amenable to detect biocatalysts targeted for a particular application. This assay is expensive, however, and limited to a few hundred samples per instrument per day in the best cases. IR- thermography might provide a general solution by monitoring the heat evolution of reaction in microtiter plates. Techniques recently reported such as flow cytometry used in combination with single-cell sorting can be a powerful method for the identification and isolation of microbial cells with particular characteristics, especially when such cells grow more slowly than other ones in a large heterogeneous population.

3.5.2.2. Selective substrates

The most convenient reactions used as assay in high-throughput screening are those involving chromogenic or fluorogenic substrates. Many such substrates are selectively taken up by live cells and can be used to monitor catalysis *in vivo*. Moreover, fluorogenic substrates enable the assaying of a variety of enzymes in enantioselective and stereoselective manner.

Another possibility is the preparation of product-specific monoclonal or polyclonal antibodies (cat-ELISA) either on solid phase or by direct reaction monitoring in solution (homogeneous cat-ELISA). Unfortunately the preparation of antibodies is not always possible. A well-developed database on the methodologies to be used for new biocatalysts identification has recently been described by Demirjian et al (2001) and Whaler & Reymond (2001b).

3.6. Conclusions

Today, massive screening of diversity is perceived as the most efficient way to find new enzymes. Diversity is either generated artificially by randomizing the gene that encodes an existing enzyme, or collected directly from the biosphere, which is a rich reservoir for new enzymes. New assays afford a lot of possibilities to determine biocatalysts activity and allow to discover new classes of organisms and facilitate the development of new biotransformations.

4. BIOCATALYST TREATMENT AND MODE OF OPERATION

4.1. Biocatalyst form

When a biocatalyst has been found for a target reaction (see below), the main question is how to ensure maximal activity and stability at the lowest cost. The first step is then to decide if the entity to be used will be under the form of a purified enzyme or of whole cells. The first criterion for the choice is the complexity of the reactions to be performed. Hence, it remains difficult to obtain good results when more than two purified enzymes are involved in the same medium. An example of this situation is methyl ketone synthesis from fatty acids, which can be performed only by whole cells (see paragraph 6). Also, use of whole cells can be advantageous to carry out oxidation reactions since the respiratory chain will provide an efficient tool to re-oxidize reduced cofactors. It should be noticed that this may not be true for the reverse process (reduction) because the supply of reduced cofactors can be rate-limiting. Main features belonging to each biocatalyst form are summarized in Table 4.

If it is a one-step process, an enzymatic approach can be envisaged. The main question at this stage is to check whether the enzyme is commercially available or not. In the later case, the necessity of enzyme

Table 4: Main characteristics of purified enzymes and whole cells when used as biocatalysts

Purified enzyme	Whole cell
– maximal specific activity – generally good selectivity of the reaction (one product from a given precursor) – generally good enantio-and/or regio selectivity – commercially available ? – purification cost	– able to carry out complex reactions involving several enzymes – no enzyme purification cost – useful in oxidation reaction (cofactors readily re-oxidized by therespiratory chain) – yields can be lowered by • incorporation of the precursor in biomass (carbon accumulation). Especially true with fungi • lowering of the selectivity (synthesis of by-products) • lowering of the enantio/regio-selectivity if several enzymes are able to carry out the same reaction within cells. – transport of substrate/product across cell wall/membrane can be rate-limiting – cells are often more sensitive to inhibition phenomena than purified enzymes

purification has to be proven. Key points for that are of course an analysis of separation costs, but also parameters such as selectivity of whole cells for the target reaction, specific activity, need for cofactors, etc (Table 4).

4.2. Biocatalyst treatment

Although some special procedures, especially freeze-drying, can be useful for enzymes to be used in organic media (see chapter on this topics), this section applies mainly to whole cells. The goal is again to find the way to achieve the best rates and yields. The first point to be addressed is to decide if the biocatalyst has to be used as growing or resting cells, which has a tremendous influence on the process which will be used. Hence, the first approach will need a complex medium including all nutrients for growth, and the entire process will be one-staged.

The second one will involve two steps, the first being the biocatalyst production itself, followed by the biotransformation in a simple medium (generally a buffer containing the precursor and sometimes an energy source).

The need for growing cells often means that the precursor is metabolized by the so-called cometabolism phenomenon. It corresponds to the situation where a micro-organism uses the enzyme apparatus elicited by growth on a given carbon source for simultaneous metabolism of related or even quite unrelated compounds which act mainly as energy suppliers. This phenomenon is quite common in xenobiotic

degradation and allows full oxidation of the precursor to carbon dioxide and water (Engesser & Plaggemeier 2000). More interesting here is the incomplete cometabolism, which allows a metabolite to be accumulated, as in the case of β-ionone hydroxylation by *Aspergillus niger* (see the chapter on bioreactor analysis and design).

The use of resting cells, when possible, offers the possibility to separately optimize growing conditions (for biocatalyst production) and biotransformation parameters. As pointed out in Table 4, precursors/products have to enter/go out of the cells, i.e. they must cross the membranes. This transport phenomenon, which is often of passive nature for most of biotransformation cases, may be rate-limiting. A way to improve this point can be cell permeabilization. This operation can allow both better solute entrance in the cells and enzyme efflux, i.e. the reaction can take place inside and outside the biocatalyst. It is also possible to make cells permeable to low molecular weight molecules while enzymes and other macromolecules remain within cells at unchanged concentrations (Freire et al 1998).

Possible treatments can be of physical and/or chemical nature. Chemical methods mainly involve the use of polar organic solvents such as diethylether, chloroform, toluene (Felix 1982, Jackson & Demoss 1965) or detergents like Triton® X-100 (van der Werf et al 1995, Felix 1982, Galabova et al 1996). Other compounds such as antibiotics, enzymes chelating agents (EDTA) can be useful in some cases (Freire et al 1998, Ferrer et al 1996). The main difficulty associated with detergents is that those compounds are very hard to eliminate from the medium, giving a more difficult further product purification. This drawback does not exist with organic solvents (Fontanille & Larroche 2003).

Physical treatments include osmotic or thermal shocks, or sonication. Thermal treatments are only applicable to thermostables enzymes (Sangiliyandi & Gunasekaran 1998, 2000). Ultrasounds are an efficient tool to disrupt cells and to obtain crude cell extracts (Sangiliyandi & Gunasekaran 2000), but this technology is difficult to scale-up. Application of freezing and thawing cycles can allow the release of low molecular weight proteins (Johnson & Hecht 1994, Fontanille & Larroche 2003). Combination of two operations can sometimes give an additive effect (Felix 1982, Griffith & Wolf 2002).

It should be noticed that this kind of treatment (permeabilization) appears to be useful when enzyme(s) to be used are autonomous, i.e. they do not require any cofactors. In the last case, it appears to be very difficult to improve membrane transports without lowering the metabolic activity of the biocatalyst, which decreases the rate of cofactor regeneration processes.

4.3. Mode of operation

Most of modern biotransformation processes can be considered as being immobilized systems. Immobilization deals with any situation where a component of the system cannot readily access to some part of the medium. This very wide definition considers that the "component" can be either the biocatalyst or a solute (substrate and/or product)

4.3.1. Biocatalyst immobilization

A wide range of basic immobilization procedures with their specific variations has been described in a large number of reviews (Cabral 2001, Rosevear 1984, Hartmeier 1985, Bucke 1983, Larroche & Gros 1997, Willaert & Baron 1996). A series of papers dealing with immobilized cells applications in the food area has also to be pointed out (Norton & Vuillemard 1994, Divies et al 1994, Masschelein et al 1994, Champagne et al 1994, Groboillot et al 1994).

Several classification schemes have been proposed. A simplified and broad version close to that in Cabral (2001), is proposed in Figure 1. It should be noticed that all methods are not useful for all kinds of biocatalysts. For example, the carrier binding technique is practically restricted to isolated enzymes, while entrapment within a matrix is useful only for whole cells. The most popular support for this last approach is Ca-alginate, obtained from Na-alginate by ionotropic gel formation in the presence of $CaCl_2$ (other divalent cations can also be used) (Smidsrød & Skjåk-Braek 1990). Several devices are commercially available to produce beads with defined diameter (see for example http://www.nisco.ch, http://www.instech.ch).

It should be pointed out that the frontier between entrapment and encapsulation is not always obvious in the literature. Hence, many authors consider that microcapsules (encapsulation technique) and microspheres (entrapment) both belong to the microencapsulation technology, at least in the case of controlled release of molecules (Richard & Benoit 2000).

Another remark deals with systems involving a water immiscible organic solvent, being thus of biphasic nature. They are considered as an immobilization technique, since the biocatalyst is confined in the aqueous layer. Use of whole cells in this kind of medium sometimes affords an emulsion, which is naturally stabilized by surfactants or proteins present in the biocatalyst (Fontanille & Larroche 2003).

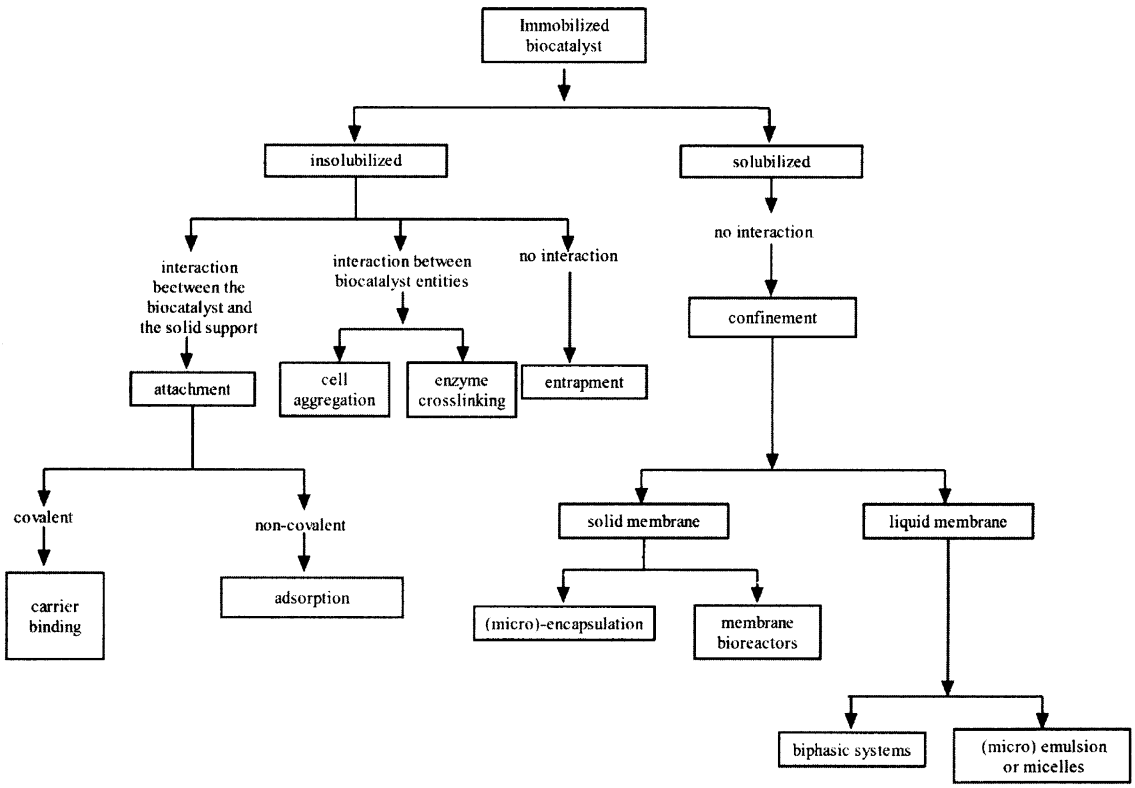


Figure 1: General methods for biocatalysts immobilization

This point again enphasizes the difficulty (and the variety) while attempting to classify immobilization procedures.

Among problems associated with the use of organic solvents, we can consider their generally non-negligible volatility, which can be an environmental concern. New developments in this area are room-temperature ionic liquids (RTILs), especially those based on the n-alkylimidazolium cation. These environment friendly solvents have many useful properties, among them negligible vapor pressure and excellent chemical and thermal stabilities (up to 400°C). Additionally their physical properties, such as density, viscosity, melting point, polarity, and miscibility with water or organic solvents, can be finely tuned by changing either the anion or the substituents in the cation or both (Lozano et al 2001, Brennecke & Maginn 2001). It has been shown that RTILs can be used in biotransformation processes (Lozano et al 2001, Howarth et al 2001, Erbeldinger et al 2000). The more widely used compound in this area is 1-butyl-3-methylimidazolium hexafluorophosphate (bmim) PF_6 (Fig 2). Their quite high cost yet prevents the generalization of their use.

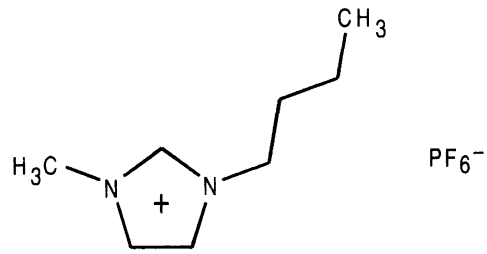


Figure 2 : Chemical structure of (bmim)PF_6

4.3.2. Solute immobilization

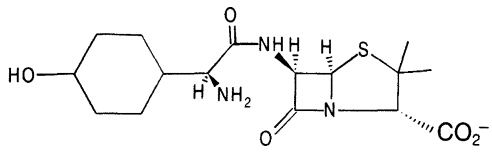
The biocatalyst can here occupy nearly all the liquid reaction medium, while the substrate and/or the product are preferentially located elsewhere. This situation arises in mainly two cases. The first deals with the use of substances poorly soluble in water, at concentrations higher than their solubility. It exists then an organic layer made of nearly pure organic solute, which can be present under the form of droplets if the volume is too low to form a continuous layer. An example of such situation is illustrated in Grivel et al (1999). Another possibility is the use of solid adsorbents. These materials are devoted to act as scavengers, generally for a product. Improvements of reaction productivities, due to prevention of metabolite(s) inhibition have been reported (Bais et al 2001, Zorn et al 2004).

5. BIOTRANSFORMATIONS

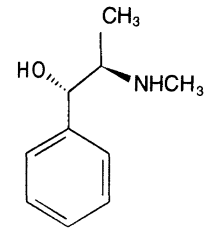
5.1. Asymmetric synthesis

Biotransformations often are preferred to chemical processes when high specificity is required, to attack a specific site on the substrate and to prepare a single isomer of the product. While chemical methods usually lead to the formation of a mixture of isomers and by-products, biotechnological methods are suited to achieved this type of transformation, as enzyme generally show a pronounced regio- and stereoselectivity.

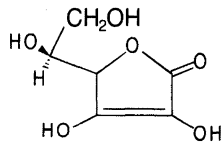
Methods in chiral chemical synthesis have progressed remarkably, but enzymes are still the catalyst of choice when a product is needed in 100% enantiospecificity. For example the drug thalidomide has important medicinal value only in one specific enantiomeric configuration, the non-active one leading to birth defects. Lot of pharmaceutical or fragrance products have one or more chiral carbon atoms in the structure (Figure 3). In those cases, decision to use a biocatalyst in synthesis was driven by the desire to obtain an enantio-specific product. Many reviews give a general idea on the importance of chiral carbon in medicine and a way to obtain chiral building blocks or synthesis of drugs and other organic compounds (Souza de Peirera 1998, Patel 2001)



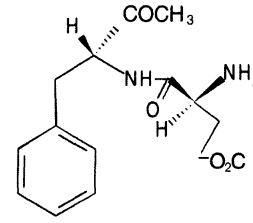
Amoxicillin
(Antibiotic)



D-Ephedrine
(CNS stimulant)



L-Ascorbic acid
(Vitamin C)



Aspartame
(Sweetener)

Figure 3: Examples of chiral commercial compounds produced by combined microbial-transformation and organic-synthesis steps

5.2. Steroids

The production of steroid drugs and hormones is one of the best example of the successful application of microbial technology in large scale industrial processes. Importance of microbial production of steroids, which are compounds structurally derived from cyclopentanoperhydrophenantrene, was realized around 1950 with the announcement of the pharmacological effects of two endogenous steroids, cortisone and progesterone.

Since the patent of Murray and Peterson in 1952 about process of 11α-hydroxylation of progesterone by a *Rhizopus* species, many researchs have been carried on microbial transformation steps focusing mainly on steroid hydroxylations, Δ^1-dehydrogenation and sterol side chain cleavage. An entire book would be necessary to review steroids biotransformation history. Overview of recent advances in microbial steroid biotransformations has been recently published by Mahato & Garai (1997) and Fernandes et al (2003).

5.3.Terpenes and terpenoids

Terpenes and terpenoids are the primary flavor and fragrance impact molecules found in the essential oils of higher plants. They are naturally branched chain C-10 hydrocarbons formed from isoprene units and are widely distributed in nature (Berger 1995). Terpenes are traditionally isolated from essential oils. They are extracted or distilled from odorous plant material but this source of natural products has several serious drawbacks. It's actually difficult to control seasonal variation or variability in the composition and yield of the final product. As a result the price of the given essential oil is a highly fluctuating variable. Biotransformation is an efficient way to avoid these inconvenients.

The first work dealing with microbial transformation of terpenes can be attributed to Mayer and Neuberg who reported in 1915 the synthesis of (+)-citronellol from (+)-citronellal by yeast (van der Werf et al 1997). Since this time, many research has been performed, and some recent reviews have summarised results obtained (van der Werf et al 1997, Schrader & Berger 2001).

These compounds are generally both volatile and lipophilic, which poses several problems. Their low water solubility often leads to the presence of an excess substrate that gives an organic layer. They also often exhibit a rather low chemical stability when poured into an aqueous environment, and undergo spontaneous autooxidation processes. Their high volatility can additionally give rise to loss by air stripping, especially when the reaction is performed aerobically (Grivel et al 1999; Grivel & Larroche 2001). Another difficulty, encountered when whole cells are used as biocatalyst, is their high toxicity. This feature is generally attributed to a high accumulation in the cytoplasmic membrane, where hydrophobic compounds preferentially accumulate (Sikkema et al 1995, Berger et al 1999).

As a consequence, only a few biotransformation processes have been proposed up to now at an industrial scale in the area of terpenes. Nevertheless, this kind of biotransformations remains of great commercial interest for the food and perfume industry since one of the main advantages in using biotechnological methods for the production of flavor and fragrances is that products can be labelled as natural. This label is particularly attractive for customers and more and more used in food products.

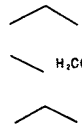
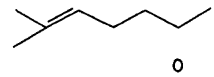
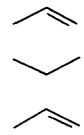
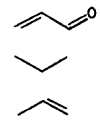
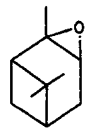
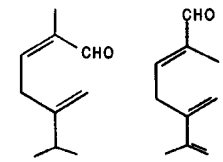
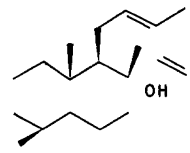
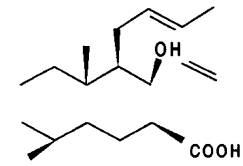
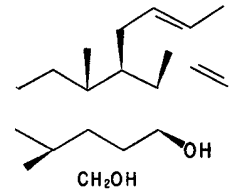
Comprehensive reviews by Krasnobajew (1984) and more recently by Schrader and Berger (2001) focussed on many transformations of terpenoids of interest to the flavor and fragrance industry. These reactions can be carried out by bacteria, fungi or yeasts. A few examples of microbial transformations are summarized in Tables 5a and 5b.

Here we will focus on monoterpenes which represent the main starting material for the majority of microbial transformation of terpenes. These compounds like limonene, α-pinene or β-pinene and their derivatives (terpenoids), are widely occurring in nature and have a strong and pleasant odour which make them important components in the manufacture of flavors and fragrances.

5.3.1. Acyclic monoterpenes

Among this class of compounds, we will consider on geraniol, nerol and citral which are some of the most studied acyclic monoterpenoids. These compounds are generally transformed by fungi and Demyttenaere et al (2000) recommend the use of filamentous fungi culture of *Aspergillus niger* and *Penicillium* species. In this case, the main bioconversion products obtained from geraniol and nerol by liquid cultures of *A. niger* are linalool and α-terpineol. Linalool, α-terpineol and limonene are the main

Table 5 a: Examples of terpenoids biotransformations with bacteriael strains

Microorganisms	Substrate	Biotransformation products	References
BACTERIA *Pseudomonas putida*	 Geraniol	 6-methyl-5-heptene-2-one	Vanderbergh et al 1989 Demyttenaere & de Pooter 1998
Corynebacterium hydrocarboclastum FERM-P 401	 (+)-Limonene	 (-)-L-Carvone	Hasekawa 1972
Pseudomonas gladioli *Escherichia coli*		 α-Terpineol	Cadwallader et al 1989 Chang et al 1995 Tan & Day 1998 van der Werf et al 1999
Nocardia p18.3 *Pseudomonas fluorescens* NCIMB 11671 *Pseudomonas rhodesiae* CIP 107491	 α-Pinene oxide	 Isonovalal Novalal	Griffiths et al 1987a Best et al 1987 Fontanille & Larroche 2002
Nocardia restricta JTS 162 *Rhodococcus erythropolis* JTS 131	 Cis-abienol	 (1)  (2)	Mikami 1988

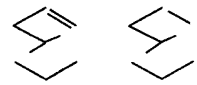
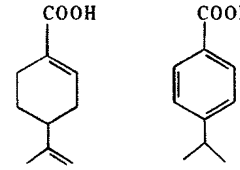
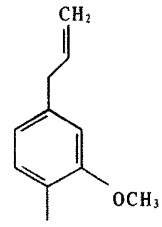
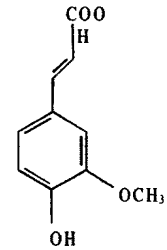
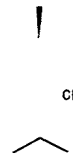
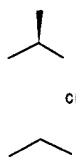
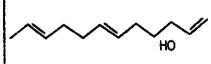
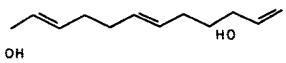
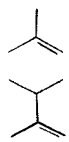
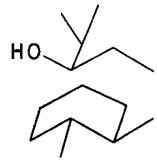
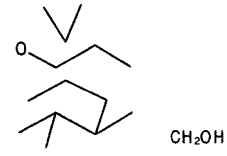
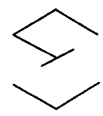
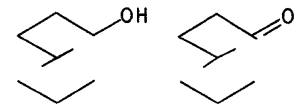
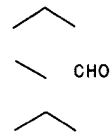
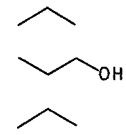
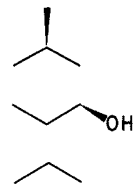
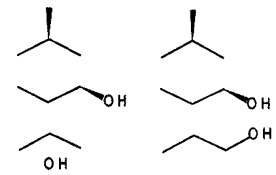
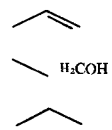
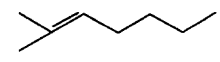
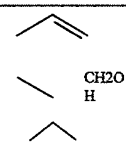
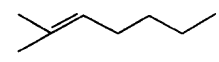
Microorganisms	Substrate	Biotransformation products	References
Pseudomonas sp. Strain PIN	 α-Pinene β-Pinene	 Perillic acid Cumic acid  α-Terpineol	Yoo & Day 2002
RECOMBINANT BACTERIA *Escherichia coli* XL1- Blue	 Eugenol	 Ferulic acid	Overhage et al 2003

Table 5 b: Examples of terpenoids biotransformations with yeast and fungi strains

Microorganisms	Substrate	Biotransformation products	References
YEAST *Rhodotorula minuta* *Candida reukaufii* AHU 3032	 β-(-)-Citronellal	 β-(-)-Citronellol	Kieslich 1976
FUNGI *Aspergillus niger* ATCC 9142	 Trans-nerolidol	 ω-hydroxy-trans-nerolidol	Abraham et al 1988
Penicillium digitatum DSM 62840 *Penicillium digitatum* strains *Cladosporium*	 R-(+)-Limonene	 α-Terpineol	van der Werf et al 1997 Adams et al 2003

Microorganisms	Substrate	Biotransformation products	References
Calonectaria decora ATCC 14767 *Cephalosporium coremioides* NRRL 11003 *Penicillium rubrum* FERM P-3 796 *Paecilomyces carneus* FERM P-3 797	 Patchoulol	 10-Hydroxy-patchoulol	Krasnobajew 1984
Aspergillus niger NCIM 612	 α-Pinene	 Pinocarveol Pinocarvone	Bhattacharyya & Ganapathy 1965
Penicillium digitatum	 Citronellal	 Menthol (93%)	Babicka & Volf, 1955
Aspergillus niger	 L-Menthol	 (31%) (35%) Hydroxymenthol	Harayama et al 1992
Aspergillus niger	 Geraniol	 6-methyl-5-heptene-2-one	Demyttenaere et al 2000
	 Nerol	 6-methyl-5-heptene-2-one	Demyttenaere et al 2000

products obtained from nerol and citral by sporulated surface cultures, whereas geraniol is converted predominantly to linalool. Bioconversion of nerol with *Penicillium chrysogenum* yielded mainly α-terpineol and some unidentified compounds. With *P. rugulosum* the major bioconversion product from nerol and citral is linalool (Figure 4).

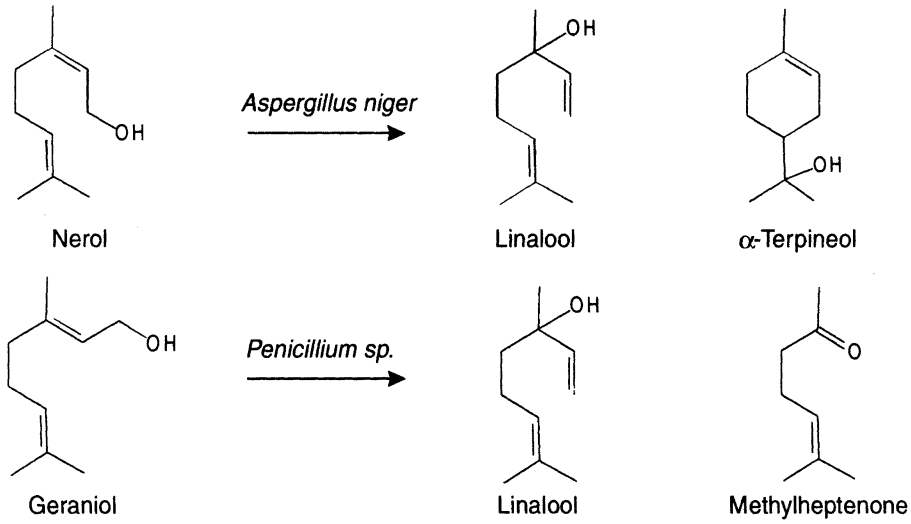


Figure 4: Main products obtained by conversion of nerol and geraniol by various microorganisms (adapted from Demyttenaere et al 2000 and Schrader & Berger 2001).

Methylheptenone is also the main product in biotransformation experiments with *Penicillium digitatum*, starting from both the alcohol nerol and the aldehyde citral, of which the latter is the racemate consisting of the cis and trans isomers geranial and neral (Demyttenaere et al 2000).

5.3.2. Monocyclic monoterpenes: limonene

The two enantiomers of limonene are the most abondant monocyclic monoterpenes in nature. They are the major constituents of citrus essential oils and by-products of the citrus processing industry, amounting to about 50 million kg year^{-1} (Schrader & Berger 2001). The biosynthesis of limonene and other monoterpenes in plants was recently reviewed by Wise & Croteau (1999). Consequently, this terpene is a very interesting substrate for the production of a variety of terpenoid flavor and fragrance compounds and is probably one of the most studied in biotransformation research. Its biotransformation, especially with regard to the regiospecificity of microbial biocatalysts, has known significant progress during the past five years (van der Werf et al 1999, Chatterjee & Bhattacharyya 2001, Schrader & Berger 2001, Duetz et al 2003). On the basis of literature, five different microbial biotransformation pathways for limonene have been proposed (Fig 5).

Whereas earlier only regiospecific biocatalysts for the 1,2 position (limonene-1,2-diol) (Dhavalikar & Bhattacharyya 1966) and the 8-position (α-terpineol) (Abraham et al 1988) were available, recent reports describe microbial biocatalysts specifically hydroxylating the 3-position (isopiperitenol) (van Dyk et al 1998), 6-position (carveol and carvone) (Duetz et al 2001a) and 7-position (perillyl alcohol, perillylaldehyde, and perillic acid) (Duetz et al 2001b). Recently, a novel pathway by *Xanthobacter* sp. C20 has been described by van der Werf et al (2001). This strain converted both enantiomers of limonene quantitatively into limonene-

8,9-epoxide, a not previously described bioconversion product of limonene. More informations about this topics are contained in an interesting and complete paper by Duetz et al (2003) who reviewed recent progress in biotranformation of limonene by bacteria, fungi, yeasts and even plants.

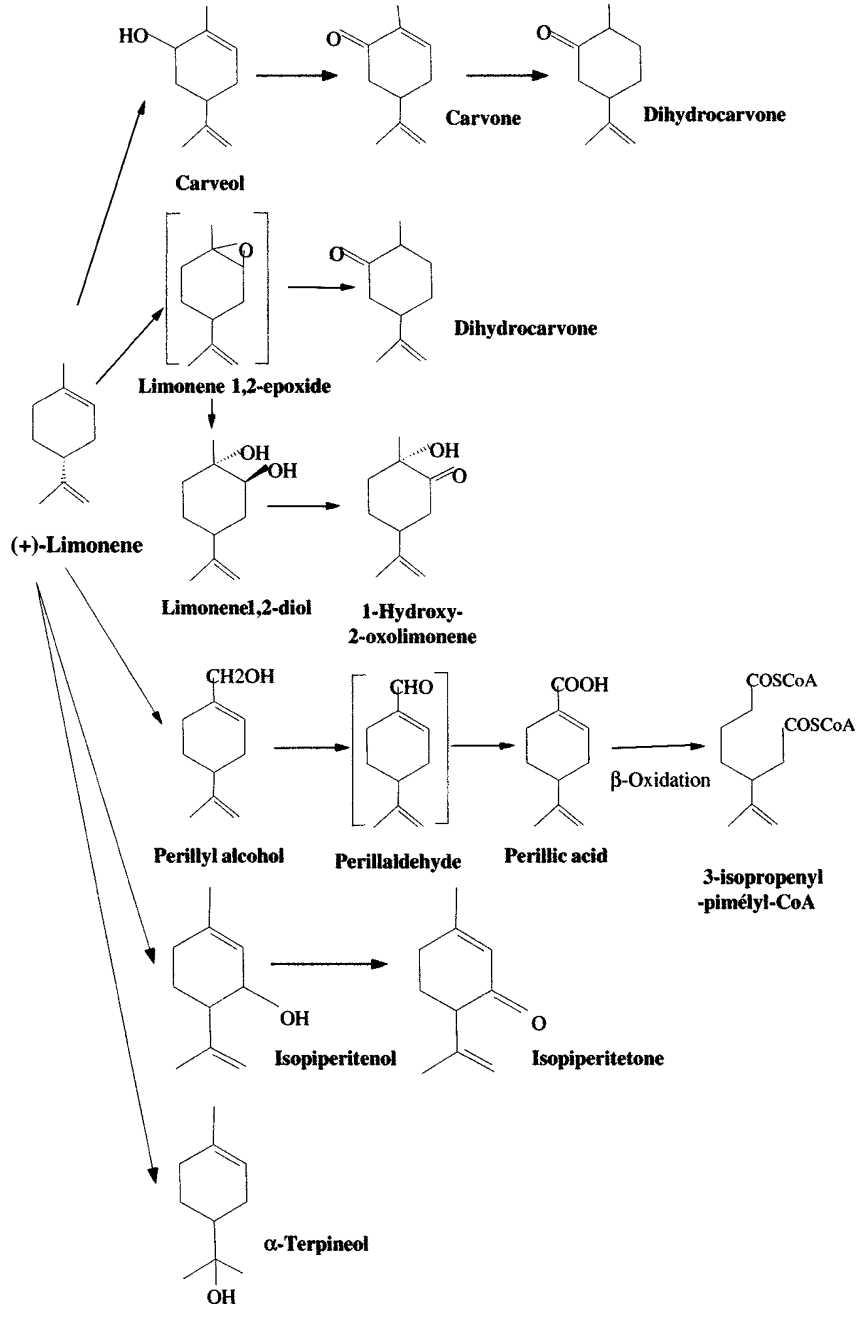


Figure 5: Microbial conversion pathways for limonene (adapted from van der Werf et al 1999)

5.3.3. Bicyclic monoterpenes : α-pinene and β-pinene

Pinene, the principal constituent of turpentine, is one of the most popular bicyclic monoterpene hydrocarbon, and has a great commercial importance as a raw material for several industries (Schrader & Berger 2001). It has been known that α- and β-pinenes can be hydrated and rearranged into bornane, camphane, and *p*-menthane derivates in the presence of acid catalysis.

Research on microbiological transformation of α-pinene was initiated by Bhattacharyya and his colleagues (1965) who reported that oxidation of this compound by the fungus *Aspergillus niger* yielded *cis*-verbenol, verbenone and *trans*-sobrerol. Later, Draczynska et al (1985) reported the transformation of both isomers by *Armillariella mellae* into verbenol, verbenone and 7-hydroxy α-terpineol (Figure 6).

The first metabolic pathway of α-pinene degradation was reported by Shukla et al (1968a, b) who showed that a soil pseudomonad capable of utilizing α-pinene as sole carbon source metabolised this compound to a wide variety of neutral and acidic compounds, including myrtenol, myrtenic acid, borneol, perillic acid and β-isopropyl pimelic acid. Microbial oxidations of α-pinene accompanied by ring cleavage were also demonstrated with *Pseudomonas putida* (Gibbon & Pirt 1971, Tudrozen et al 1977).

More recently, Yoo & Day (2002) have isolated a new soil pseudomonad strain able to transform both α- and β-pinene to several bicyclic and *p*-menthene derivates such as limonene and *p*-cymene (Figure 7).

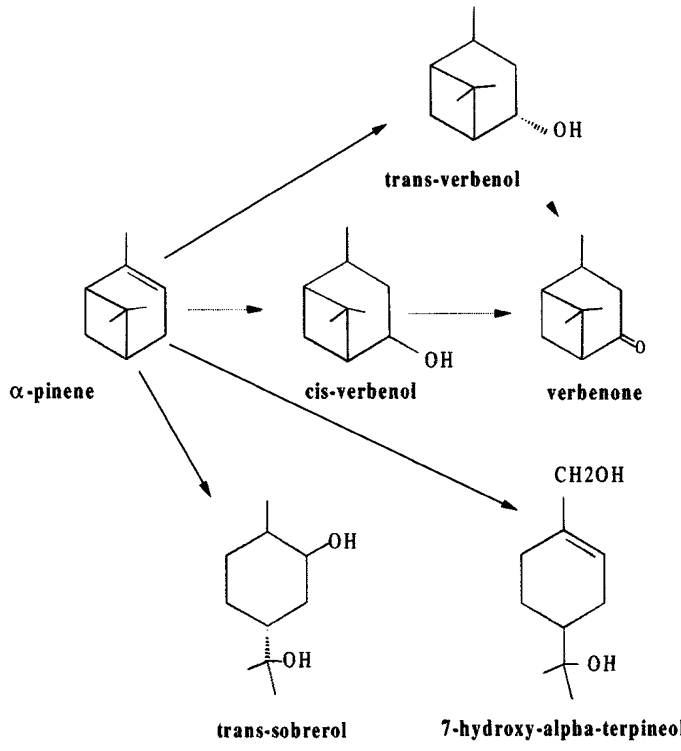


Figure 6 : Biotransformation of α-pinene by strains of *Aspergillus niger* and *Armillariella mellae* (adapted from Draczynska et. al. 1985)

Microbial catabolic pathways leading to acyclic compounds, isonovalal and novalal were described too (Trudgill 1986, Trudgill 1994, Colocousi et al 1996). The route to aldehydes (Figure 8) starts with α-pinene oxide (Best et al 1987; Griffiths et al 1987a) and is claimed by several patents (Harries et al 1989, Burfield et al 1989). The initial double ring cleavage step is lyase catalysed, and the respective enzymes from *Nocardia* sp P18.3 (Griffiths et al 1987b) and from *Pseudomonas putida* NCIMB 10684 (Ratledge, 1994) have been characterized. In a recent review, Zorn et al (2004) even proposed a complete degradation pathway of α-pinene by *Pseudomonas fluorescens* NCIMB 11671. Recently, Fontanille & Larroche (2002), have isolated a new pseudomonad strain, *Pseudomonas rhodesiae* PF1, able to growth on α-pinene as sole

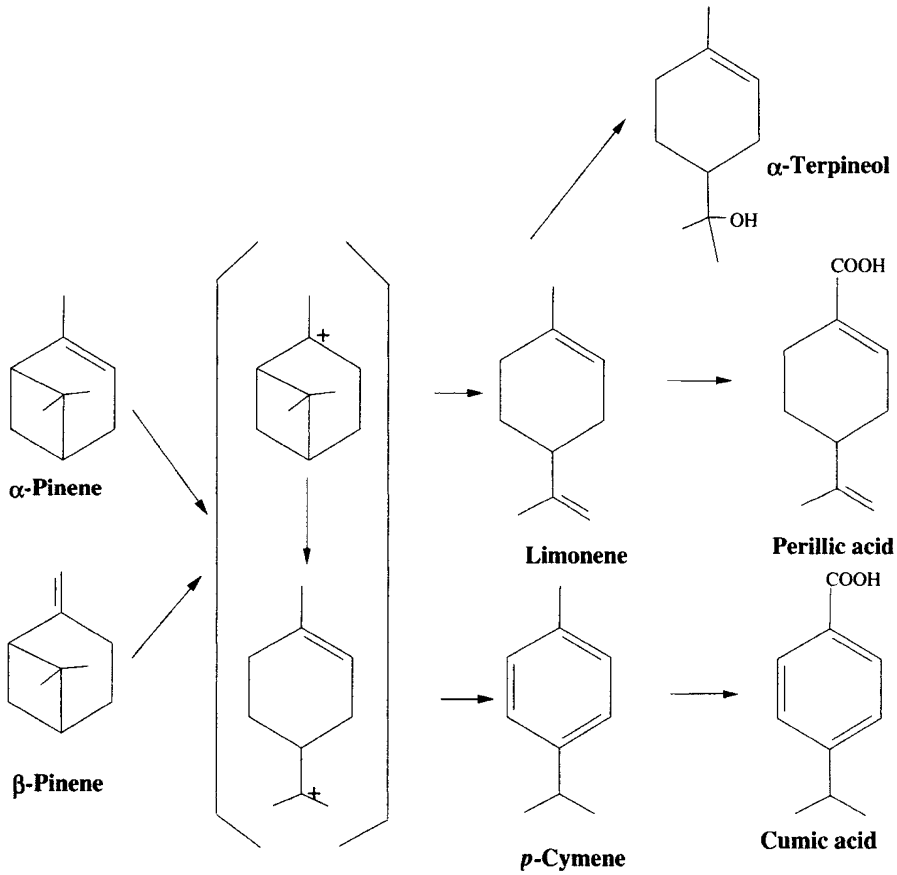


Figure 7 : Degradation pathway of α and β-pinene by a strain of *Pseudomonas* PIN (adapted from Yoo & Day 2002)

carbon source, and very efficient for production of isonovalal from α-pinene oxide. The best results achieved allowed recovery of ca. more than 800 g/l organic solvent of isonovalal in 5 h operation, which is the most efficient process to date in the area of terpene biotransformations (see later).

6. PROCESSES OF PRODUCTION OF MOLECULES WITH FLAVORING PROPERTIES BY BIOTRANSFORMATION: CASE STUDIES

Design, control and improvement of processes of production of molecules with property of flavors by microorganisms can only result from a global approach integrating, in term of strategy of culture and separation, the constraints imposed by the microorganisms used and those related to their environment,

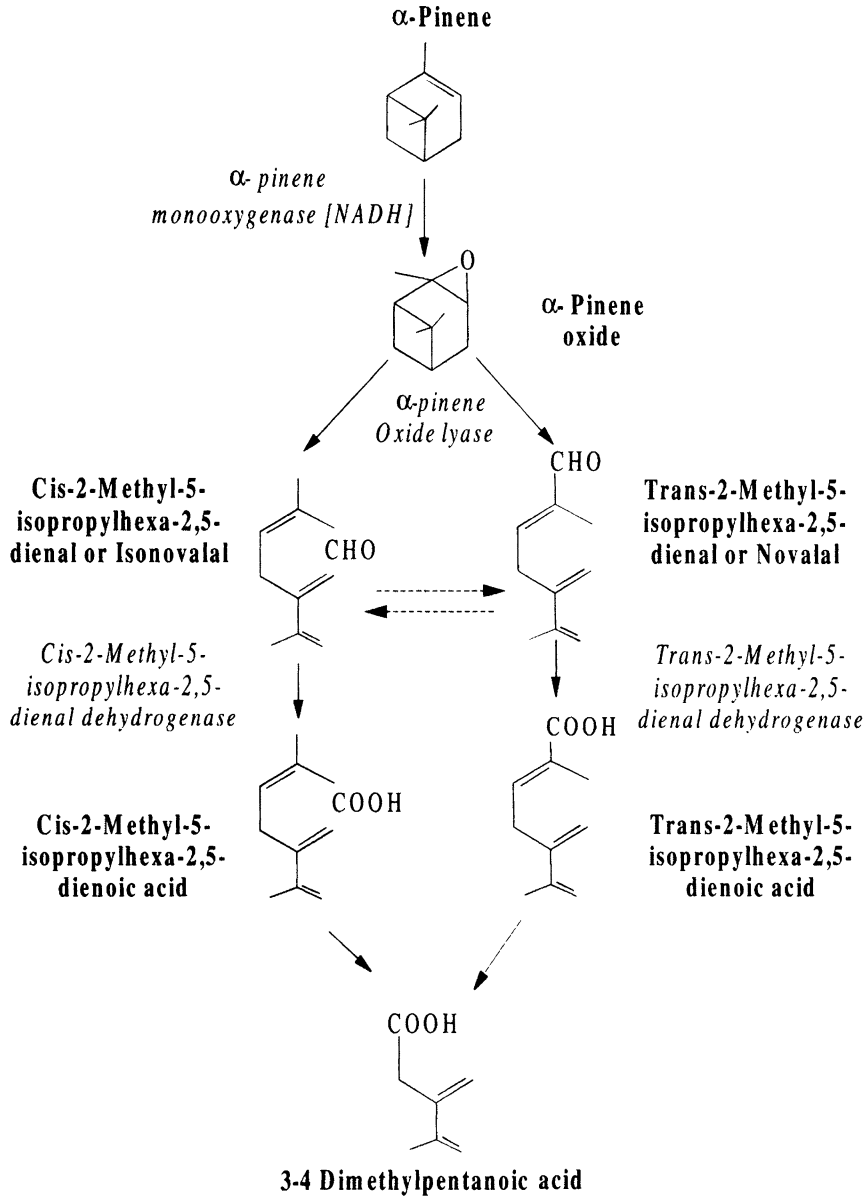


Figure 8 : Proposed degradation pathway of α-pinene by *Nocardia* P 18.3 and *Pseudomonas fluorescens* NCIMB 11671 (Best et al 1987; Griffiths et al 1987b). The same scheme is probably valid for *Pseudomonas rhodesiae* CIP 107491 (Fontanille & Larroche 2002)

and taking account of legislation. This part will give some recent results on the analysis of the biological constraints, the characterization of the physicochemical environment and the determination of the limiting steps at the time of the design or the operation of these processes. The production of methylketones, alkylpyrazines and terpenoïds will be used as illustration.

6.1. Production of methylketones

Methylketones formation from fatty acid by spores of *Penicillium roquefortii* was reported for the first time in 1958. This pioneering work concerned the formation of heptanone-2 from octanoïc acid. Since this date, many works were devoted to this reaction and more generally to the formation of ketones in C5, C9, C11, in consequence of their potential interest in the production of blue cheese flavors. The general reaction is as follows:

$$R\text{-}CH_2\text{-}CH_2\text{-}COOH + O_2 \Longrightarrow R\text{-}CO\text{-}CH_3 + CO_2 + H_2O$$

The process is aerobic and involves an enzymatic chain (7 reactions) whose first links are common to the classical fatty acids β-oxidation. It is possible to observe a reduction of ketones to alcohols, but this reaction is strongly inhibited when the biotransformation is carried out in a simple synthetic medium. The energy balance of the whole set of biosynthesis reactions to a methylketone shows that the system is autonomous and thus likely to function with an output of biotransformation equal to 1, without contribution of energy substrate (glucose), provided the ratio of rates of energy-rich intermediates production to oxygen uptake - i.e. the P/O ratio is equal to or higher than 1. This can be achieved if the oxygen transfer is sufficient so that the dissolved oxygen concentration is not limiting.

Table 6 recapitulates the series of the limiting steps which were met when designing and operating the process of production of methylketones by *P. roquefortii*. It also shows how the process was changed to be freed from these limitations.

Table 6: List of the limiting steps and improvements made to raise these limitations in the case of biotransformation of fatty acids in methylketones

Limiting step	Process improvement
Mycelial proliferation Transfer of oxygen in the viscous medium	Use of spores as biocatalyst
Substrate inhibition	Controlled sequential feeding of fatty acid
Low solubility of ketones in aqueous medium Stripping of ketones by the aeration gas	Use of a biphasic organic solvent-water system

The organic solvent must obey the classical rules of choice of a solvent compatible with a microorganism. The organic phase must thus have a high molar mass and a $\log_{10}K_{ow} > 4$. The solvent selected is a heavy, isoparaffinic industrial solvent. The reaction medium was made up to 85% by this solvent and 15% (v/v) by the aqueous phase containing the spores. It was possible to obtain at the end of the reaction approximately 80 to 100 $g.L^{-1}$ of 2-heptanone with an apparent yield of 80%. One can also obtain 20 $g.L^{-1}$ 2-pentanone starting from hexanoic acid, or 60 $g.L^{-1}$ 2-nonanone starting from decanoïc acid (Larroche et al 1992). The ketones are recovered by simple distillation of the organic phase.

6.2. Production of alkylpyrazines

Pyrazines are nitrogen heterocycles discovered during the 1960's responsible for the taste and flavor of roasts or roasted foods. It is in 1962 that one showed for the first time that microorganisms, especially *Bacillus subtilis*, could produce alkylpyrazines (Seitz 1994). Their threshold of smell is relatively low (about 500 $\mu g.L^{-1}$ in water at 20°C) and they are produced in general in very small quantities. In this example, the goal was to make maximum the dimethyl- and tetramethyl-pyrazines concentrations. The limitations, here, were initially of biological nature and the research effort focussed on the determination of culture medium composition and on the search for the precursors of these two pyrazines. The strain chosen, *Bacillus subtilis* IFO 3013, was cultivated on a medium containing soya flour (5 to 10%, w/w). The study of the bioconversion pathways showed that dimethylpyrazine was produced according to the following stoichiometry:

and tetramethylpyrazine according to:

The two pathways were distinct (Figure 9) and corresponded to two physiological states of the cells. One could thus plan to separately maximize the concentrations of dimethyl- and tetramethyl-pyrazines, or to obtain a given distribution of the two compounds in the reaction system. In addition, the precursors of a pathway were inhibitors of the other one. Conversion of threonine into 2,5 dimethylpyrazine was carried out by growing cells, while that of acetoin to tetramethylpyrazine was the fact of resting bacteria. This information resulted in adopting a culture strategy in a reactor, with controlled pH, which was as follows:

- addition of threonine at the beginning of a culture, possibly followed-up by sequential additions;
- then, addition of acetoïn and ammonium in the form of ammonium acetate.

This allowed to recover 4 $g.L^{-1}$ 2,5 dimethylpyrazine and 1 $g.L^{-1}$, which were recovered by extraction and distillation (Gros et al 1996, Larroche et al 1999). This process was patented by Sanofi-Bio-Industries, now Degussa Flavors & Fruit Systems. It should be noticed that this patent claimed the possible use of two microorganisms, *Bacillus subtilis* and *Brevibacterium linens*.

6.3. Production of isonovalal in a biphasic medium

Terpenes structures have been previously described. One of the difficulties encountered in their biotransformations, particularly of mono- and sesqui-terpenes is partly due to their strong antimicrobial activities. Compounds such as citral, geraniol, or thymol are respectively 5, 7, 5 and 20 times more efficient that phenol. This is why a preliminary phase of biomass production is necessary before the biotransformation itself. Two procedures can be used:

- the precursor is added directly to the culture medium at the end of a given time;
- biomass is separated from the growth medium and then fed to the biotransformation medium, which has a different composition. This solution makes it possible to adjust the biocatalyst concentration.

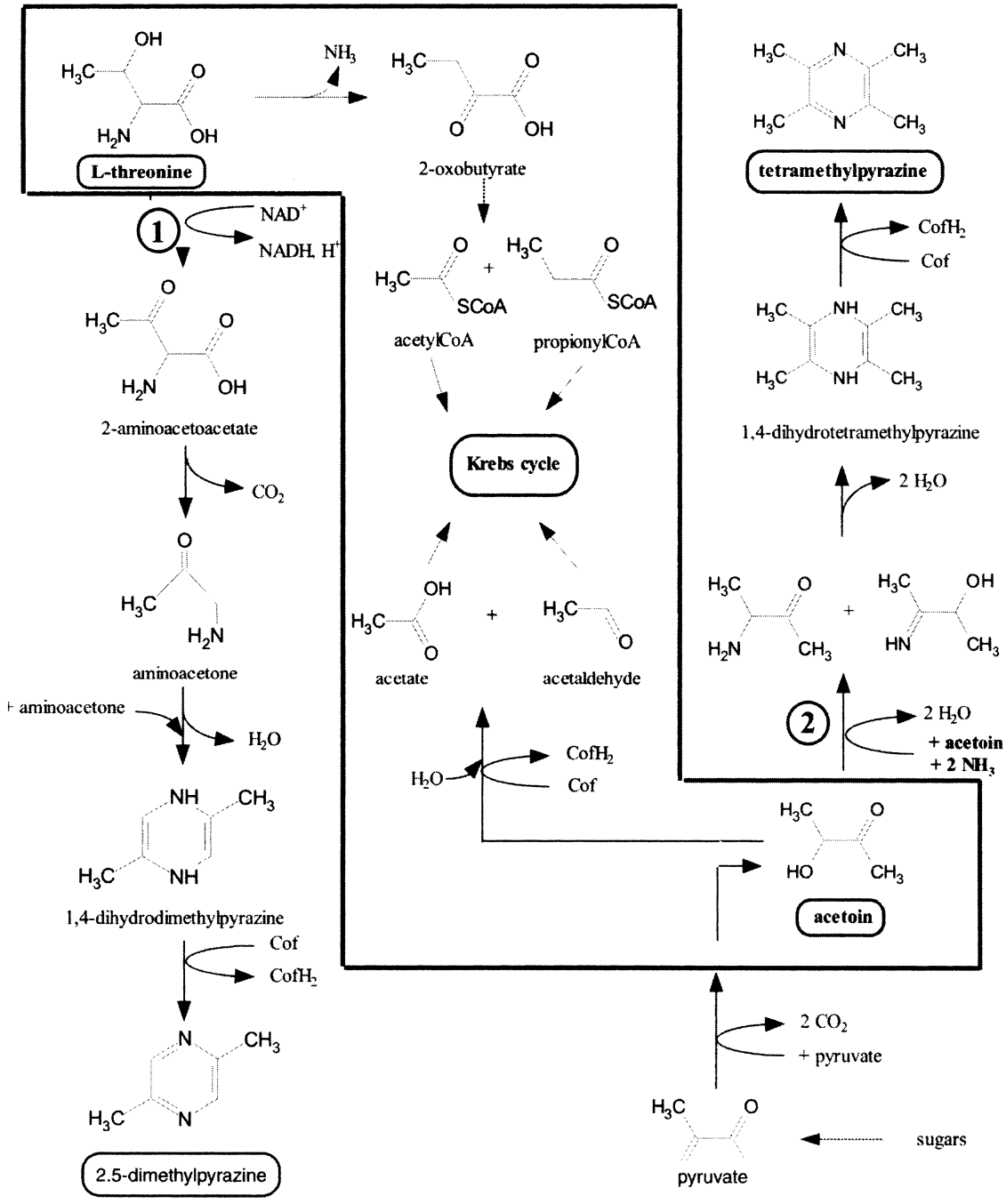


Figure 9: Metabolic pathway of methylpyrazines production

Isonovalal (Z-2-methyl-5-isopropyl-hexa-2,5-dien-1-al) is a molecule whose odor is described as "citrus, spiced, timbered, resinous". It can be obtained from α-pinene oxide by a rearrangement catalysed by a specific decyclase produced by *Pseudomonas rhodesiae* PF1, which breaks the three cycles of α-pinene oxide and gives an acyclic aldehyde (Figure 8).

One found in this process the constraints already quoted for the other treated examples: strict aerobic microorganism, therefore requiring aeration during its growth; toxicity, significant volatility, and low water solubility of the precursor; delicate strain storage. Nevertheless a process was proposed. It included the following steps:

1. Preculture for the production of the inoculum used in the following phase.

2. Production of the catalyst. Growth occurred in an aerated stirred-tank reactor, in a complete medium with α-pinene as the carbon source at 30°C. During growth, α-pinene caused the induction of enzymes. Before end of growth, the medium was recovered, concentrated (25 $g.L^{-1}$ of biomass) by centrifugation and resuspended in a phosphate buffer (pH = 7.5). The suspension was frozen and stored at -20°C until use.

3. Biotransformation step. It was carried out in a stirred-tank reactor, without aeration, in a biphasic organic water-solvent medium. The volume ratio of the aqueous to organic phases was 1:1. The organic layer initially contained 600 $g.L^{-1}$ α-pinene oxide, and a fed-batch technique was applied to αprovide further precursor amounts. Cells were permeabilized before use by means of two procedures. At first, the biomass was freezed and thawed, then treated by an organic solvent such as diethylether or chloroform (5%, v/v) during one hour. This treatment allowed the enzymatic activity to be recovered outside the cells, and the biotransformation was in fact carried out with a crude enzymatic system.

One could obtain more than 800 $g.L^{-1}$ isonovalal in five hours, with a molar yield close to 80% (Figure 10). It is, at our knowledge, one of the most efficient process in the area of terpene biotransformations.

The development and optimization of production processes for molecules with aroma properties goes, as for all the processes, through a fine analysis of limiting steps. As in the chemical or oil industry processes, the study of catalyst - here biocatalyst - is of primary importance and its environment must be controlled perfectly.

7. CONCLUSIONS

Optimization of a biotransformation process must take into account all components of the system, i.e. the biocatalyst itself and environmental parameters. The first issue to address is the finding of the so-called limiting step, which can be of biological or physical nature. In the first case, the biocatalyst has to be improved by using techniques such as screening for a new microorganism or genetic engineering. In the second one, it is an engineering problem, the aim being here to be sure to be able to express the full biocatalytic activity in the system. It is, thus, an integrated, multidisciplinary issue that must optimize the coupling between the biocatalyst, the reaction medium and the mode of operation. This approach, if systematically used, would allow a better industrial development of this kind of operation for biomolecules production.

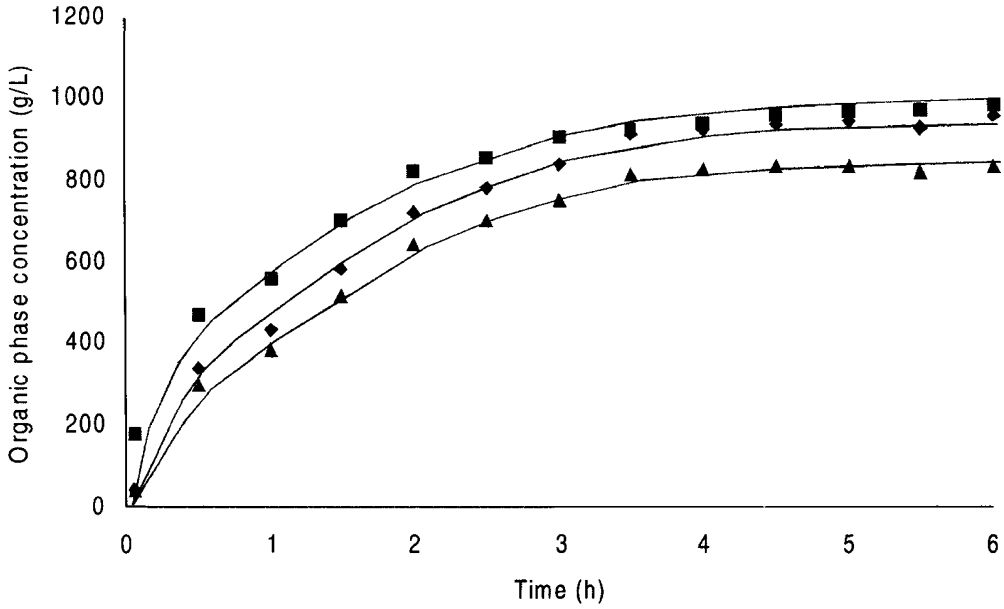


Figure 10: Time-course of α-pinene oxide (■), total products (◆) and isonovalal (▲) concentration during a biotransformation of α-pinene oxide in a biphasic system (hexane/phosphate buffer, volume ratio 1:1) with permeabilized cells. Total volume 500 mL, stirring rate 800 rpm, temperature 30°C, initial precursor concentration in the organic phase 600 $g.L^{-1}$

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Enzymes as Tools for the Stereospecific Carbon-Carbon Bonds Formation in Monosaccharides and Analogs Synthesis

Jean Bolte, Virgil Hélaine, Laurence Hecquet and Marielle Lemaire

1. INTRODUCTION

The control of the absolute configuration of asymmetric centres is a major challenge in organic synthesis. Among the advantages provided by the use of enzymes compared to chemical catalysts, (regioselectivity, green chemistry conditions), stereospecificity is of special interest for carbon-carbon bond formation. For this purpose, every enzymes acting in the biosynthesis of natural products could be useful. However, up to now, only a few of them have led to preparative scale applications. Indeed, some special characteristics are needed for an enzyme to be a good catalyst for organic synthesis: it has to display a large substrate specificity to extend its application field; it is better if it is not dependent on a coenzyme difficult to provide, like PEP, ATP, NAD or acetylCoA; and, moreover, it has to be available in large amount. These requirements are not always easy to fulfil so that many potentially interesting enzymes, especially those acting in the secondary metabolism, for the biosynthesis of terpenes, steroids, alkaloids or antibiotics have been sligthly used. These last years, however, due to the progress of the molecular biology technology, new enzymes have appeared in organic syntheses: cloning and overexpression of the genes, easily provide large amounts of enzymes of high purity. Moreover, mutagenesis methods allow to extend their substrate specificity, or to improve their stability in non conventional conditions.

In this review, we will focus on C-C bond forming enzymes used for the synthesis of monosaccharides, where the structure complexity is a major challenge. They catalyze nucleophilic addition reactions onto aldehydes. They are aldolases belonging to the class of lyases, transferases or thiamine pyrophosphate dependent ligases (Gigsen et al 1996, Machajewski et al 2000, Wymer & Toone 2000).

2. DHAP aldolases

Aldolases are enzymes which catalyze reversible or irreversible addition of a ketone (donor), here dihydroxyacetone phosphate (DHAP) on an aldehyde (acceptor). Aldolases are classified in two groups depending on their reaction mechanism. Type I aldolases, found mainly in plants and animals, are activating the donor by forming a Schiff base intermediate. Type II aldolases need a Zn^{2+} cation in the active site to facilitate enolate formation on the donor (Fig 1).

The most widely used DHAP aldolase is the fructose 1,6-diphosphate aldolase (FDP aldolase). This enzyme is a key enzyme of the glycolytic pathway, reversibly catalyzing the clivage of fructose diphosphate into two three carbon units, DHAP and glyceraldehyde-3-phosphate (G3P). *In vitro*, the equilibrium is shifted to the condensation reaction and the enzyme creates the C3-C4 bond with *(3S,4R)* stereochemistry.

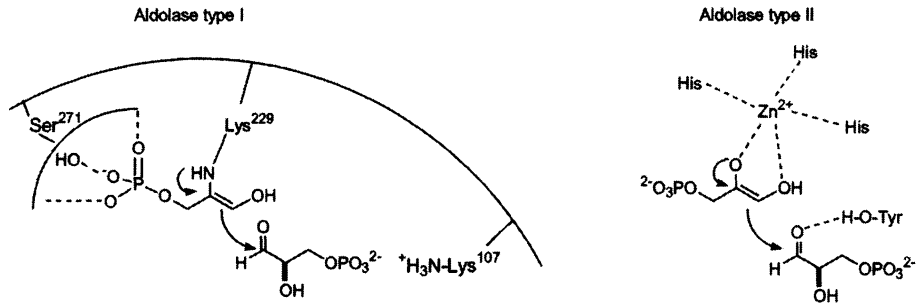


Fig. 1. Reaction mechanism for Class I and Class II aldolases

The three other stereoisomers are accessible using other aldolases which are forming C3-C4 bond with a specific stereochemistry (Fig 2).

Commercial FDP aldolase is extracted from rabbit muscle. Whitesides, who initiated the name RAMA (for RAbbit Muscle Aldolase), published in 1983 together with Wong the first papers illustrating the synthetic potential of this enzyme (Wong & Whitesides 1983). They have established that the enzyme is very specific towards its donor substrate, tolerating only very close DHAP analogues but accepting a large number of aldehydes as acceptors. Up to now, a great number of strategies for obtaining sugars and analogues using RAMA catalyzed reaction for the stereochemistry control, have been published (Fig 3).

Other FDP aldolases from plants or micro-organisms have been also proposed. The most promising has been extracted from *Staphylococcus carnosus*. This enzyme belongs to type I aldolase and has Shown the same stereospecificity and substrates tolerance as RAMA does. In addition the enzyme is remarkably stable under the synthetic reaction conditions (pH and temperature). This powerful enzyme has been cloned in *E. coli* (Zannetti et al 1999). Two other DHAP aldolases, rhamnulose-1-P and fuculose-1-P aldolases are also of interest. These enzymes from micro-organism are difficult to produce from wild strain so they were not used until they were cloned and overexpressed.

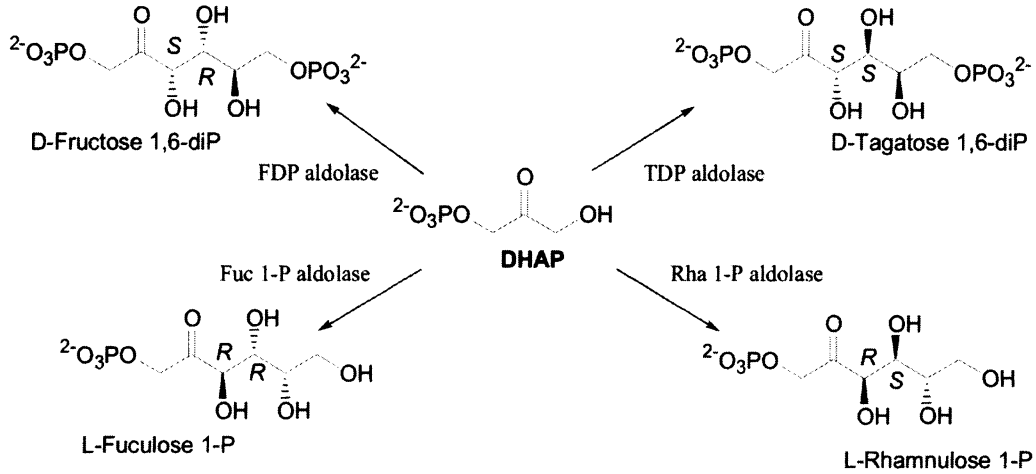


Fig. 2. Stereospecificity of DHAP aldolases

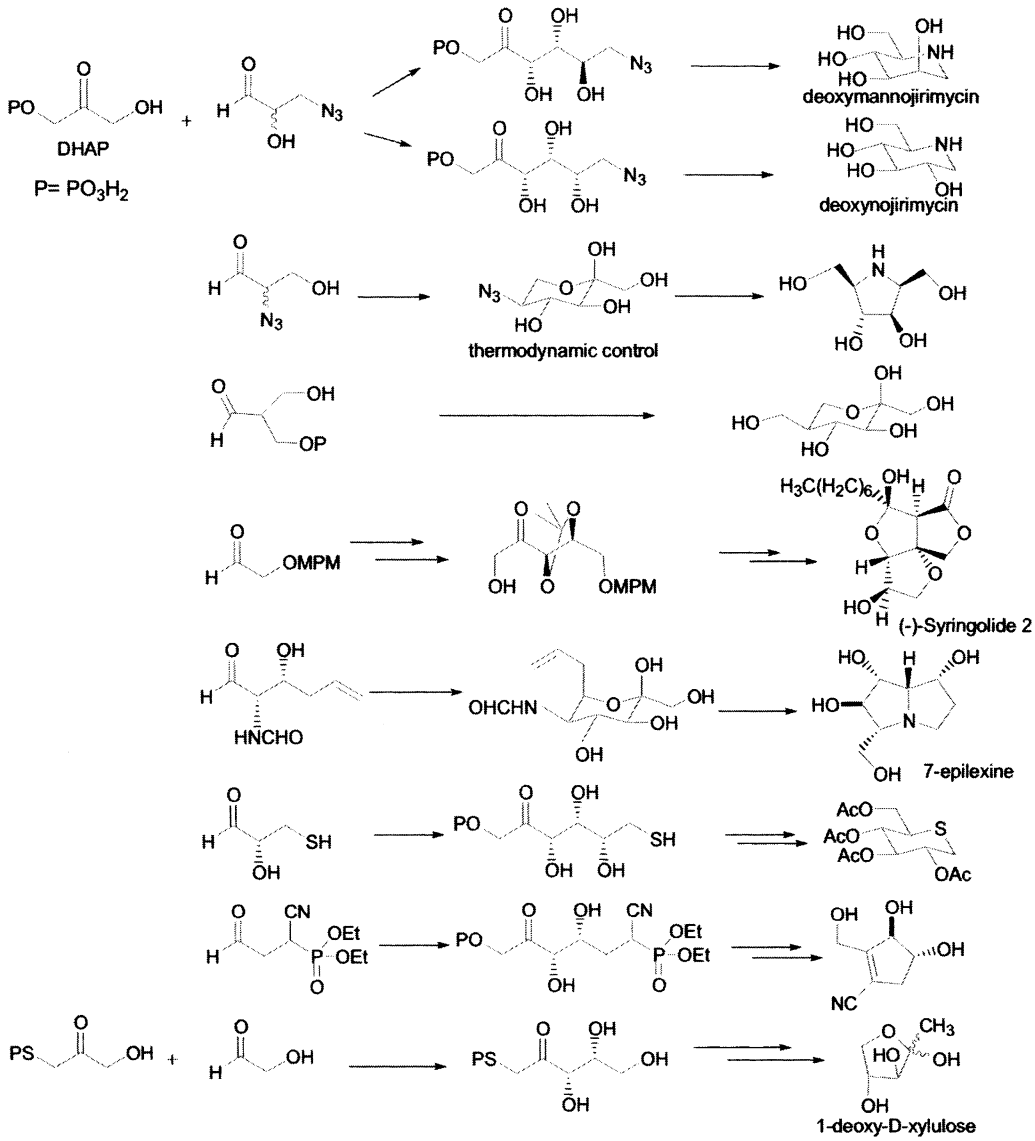


Fig. 3. FDP aldolase catalyzed syntheses

For chemical use, DHAP is required. But this unstable compound must be prepared just before being the emzymatic reaction. The possible routes for DHAP preparation are depicted in Figure 4 (Ferroni et al 1999). The two first are improvement of the Colbran's original strategy published in 1967. We have proposed a method starting from dibromoacetone (Gefflaut 1997). DHAP can also be synthesized by enzymatic reaction where L-glycerolphosphate is oxidized into DHAP by a glycerolphosphate oxidase (Schoevaart et al 1999).

In Figure 5, some examples are illustrating syntheses *via* a DHAP aldolase. Among them, numerous iminocyclitols as glycosidases inhibitors are found. Glycosidases are playing a role in different pathologies.

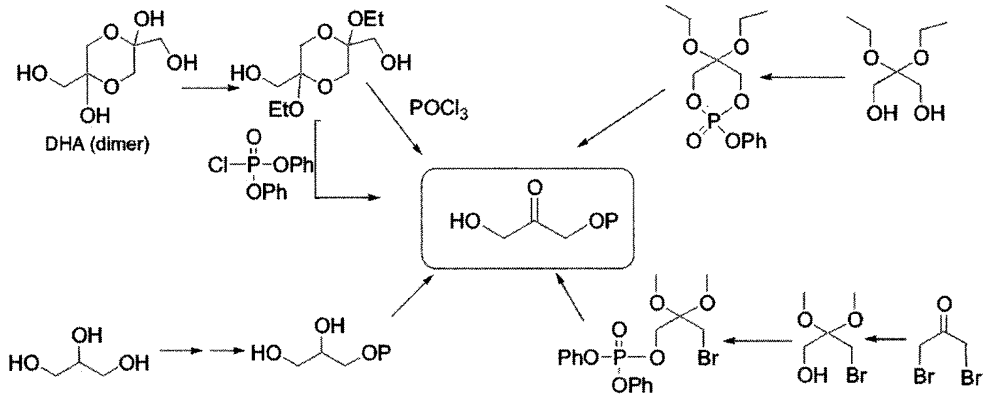
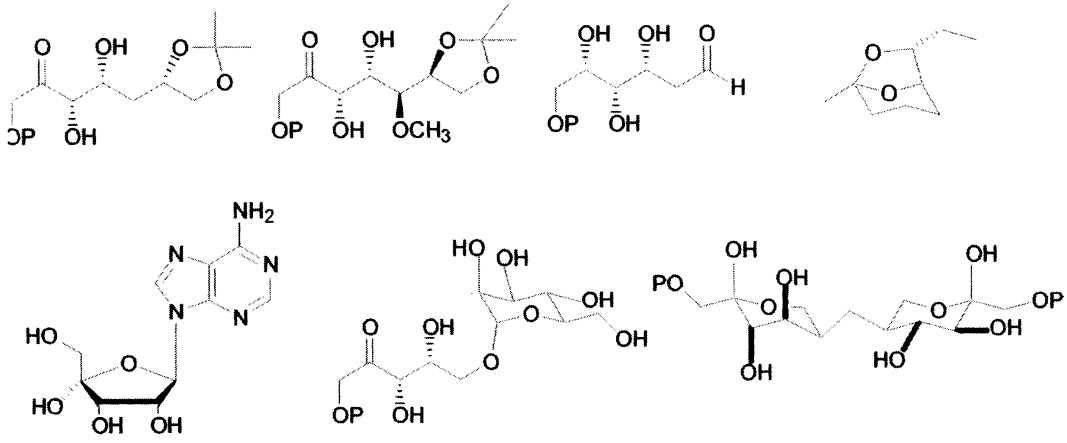


Fig. 4. Dihydroxyacetone phosphate (DHAP) syntheses



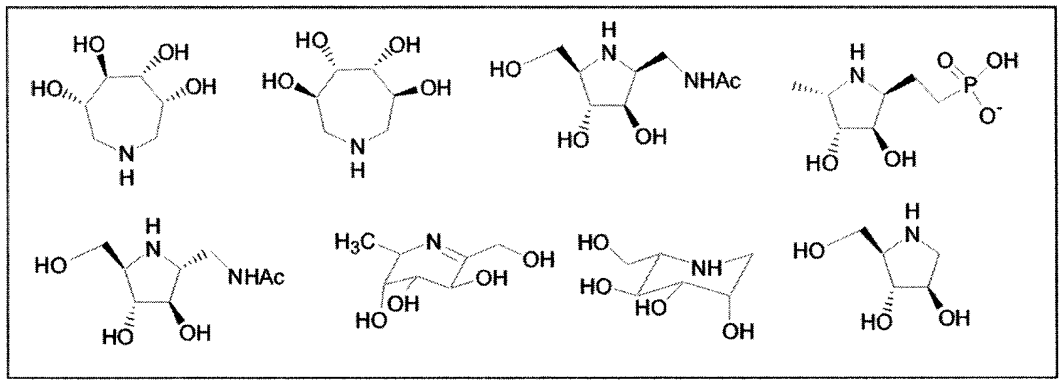


Fig. 5. Compound provided by DHAP aldolase catalysis: in blue pattern form DHAP, in red, the bond biuld by the enzyme, in the blue frame the glycosidase inhibitors.

Effenberger (Ziegler et al 1988) and Wong (Pedersen et al 1988), who respectively initiated this work, have published the first papers on deoxynojirimycin and deoxymannojirimycin syntheses, demonstrating by this way that DHAP aldolases are one of the most versatile tools to prepare such compounds.

3. PYRUVATE ALDOLASES

Different recent reviews have been published about pyruvate aldolases (Augé & Crout 1998, Augé & Gautheron-Le Narvor 1997). Pyruvate aldolases have been used for obtaining α-ketoacids with 2-oxo-3-deoxyulosonic acid skeletons (heptulosonic: DAH, octulosonic: KDO, nonulosonic: KDN or N-acetylneuraminic acid: sialic acid). *In vivo*, these enzymes are working in the degradation way, whereas phosphoenolpyruvate aldolases (belonging to ligase class) give the same products using a biosynthetic

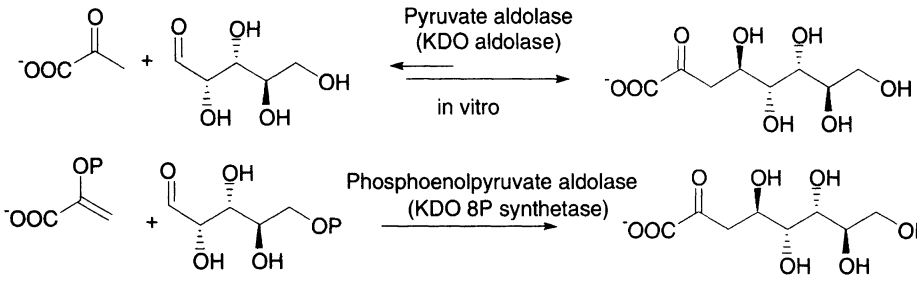


Fig. 6. Aldolisation reactions from pyruvate

pathway (Fig 6). These latter are less often used due to their low substrate specificity.

Commercially available N-acetylneuraminic acid aldolase (sialic acid aldolase) was the first studied. Like most aldolases, it's very specific for the donor substrate (here pyruvate) and able to accept many aldehydes. The first enzymatic synthesis of sialic acid was carried out by David and Augé (Augé et al 1984). Other syntheses of this compound or analogues were published (Fitz et al 1995) (Fig 7). Surprisingly, the stereospecificity of this enzyme depends on the aldehyde substrate used: if C3 is of natural configuration (*S*), carbonyl attack is performed on the *si* face rising to a new asymmetric center of *S* configuration whereas attack takes place on the *re* face in the other case.

KDO aldolase leads to ulosonic acids of *4R* configuration on reaction with its natural substrate, D-arabinose, as well as with D-ribose, D-xylose, D-lyxose and L-arabinose (Fig 7). *Aspergillus terreus* whole cells were used for preparative purposes. KDPG aldolase, having the same stereospecificity for the newly created C4 asymmetric center, was also used in organic synthesis (Henderson et al 1998).

4. THIAMINE PYROPHOSPHATE DEPENDENT ENZYMES (SPRENGER & POHL 1999, SCHÖRKEN & SPRENGER 1998).

Enzymes using thiamine pyrophosphate (ThDP) such as α-ketoacid decarboxylases, first subunit (E1) of α-ketoacid dehydrogenase complexes, transketolase and acetolactate synthase catalyze both C-C bond cleavage and formation. In the condensation way, these reactions are biological equivalents of aldehydes coupling reactions catalyzed by thiazolium salts (Fig 8).

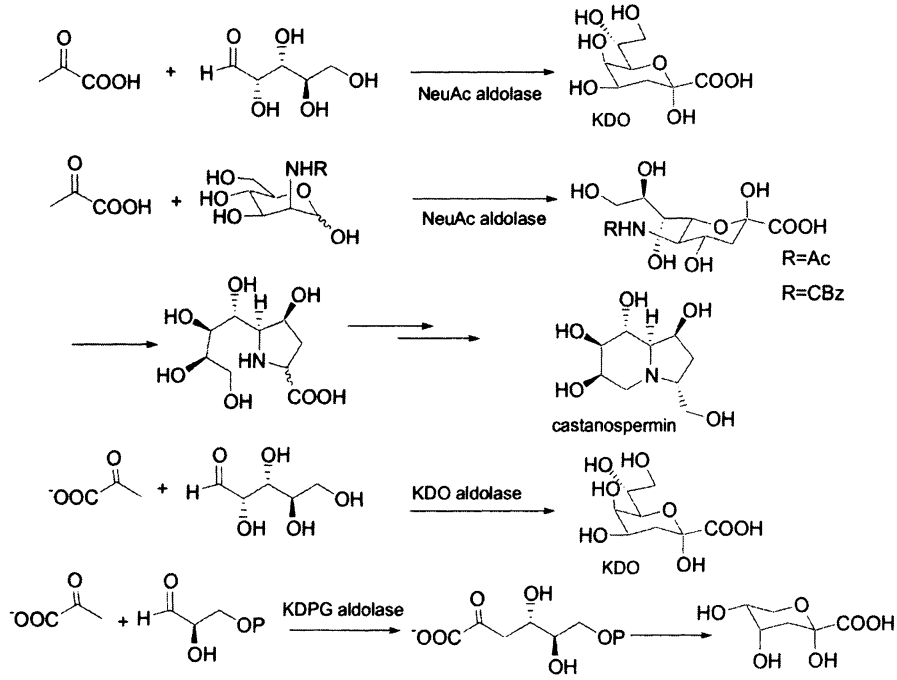


Fig. 7. Pyruvate aldolase catalyzed syntheses

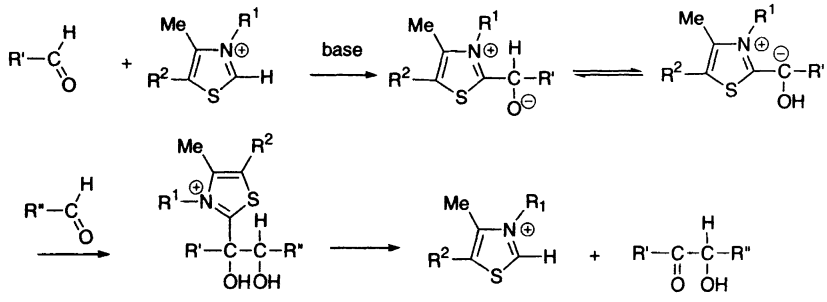


Fig. 8. Coupling reaction catalysed by thiazolium salts

The carbanion obtained by deprotonation of ThDP reacts with the carbonyl group of a α-ketoacid (case of decarboxylases, transketolases) or a ketol (case of transketolase). After decarboxylation, the carbanion thus obtained can evoluate in three different ways: a) be protonated (rising to a new aldehyde: decarboxylation reaction) ; b) be transferred to another subunit (rising to a carboxylic acid – case of α-ketoacid dehydrogenase complexes) ; c) be added to an aldehyde to lead to a ketol (which is the transketolase reaction) (Fig 9).

It's noteworthy that, for decarboxylases, protonation is the "normal" biological reaction and that ketol formation is a side reaction (they are so called lyases because there is a C-C bond cleavage), whereas for

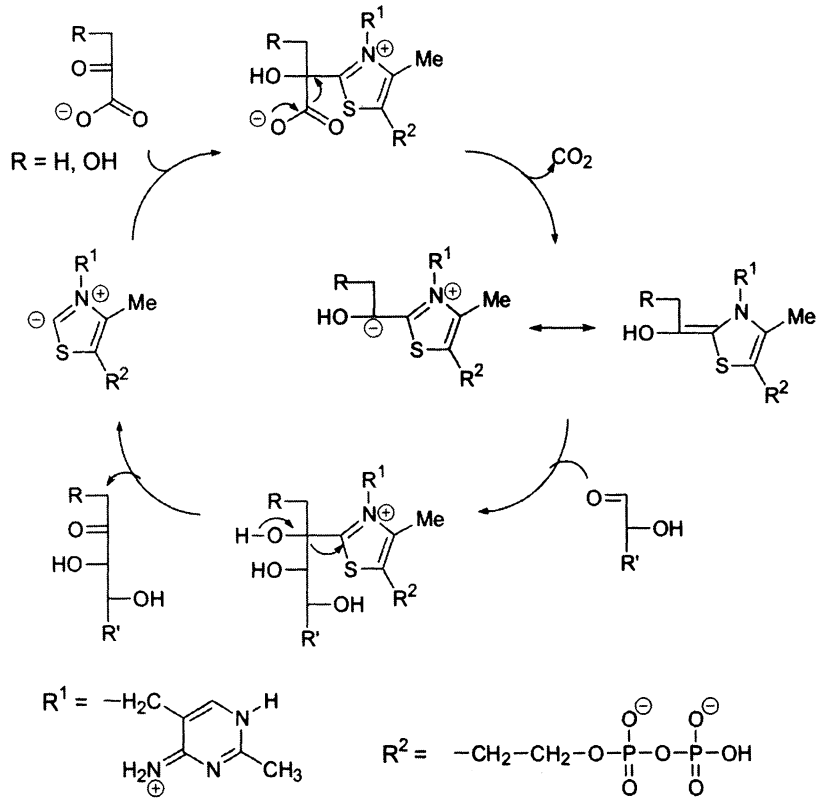


Fig. 9. Reaction catalysed by thiamine pyrophosphate dependent enzymes

transketolase, although it's using α-ketoacid substrate which is decarboxylated, C-C bond cleavage is followed by formation of a new C-C bond, so it's belonging to transferase class of enzymes.

5. TRANSKETOLASE (TK)

This enzyme has led to many applications for 15 years. Although its activity towards unnatural substrates (unphosphorylated aldoses) has been noted since 1955, we first proposed its use for preparative scale syntheses of ketoses and analogues (Bolte et al 1987). For synthesis, we often used spinach enzyme extracts, easy to isolate. Enzyme from yeast was also used, we have started to produce and use it after its overexpression (André et al 1998). Transketolase from *E. coli* has also been overexpressed and is a versatile tool for organic chemistry.

Transketolase transfers α-hydroxyacetyl group from either hydroxypyruvate or a ketose on a *2R* configuration α-hydroxyaldehyde (*in vivo* erythrose-4-phosphate) leading to a ketose with (*3S,4R*) configuration, found in fructose. Giving compounds with the same stereochemistry as these obtained with FDP aldolase, transketolase is an interesting alternative for obtaining a ketose with n carbons, FDP aldolase will need a n-3 carbons aldehyde whereas TK will need a n-2 one. Depending on the ease of getting the aldehydes, and on the enzyme activities towards these particular substrates, one or the other possibility will be retained (Fig 10).

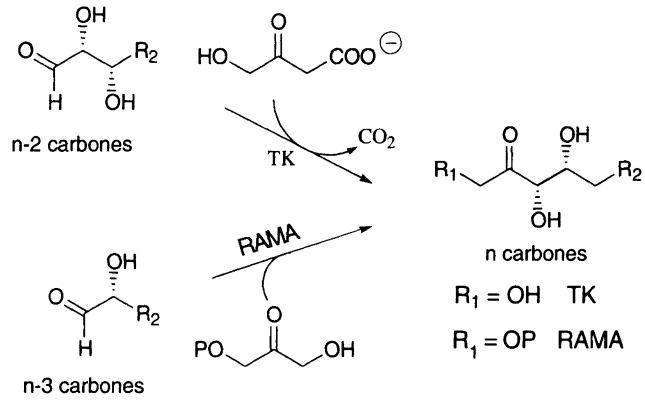


Fig. 10. Transketolase (TK) and fructose-1,6-disphosphate aldolase (RAMA) are complementary tools

Few syntheses carried out using TK are reported in Figures 11 and 12.

In these syntheses, hydroxypyruvate has been used in almost each case instead of a ketose donor because in that case the reaction becomes irreversible. The enzyme is enantioselective towards α-hydroxyaldehyde acceptor, which accordingly can be employed as a racemic mixture for kinetic resolution.

6. α-KETOACID DECARBOXYLASES AND DEHYDROGENASES

Pyruvate decarboxylase (PDC) has been the most studied enzyme for C-C bond formation. Obtaining (*R*)-phenylacetylcarbinol from benzaldehyde and glucose with yeast is one of the oldest industrial

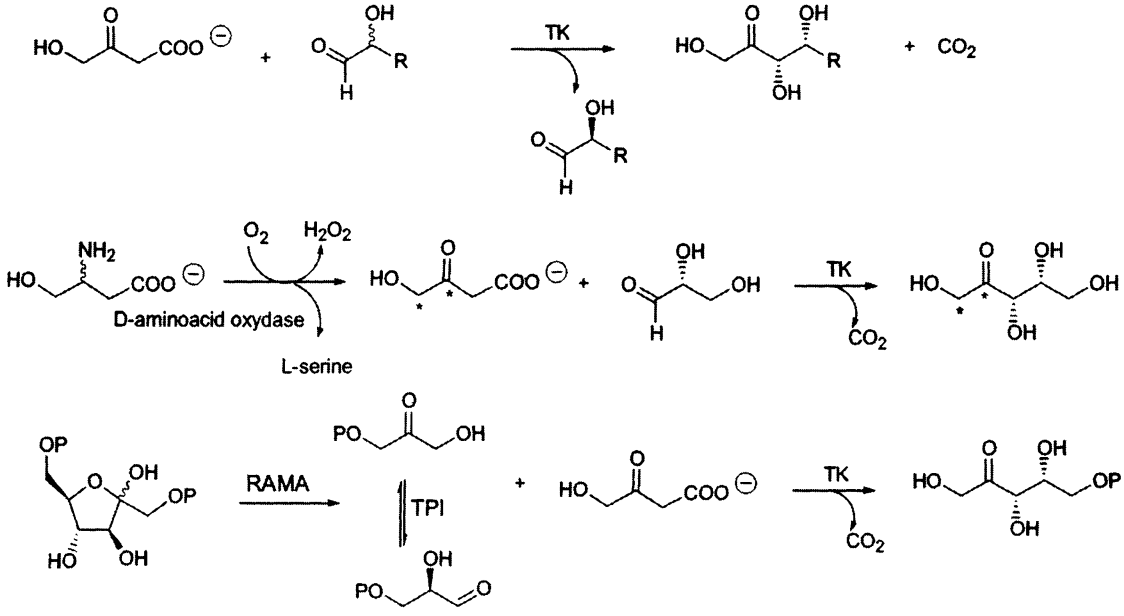


Fig. 11. Transketolase (TK) catalyzed syntheses

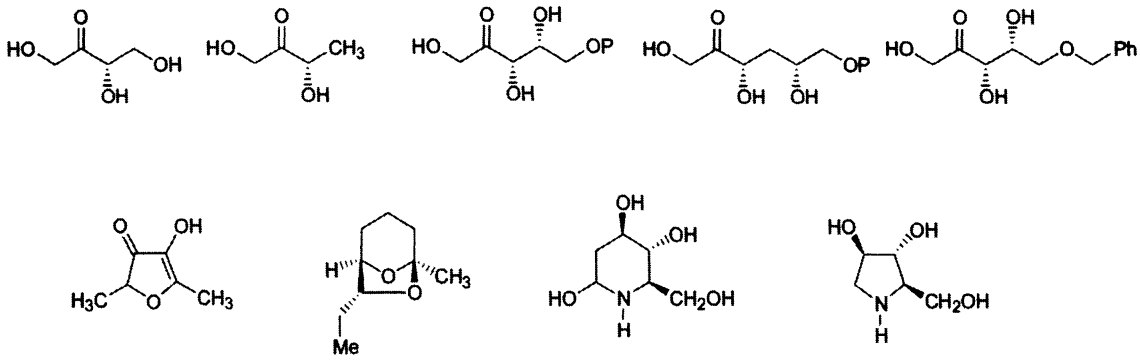


Fig. 12. Compounds synthetized *via* transketolase (TK), in blue, the pattern from hydroxypyruvate, in red the enzymatically built bond

bioconversion reaction: it was performed for synthesis of ephedrine (Fig 13) (Hildebrandt & Klavehn 1932, Idin et al 1998).

This reaction consists of an acetyl group transfer from pyruvate obtained during fermentation process to benzaldehyde, catalyzed by pyruvate decarboxylase. Baker's yeast (*Saccharomyces cerevisiae*) was widely used as catalyst in this reaction. Nevertheless, the presence of other enzymes, particularly alcohol dehydrogenase, leads to by-products. The way of solving this problem is a good example of biocatalysis development: obviously in this case, purified PDC is required. PDC was isolated from many sources: yeasts, fungi, bacteria or plants. The enzyme from *Zymomonas mobilis* has many advantages considering enzyme stability and activity. But, for ligase activity, it is less powerful compared to *S. cerevisiae* homolog. A comparison between structures of these two enzymes showed that the residue 392 is an alanine in PDC from *S. cerevisiae* whereas it is tryptophane in *Zymomonas mobilis* one. Mutating Trp 392 into Ala or Ile has given a carboligase activity four times higher without lowering neither catalytic activity nor stability (Idin et al 1998).

Other aromatic aldehydes were also substrates in this reaction (Fig 13).

7. INCREASING SUBSTRATE SPECIFICITY OF A GIVEN ENZYME BY MUTAGENESIS

Determining crystalline structure of an enzyme allows to know which aminoacid residues are present in the active site, enabling to have an idea on their respective role in the formation of the enzyme-substrate complex and in the reaction itself. Replacing a residue by another using mutagenesis is a routine task for molecular biologists. Thus, by this technique, the type of reaction catalyzed, the reaction stereospecificity or the substrate specificity can be modulated.

Yeast transketolase, the enzyme we used, was crystallized and its structure analyzed by Schneider and Coll. The structure showed that Asp177 could interact with the hydroxyl group in the second position of the acceptor aldehyde and therefore could be responsible for enantioselectivity of the enzyme towards substrate with *2R* configuration (Fig 14). Avoiding this interaction could enable the enzyme to catalyze reactions with *2S* configuration substrates and thus could lead to ketoses with (*3S,4S*) configuration (the same as D-tagatose). Mutation of Asp477 to Ala was carried out and confirmed our hypothesis: the

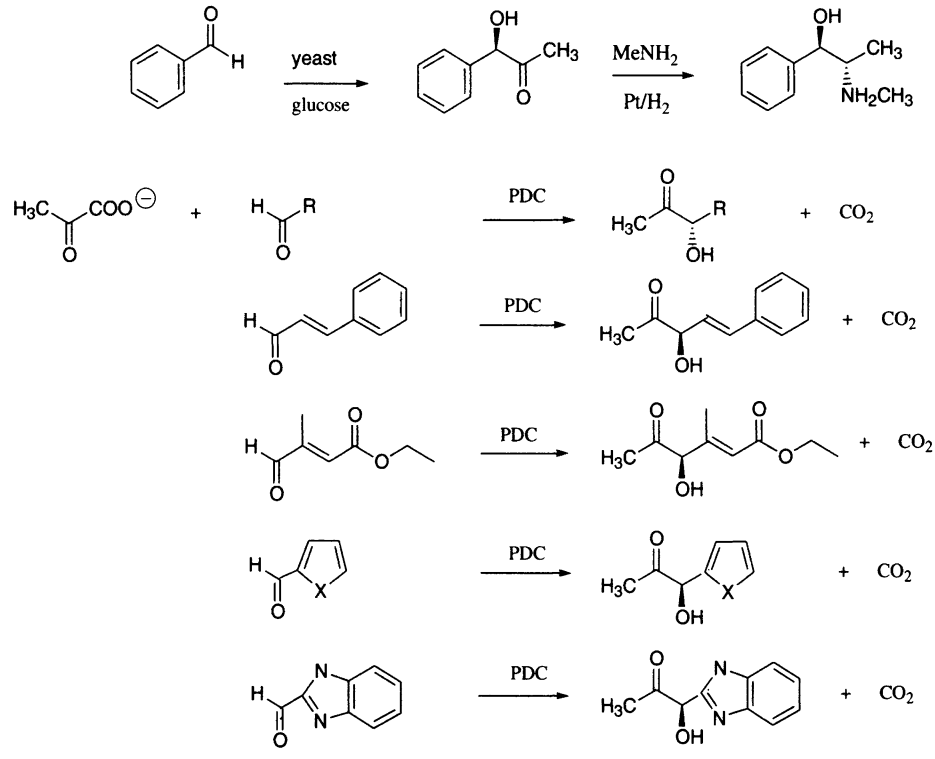


Fig. 13. Pyruvate decarboxylase catalyzed reaction

enzyme has lost its enantioselectivity, but its efficiency was lowered 100 times, limiting its use for synthetic applications (Nilsson et al 1998, Hecquet et al 2001).

This loss of activity is very often occurring during site-directed mutagenesis experiments. This was due to other modifications hard to foresee. Most of the modifications enabling to improve efficiency of an enzyme were obtained by random mutagenesis also called directed evolution (Arnold 1998, Bornschener & Pohl 2001).

In this technique, the modification is carried out *in vitro* randomly on one or two nucleotides in the enzyme gene. Mutated genes are introduced in *E. coli* cells and library of clones was thus obtained, able to express a modified protein. A screening test is then achieved on this library enabling to select the cell expressing the enzyme with the best improvement. This mutant will be submitted to another cycle of mutation and so on till obtaining a satisfying enzymatic activity.

This method is suitable if the screening test is efficient to select the right clones over thousands obtained each generation. It was used recently on a pyruvate aldolase (Fong et al 2000): 2-oxo-3-deoxy-D-phosphogluconate aldolase (KDPG aldolase) which was very specific for D-glyceraldehyde, but was able to catalyze reaction of L-glyceraldehyde leading to sugars from the L series (Fig 15).

More recently, the N-acetylmeuraminic aldolase has been altered to improve its catalytic activity towards enantiomeric substrates including N-acetyl-L-mannosamine and L-arabinose to produce L-sialic acid and L-

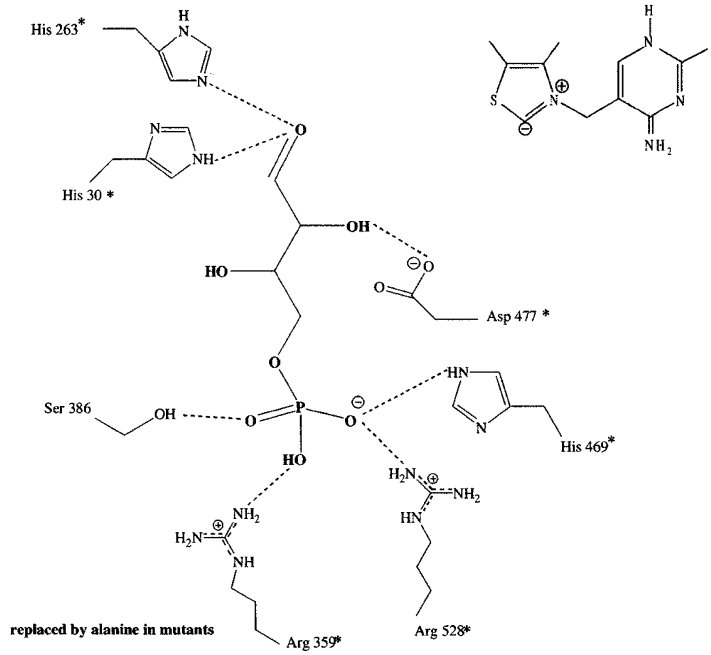


Fig. 14. Active site of yeast transketolase

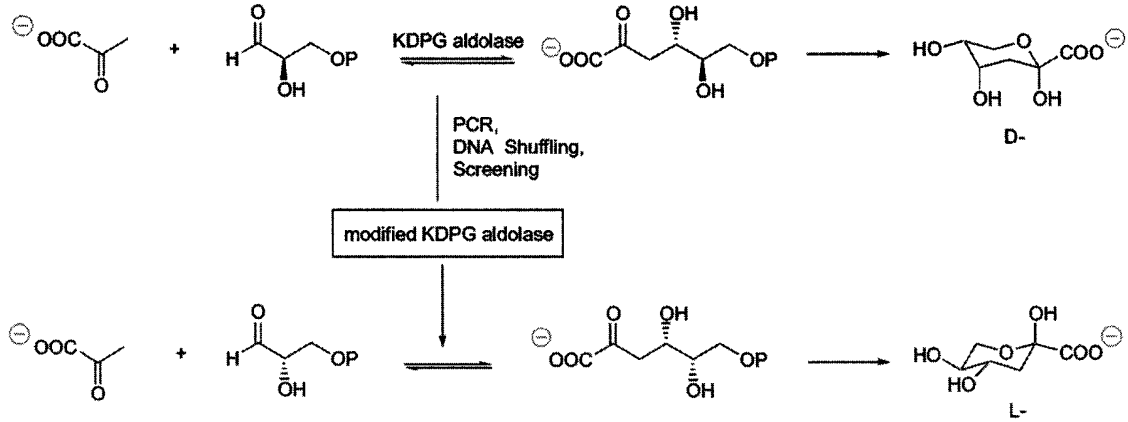


Fig. 15. Inversion of the KDPG aldolase enantioselectivity by directed evolution

KDO the mirror-image sugars of the corresponding natural D-sugars (Figs 16 and 17) (Wada et al 2003).

8. EXPLORING THE BIODIVERSITY TO FIND NEW CATALYSTS

Progress in screening methods to find new microorganism, new biotechnological tools to produce enzymes slightly expressed, as well as the decoding of the genome of many species will allow new enzymes to emerge as useful catalysts for enzymatic synthesis. Three recent examples from the literature look promising (Fig 18).

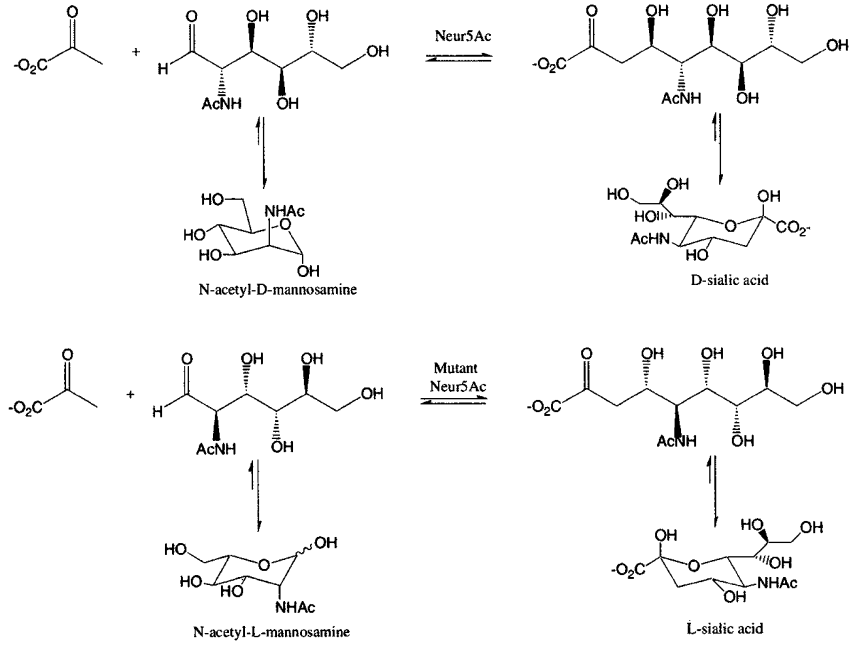


Fig. 16. Neu5Ac aldolase catalyzed synthesis of enantiomeric sialic acid

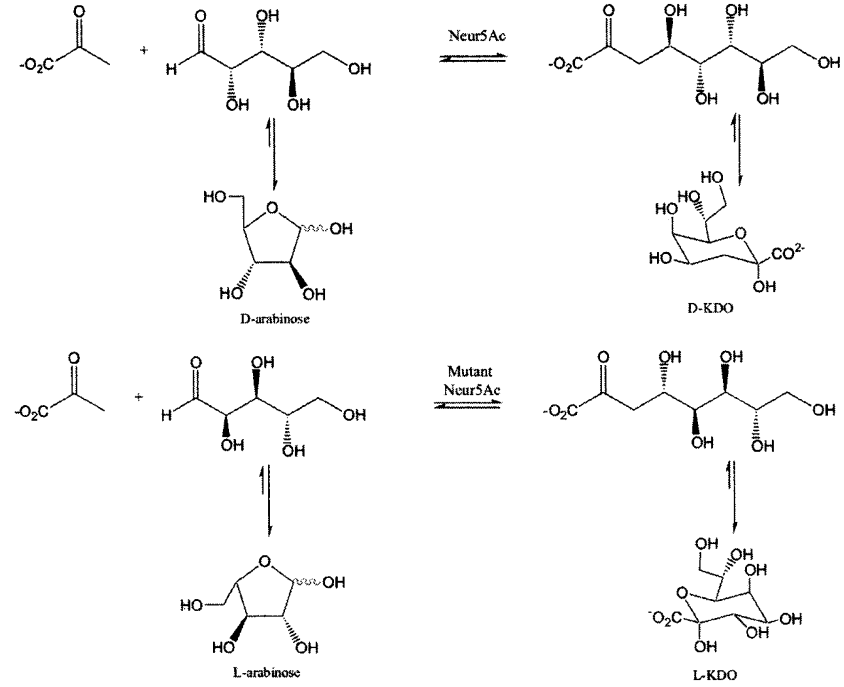


Fig. 17. Neu5Ac aldolase catalyzed synthesis of D- and L-KDO

A few years ago, Rohmer and Sahm groups showed that 1-deoxyxylulose-5-phosphate is the isopentenyl pyrophosphate in various organism, which consequently did not use the mevalonic acid pathway (Rohmer et al 1996, Putra et al 1998)

Studying this new way, Sprenger and Coll (Sprenger et al 1997, Taylor et al 1998) found in *E. coli* a hitherto unknown enzyme which catalyzes the formation of 1-deoxy-ketoses from pyruvate and an aldose. It is a thiamine pyrophosphate enzyme closely related to transketolase.

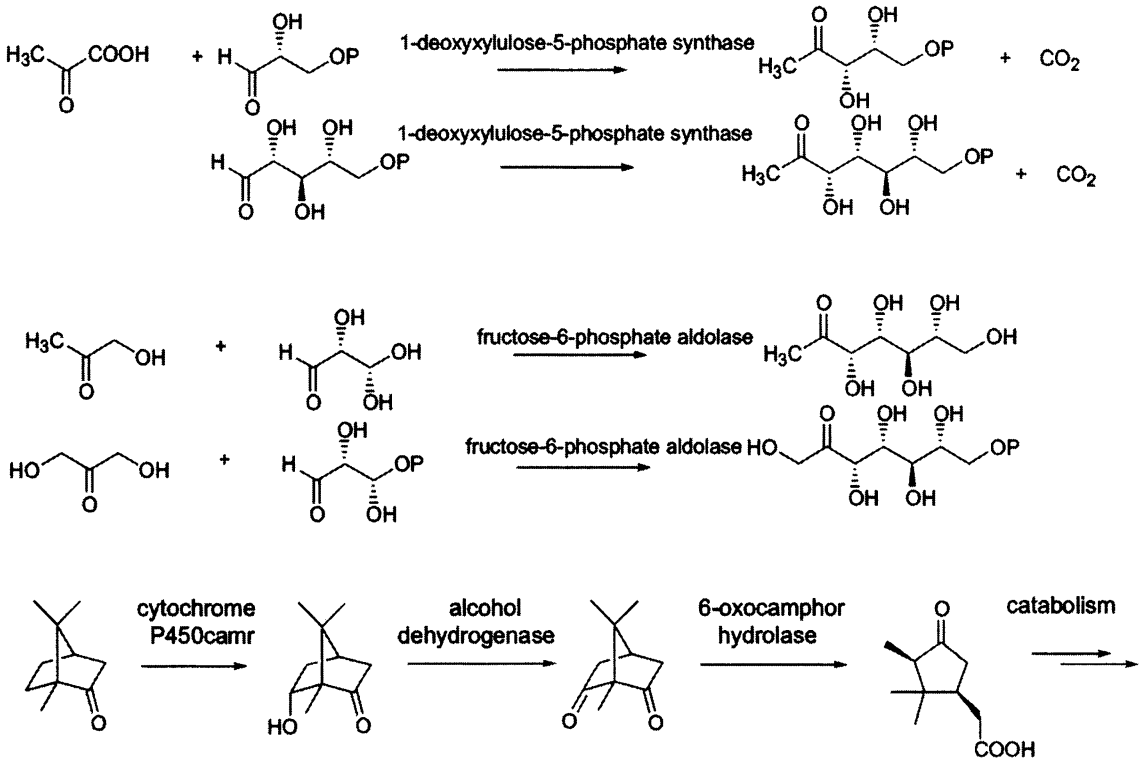


Fig. 18. New found enzymes : Deoxy-xylulose phosphate synthase, fructose-6-phosphate aldolase, and 6-oxo-norcamphorhydrolase

More recently, the same group discovered in the *E. coli* genome a non-expressed sequence, with a strong analogy with the transaldolase gene. After cloning and expression of this gene, they have shown that the protein produced had a fructose-6-phosphate aldolase activity, allowing the reversible condensation of dihydroxyacetone onto glyceraldehyde phosphate (Schürmann 2001). This new enzyme could be an attractive alternative to FDP aldolase for the synthesis of fructose analogs.

The last example is a 6-oxo-camphorhydrolase found on a *Rhodococcus sp.* grown on (+)-camphor as a sole carbon source. Camphor metabolisation pathway provides 6-oxo-camphor which undergoes a retro Claisen reaction leading to optically pure α-campholinic acid, so carrying out the desymmetrisation of 6-oxo-camphor. The enzyme responsible for this reaction has been overexpressed in *E. coli* and used for the desymmetrisation of various meso bicyclic β-diketones (Grogan et al 2001).

9. PRODUCING A CATALYTIC ANTIBODY

The catalytic antibody concept which has been developed quickly from the initial works of Schultz and Lerner (Schultz 1989, Schultz & Lerner 1995) led in the last years to new enzymes really useful for synthetic purposes. When an antibody is directed against a hapten which presents a structure mimicking the transition state of a given reaction, this antibody can be able to catalyse this reaction, since, having a better affinity for the transition state than for the fundamental one, it decreases the activation energy of the reaction. This concept was applied to aldolases (Reymond et al 1995, Barbas III et al 1997, Hoffmann et al 1998). In the works summarized in the Figure 19, antibodies recognising a diketone were produced. The selected antibody reacts with the diketone, by a lysine residue, leading to a Schiff base. The imine-enamine tautomery gives a structure very close to the reaction intermediate observed for class I aldolases (Fig 1). This antibody showed a surprising ability to catalyse stereospecific aldolisation reactions between ketones like acetone, hydroxyacetone or cyclopentanone and aldehydes, especially aromatic or unsaturated ones. These antibodies are also able to resolve racemic β-aldols, by catalysing a retro-aldolisation reaction onto only one enantiomer.

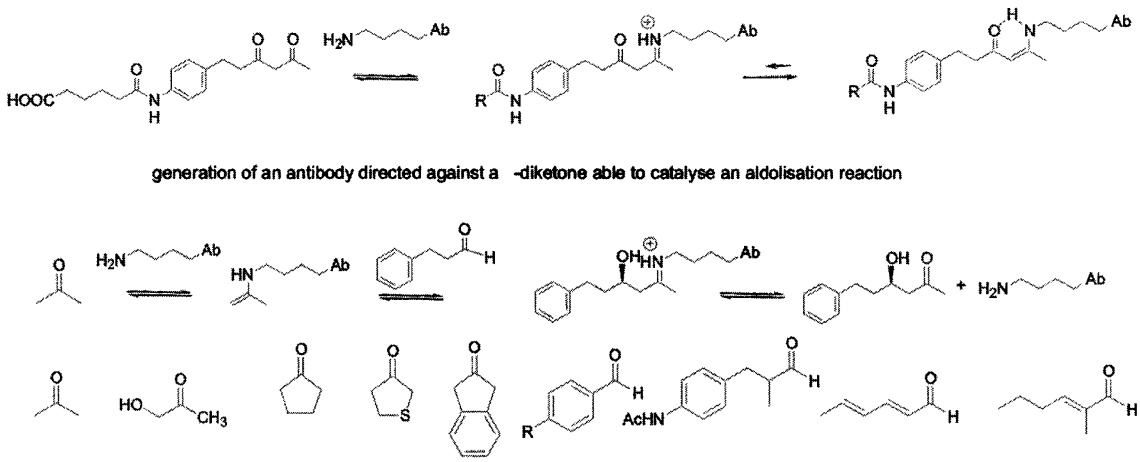


Fig. 19. Catalytic antibodies

10. CONCLUSIONS

For the last twenty years, enzymes have appeared to be useful tools for the obtention of chiral structures by organic synthesis, and very important results have been gained for the carbon-carbon bond formation. Chemists have learned how to exploit the stereospecificity of available enzymes, and tried to extend their usefulness, looking for new natural substrate analogs. The most spectacular progress have been provided by the interactivity of chemists and biologists, and there is no doubt that new important results will appear in the future for academic works as well as for industrial applications.

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Enzymes Engineered for New Reactions- Novel Catalysts for Organic Synthesis

Per Berglund

1. INTRODUCTION

Enzyme catalysis is an established powerful synthetic tool in the synthesis of complex compounds with precise and defined stereochemistry. Enzymatic processes are environmentally benign acting at ambient temperature and pressure, and many enzymatic reactions are possible to carry out in an aqueous reaction medium. Bridging the gap between molecular biology and synthetic chemistry is now a productive success in many chemistry laboratories worldwide. This has revolutionized the field of organic synthesis and much effort is being focused on tailoring enzyme catalysts in terms of stability and specificity. The progres can be seen in the area of enzyme redesign where new chemical reactions are created in the enzyme active site. These enzymes with engineered reaction specificity are of great interest in organic synthesis. Examples of this from various enzyme classes, such as the pyridoxal phosphate enzymes, thiamine diphosphate enzymes, and hydrolases will be discussed in this chapter.

1.1 Enzymes in Organic synthesis

Enzyme-catalyzed synthesis in a non-aqueous reaction medium is a standard synthetic tool in chemistry laboratories and there is extreme progress implementing enzymatic processes in multistep industrial organic synthesis (Roberts 1999, Faber 2000, Liese et al 2000, Lye et al 2002). The use of a non-aqueous reaction medium for an enzyme was considered to be a non-conventional process only twelve years ago (Tramper et al 1992). The principle of using modified substrates and substrates different from the natural substrate for an enzyme process was not standard in those days but are conventional processes today (Vulfson et al 2001). Now, the progress of the field has shifted from substrate- and medium engineering. Modern enzyme technology now offers the opportunity to alter the reaction mechanism by which a certain enzyme catalyses the transformation of its natural substrate so that a reaction, which is non-natural to the particular enzyme, can occur. This is a process that can be considered non-conventional today in the field of applied catalysis, but is a very powerful strategy to generate new enzyme catalysts for chemical transformations that are difficult in organic synthesis and where potent alternatives are needed.

1.2 Engineering reaction specificity

The opportunities that biotechnology offers in terms of redesigning and adapting enzymes for a predefined task is a challenging and exploding field today, which creates new powerful catalysts for applied chemistry (Hult & Berglund 2003). The engineering of new catalytic activities into an existing enzyme, i.e. alteration of reaction mechanisms of enzymes to catalyze formation or breaking of new bonds, is perhaps the most challenging area in this field. Engineering of new catalytic activities is the "Holy Grail" of enzyme

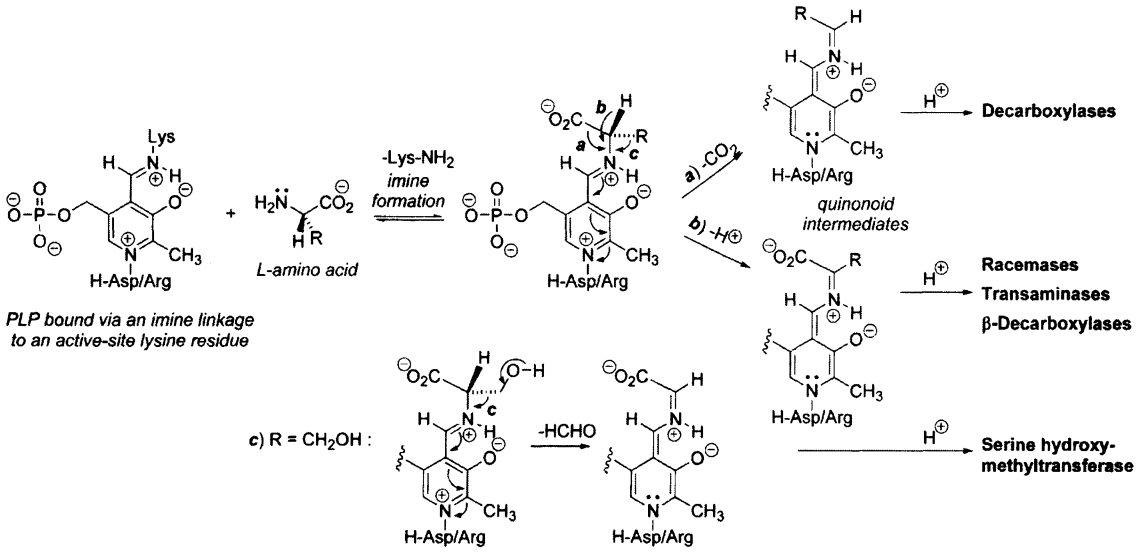
redesign (Penning & Jez 2001). A new enzyme variant with a new catalytic activity is said to possess "altered reaction specificity", a term defined by Graber et al (1999) as a new catalytic activity not inherent in the wild type and a suppressed original activity to a level significantly below that of the new one. One of the early examples of this is the engineering of subtilisin (EC 3.4.21.62) for synthesis of peptides in water (Abrahmsén et al 1991). This study illustrates the power of rational design and that careful changes of an active site based on good knowledge of both the three-dimensional structure and the mechanism of an enzyme can lead to impressive and useful changes.

There are in principle three possible routes to alter a pre-existing enzyme activity into a new one (Cedrone et al 2000). These are (i) change of substrate specificity, (ii) reinforcement of a promiscuous reaction, and (iii) change of enzyme mechanism. The latter implies engineering of the reaction specificity (Graber et al 1999). Some examples of reinforcement of promiscuous reactions and engineering of reaction specificity will be discussed below.

2. PROTEIN ENGINEERING STRATEGIES

2.1 Reactions with pyridoxalphosphate dependent enzymes

Pyridoxal-5'-phosphate (PLP) from vitamin B_6 (pyridoxine) is a cofactor utilized by nature in a wide variety of chemical transformations catalyzed by enzymes with diverse reaction mechanisms (John 1995, Paiardini et al. 2003), (Scheme 1). The formed protonated PLP-imine functions as an "electron sink" to facilitate any elimination at the α-carbon of the amino acid substrate. The resulting α-carbanion electrons are then delocalized all the way to the pyridinium nitrogen in a quinonoid structure. Although PLP enzymes use similar reaction mechanisms there are generally only two conserved amino acid residues among the various enzymes; the lysine bound to the cofactor in the resting state (Momany et al. 1995), and either an aspartate or glutamate (as in transaminases) hydrogen-bonded to the protonated pyridinium nitrogen, or alternatively an arginine (as in racemases) coordinated to the unprotonated pyridine nitrogen. There is usually also an arginine residue bound to the α-carboxylate group (John 1995). The position of this arginine is important in directing the reaction to either deprotonation or decarboxylation.



Scheme 1. The common first steps in the mechanism of PLP enzymes leading to a quinonoid intermediate. In decarboxylases the α-carboxylate leaves the α-carbon of the imine (route **a**). In racemases, transaminases and β-decarboxylases, the α-proton leaves (route **b**). In serine hydroxymethyl transferase, a retro-aldol reaction produces formaldehyde (route **c**).

The protein part of each different PLP enzyme directs the catalytic properties of PLP so that only one single reaction proceeds with unique substrate specificity. The basis behind this was rationalized for the PLP enzymes already in 1966 (Dunathan 1966) and it was suggested that the bond that breaks must lie in a plane orthogonal to the plane of the PLP-imine π-electrons for maximal σ-π-orbital overlap (Fig 1). The enzymes control the orientation around the α-carbon-imine nitrogen σ-bond by restricting its rotation by a positively charged amino acid residue, often an arginine, which forms a salt bridge to the carboxylate group. Depending on where this positively charged residue is located in the 3D structure of the enzyme, the enzyme catalyzes either α-deprotonation, α-decarboxylation, or α-dealkylation.

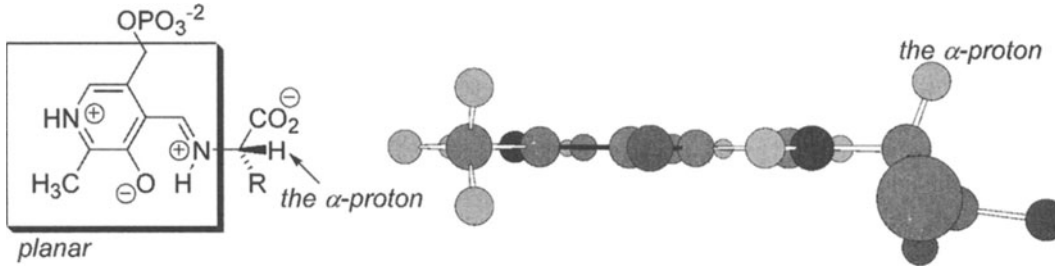


Figure 1. View of the planar aromatic pyridinium ring system, from above (left) and from the side (right), showing the orthogonal orientation of the α-proton on the stereocenter of an L-amino acid necessary for α-deprotonation. In order for α-deprotonation to occur, the proton has to be locked in an orthogonal orientation by the enzyme (racemase, transaminase or β-decarboxylase). In α-decarboxylation, the α-carboxylate is locked in an orthogonal fashion (decarboxylase) or in α-dealkylation, the R-group is locked orthogonal (such as in serine hydroxymethyl transferase) (Dunathan 1966).

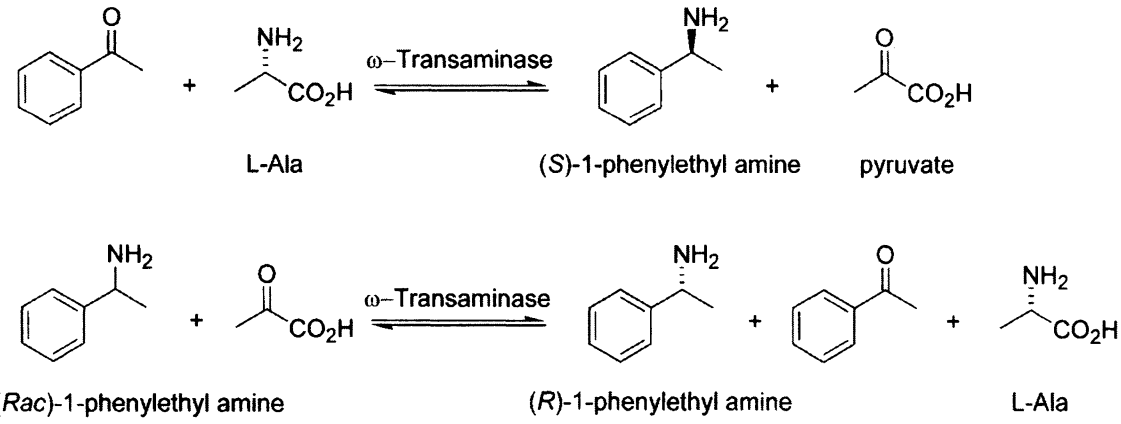
When an amino acid is heated with PLP in the absence of enzyme, both α-decarboxylation and α-racemization products, among others, can be found. Recently, Breslow's group synthesized modified pyridoxal phosphate analogs in order to block the transamination activity in preference to racemase activity in transformations of amino acids without an enzyme (Liu & Breslow 2001). They found that transamination activity, i.e. re-protonation of the quinonoid intermediate at the 4' position, is favored when the pyridine nitrogen is protonated, and that racemization, i.e. re-protonation of the quinonoid intermediate at the α-position, is favored in the unprotonated case. This agreed well with the enzymes aspartate aminotransferase (EC 2.6.1.1) versus alanine racemase (EC 5.1.1.1), where in the former case the protonated pyridinium nitrogen is hydrogen bonded to an aspartate carboxylate group, and in the latter case the unprotonated pyridine nitrogen is hydrogen bonded to a weakly acidic arginine (Scheme 1).

2.1.1 Transamination

The enzyme alanine racemase (EC 5.1.1.1) from *Bacillus stearothermophilus* is a PLP enzyme. A double active-site mutation of this enzyme altered its reaction specificity from racemization to D-amino

acid transamination (Yow et al 2003). The pyridinium nitrogen of the cofactor is hydrogen bonded to arginine 219 in this alanine racemase and the corresponding residue in the amino transferases is a carboxylate. Consequently, mutation of arginine 219 into glutamate resulted in a 5.4-fold increase in the forward half transamination activity with D-alanine and a thousand-fold decrease in the racemase activity. A second mutation, Tyr265Ala, completely eliminated the racemase activity. This study demonstrates the importance of the residue coordinated to the pyridinium nitrogen for controlling the reaction specificity of PLP enzymes.

Enantiomerically pure amines are very important building blocks in organic synthesis. Interestingly, a promiscuous reaction of a PLP wild type enzyme has been found where amines react (Shin & Kim 1997, Shin & Kim 2001, Shin et al 2001). This enzyme is a transaminase obtained from a number of different microorganisms (EC 2.6.1.18). The fact that an amine is a substrate shows that the first step in the reaction mechanism, removal of the α-proton, is possible without the α-carboxylate present in a natural amino acid substrate of a PLP enzyme. A number of amines in high stereoisomeric purity were prepared with this enzyme both in the stereoselective synthesis mode leading to *S*-amines (> 99% ee) through enantioselective amination of ketones (Shin & Kim 1999), and in the kinetic resolution mode leading to *R*-amines (>95% ee) in a biphasic aqueous-organic solvent to avoid inhibition by the acetophenone product (Shin & Kim 1997), Scheme 2.

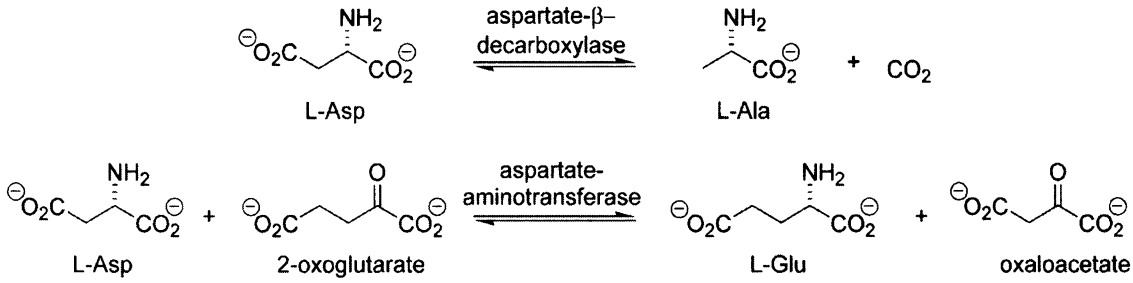


Scheme 2. The stereoselective synthesis mode of an *S*-amine (Shin & Kim 1999) and the kinetic resolution mode of a racemic amine (Shin & Kim 1997).

2.1.2 β-Decarboxylation

L-Aspartate-β-decarboxylase (EC 4.1.1.12) is a PLP enzyme catalyzing decarboxylation at the 4-position of aspartate to form L-alanine in nature (Rosenberg & O'Leary 1985), Scheme 3. The high similarity allowing for the possibility to switch reaction specificity between PLP enzymes has been elegantly demonstrated by Christen and collaborators (Graber et al 1995, Graber et al 1999). They introduced the

L-aspartate-β-decarboxylase activity into the enzyme aspartate aminotransferase (EC 2.6.1.1). The transamination activity of a triple active-site mutant decreased 18000-fold compared to the wild type and the new β-decarboxylase activity increased 1300-fold (k_{cat} = 0.08 s^{-1}) from the wild type to a level eight times higher than the remaining transamination activity (Graber et al 1999). This work is a good example of a true modification of enzyme reaction specificity.



Scheme 3. The L-aspartate-β-decarboxylase activity engineered into aspartate aminotransferase (upper part). The natural activity of aspartate aminotransferase is shown in the lower part.

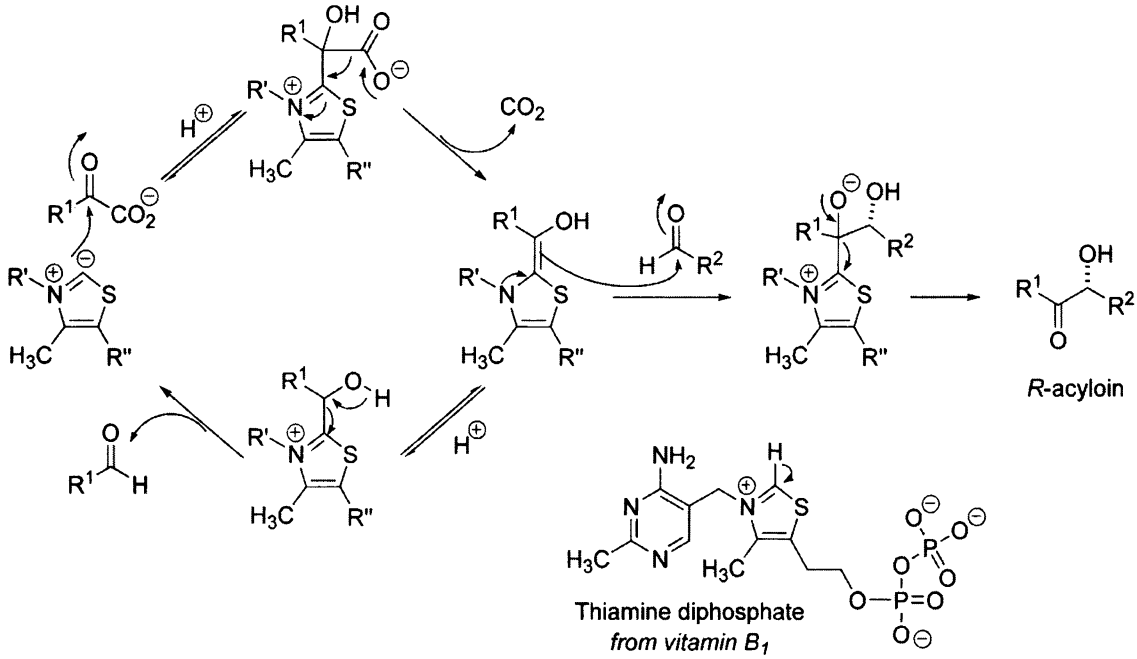
2.2. Carbon-carbon bond forming reactions

The stereoselective formation of new carbon-carbon bonds is of special interest to synthetic chemists. Enzymes catalyzing such reactions are therefore very important and this area has recently been reviewed by Breuer & Hauer (2003). The earliest commercialized example of a modified enzymatic reaction is found in this group of reactions, namely the formation of an acyloin catalyzed by a decarboxylase enzyme (Neuberg & Hirsch 1921).

2.2.1 Thiamine diphosphate dependent enzymes

Pyruvate decarboxylase (EC 4.1.1.1) is an enzyme utilizing thiamine diphosphate, from vitamin B_1 (thiamine), as a cofactor to catalyze decarboxylation of pyruvate in nature. It has been used commercially for a long time for the production of (*R*)-phenylacetyl carbinol, a precursor of important drugs such as L-ephedrine, pseudoephedrine and norephedrine. This process was discovered by Neuberg & Hirsch (1921) and is a carbon-carbon bond forming reaction called acyloin (α-hydroxyketone) condensation which is not a natural reaction for the enzyme. The process was commercialized already in 1932 and is said to be the first chiral biotransformation process commercialized (Engel et al 2003). It is a fermentative process using the yeast *Saccharomyces cerevisiae*, glucose and benzaldehyde. The presence of

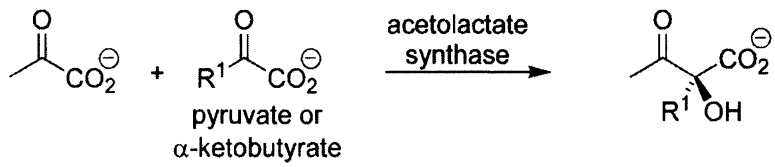
benzaldehyde directs the reaction to the carboligation product instead of the natural decarboxylation product acetaldehyde (Hanč & Kakáč 1956), Scheme 4.



Scheme 4. The reaction mechanism of pyruvate decarboxylase ($R^1 = CH_3$ for pyruvate). The benzaldehyde present (R^2 = phenyl) traps the formed enamine intermediate and an acyloin is formed instead of the natural formation of acetaldehyde.

Scheme 5. Natural reaction of acetolactate synthase forming an *S*-acetohydroxy acid. $R^1 = CH_3$ (pyruvate) or CH_2CH_3 (α-ketobutyrate) (Engel et al 2003).

An alternative process for production of (*R*)-phenylacetyl carbinol was developed by Engel et al (2003) using the isolated thiamine diphosphate enzyme acetolactate synthase (EC 4.1.3.18) from *Escherichia coli*. In contrast to pyruvate decarboxylase, the natural reaction of acetolactate synthase actually is carbon-carbon bond formation through condensation of pyruvate with another pyruvate or with α-ketobutyrate, but not condensation of pyruvate with aldehydes similar to the acyloin condensation of pyruvate decarboxylase (Scheme 5).



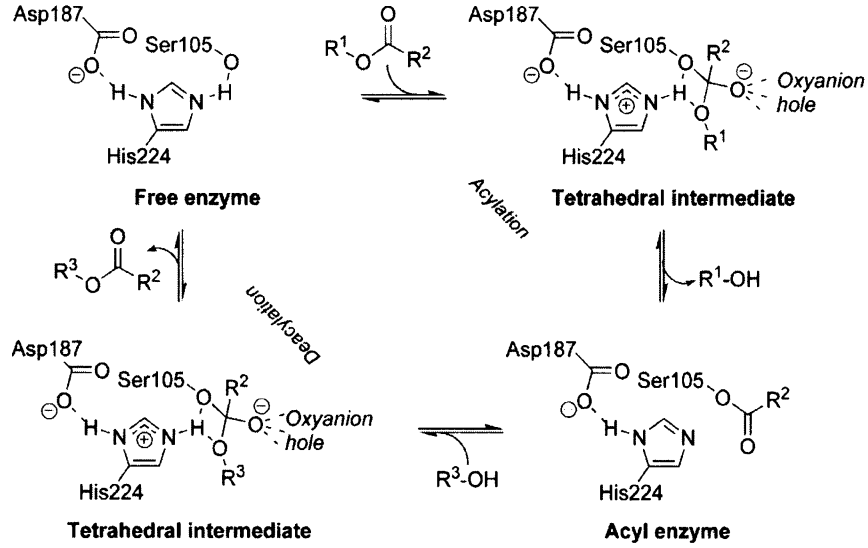
Three isoenzymes of acetolactate synthase were found, overexpressed and purified and their potentials for (*R*)-phenylacetyl carbinol production from pyruvate and benzaldehyde were investigated (Engel et al

2003). Two mutants of isoenzyme II were created. One of these, Met250Ala, suppressed the natural product acetolactate in favor of (*R*)-phenylacetyl carbinol. The other mutant, Trp464Leu, showed a low sensitivity to accumulation of the product (*R*)-phenylacetyl carbinol. All three isoenzymes and the two mutants investigated showed excellent stereoselectivity and the product was obtained with more than 97% ee. The enzymes showed broad substrate specificity. Both substituted benzaldehydes and other aromatic aldehydes have been explored. Acetolactate synthase has therefore a high potential to become a versatile and stereoselective carbon-carbon bond forming catalyst suitable for practical use.

2.2.2 Hydrolases

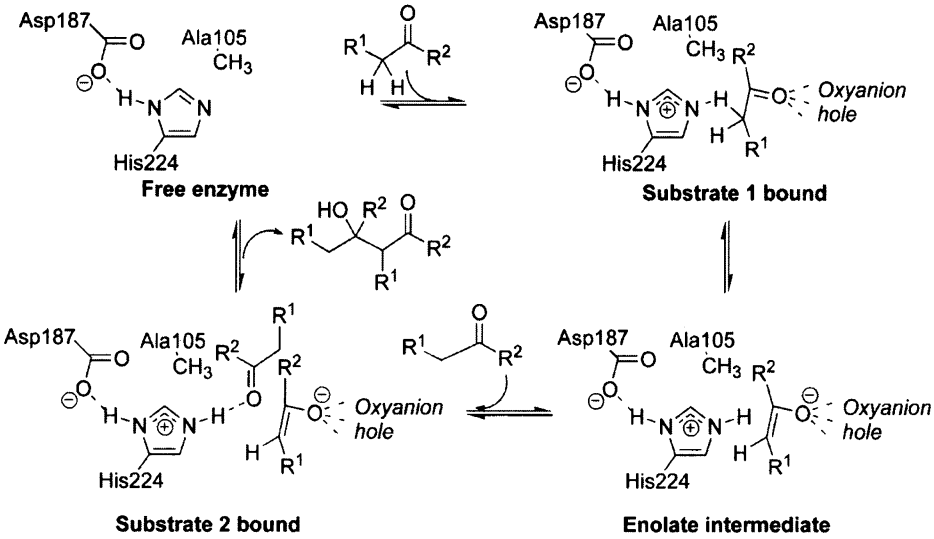
Serine hydrolases belong to the α/β-hydrolase fold superfamily of enzymes. This is an example of a superfamily with conserved mechanistic features that catalyze a wide variety of different reactions in nature (Ollis et al 1992, Holmquist 2000). Such a superfamily provides strong evidence for the important role of divergent evolution (Babbitt & Gerlt 1997, O´Brien & Herschlag 1999), and the potential to alter reaction specificity of such enzymes is therefore high. Serine hydrolases have been the major enzyme class used in organic synthesis (Bornscheuer & Kazlauskas 1999), and among them lipases are the enzymes most extensively studied.

Many lipases show broad substrate specificity, high stability in organic solvents and excellent stereoselectivity. They catalyze acyl transfer reactions of various esters and carboxylic acids and are frequently used in preparative synthesis for kinetic resolutions of racemates (Berglund & Hult 2000, Bornscheuer et al 2002). For this purpose, numerous engineering studies have been made in order to improve lipase enantioselectivity (Berglund 2001, Bornscheuer 2002, Reetz 2001). We recently explored the possibility to rationally engineer a lipase scaffold to catalyze new reactions such as aldol additions with the hope to obtain a catalyst with all advantages of a lipase including broad substrate acceptance range and high stability in an organic solvent (Branneby et al. 2003). *Candida antarctica* B lipase (EC 3.1.1.3) is one of the most well-studied lipases (Rotticci et al 2001). Its reaction mechanism is shown in Scheme 6 for a hydrolysis (R^3 = H), or transesterification ($R^3 \neq$ H). In the first steps (acylation of the enzyme), the active-site serine 105 is activated by histidine 224 and aspartate 187 and attacks the carbonyl carbon of the substrate acyl donor forming a tetrahedral intermediate which is stabilized by three hydrogen bonds in an oxyanion hole formed by the side chain of threonine 40 and the backbone amide protons of threonine 40 and glutamine 106. The leaving group then leaves and an acyl enzyme is formed. The subsequent steps (deacylation) are similar leading to the product after deacylation of the enzyme by a second nucleophile.



Scheme 6. The mechanism of *Candida antarctica* lipase in acyl transfer reactions. R^1, R^2, or R^3 can be a chiral entity.

Ab initio calculations on a minimal model system suggested that an aldol addition reaction would be possible, where the histidine acts as a general base abstracting a α-proton from a substrate carbonyl compound. The resulting enolate is stabilized in the oxyanion hole and then reacts with a second carbonyl compound leading to an aldol product after proton transfer from the protonated histidine (Scheme 7), (Branneby et al 2003). Mutants were then created with the active-site serine 105 replaced. These mutants lack the nucleophilic feature of the active site but the histidine 224 and the electrophilic oxyanion hole still remains.



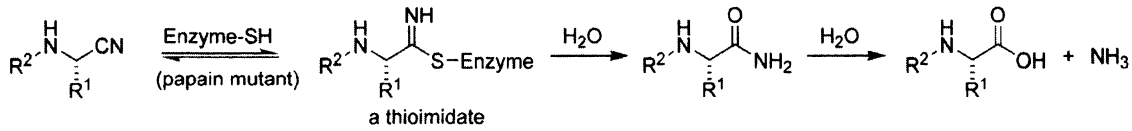
Scheme 7. The mechanism of an aldol reaction catalyzed by the mutant Ser105Ala of *Candida antarctica* lipase B. R^1 = n-butyl and R^2 = H in the case of hexanal as Substrate 1 and Substrate 2 (Branneby et al 2003).

The reaction was studied in cyclohexane with a number of aldehydes and ketones and the reaction rates were found to be in the same range as those of catalytic antibodies. Wild type lipase expressed some aldol addition activity, yet lower than that of the Ser105Ala mutant. Moreover, the Ser105Ala mutant had no measurable hydrolase activity. Lipase inhibited with the covalent inhibitor methyl *p*-nitrophenyl n-hexylphosphonate (Rotticci et al 2000) was completely inactive confirming that the active site is necessary for the reaction (Branneby et al 2003). This study shows the catalytic plasticity of an enzyme and demonstrates the advantages of a lipase (broad substrate specificity and stability in organic solvent) being utilized for a new reaction such as an aldol addition.

Reactions involving enolate intermediates have been studied with serine hydrolases before. In 1986, Kitazume and collaborators showed that conjugate addition reactions can be catalyzed by the wild type enzymes α-chymotrypsin (EC 3.4.21.1), porcine liver esterase (EC 3.1.1.1), and *Candida rugosa* lipase (EC 3.1.1.3) (Kitazume et al 1986). The reactions studied were Michael-type additions of thiols, amines and alcohols on a α, β-unsaturated carboxylic acid.

2.3. Nitrile hydrolysis

Nitriles are important intermediates used in organic synthesis. They are generally quite resistant to chemical hydrolysis, but are hydrolyzed in nature by enzymes from either of the two classes nitrile hydratase (EC 4.2.1.84) or nitrilases (EC 3.5.5.X). Nitrile hydratase produces the amide that can be further hydrolyzed to the corresponding carboxylic acid by an amidase, whereas a nitrilase directly yields the carboxylic acid. Nitrile hydratase has been successfully used industrially for production of acrylamide (Kobayashi et al 1992). The group of Ménard has demonstrated an alternative for nitrile hydrolysis in the installation of nitrile hydratase activity into the cysteine protease papain (EC 3.4.22.2) (Dufour et al 1995), an enzyme well studied under a wide range of reactions conditions and known to be active in organic solvents



Scheme 8. Hydrolysis of peptide nitriles by the papain mutant Gln19Glu. The nitrile hydratase activity of the mutant hydrolyzes the thioimidate to the amide which is hydrolyzed by the amidase activity of the mutant. R^1 = H or CH_3. (Dufour et al 1995).

Peptide nitriles are reversible inhibitors of cysteine proteases (Thompson et al 1986). They react with the active-site cysteine to form thioimidates. The structure of a thioimidate resembles that of the acyl enzyme intermediate in the natural hydrolysis reaction of papain, where the carbonyl oxygen is hydrogen bonded in the region termed the "oxyanion hole". This consists of two hydrogen bond donors on the backbone amide nitrogen of cysteine 25 and the side chain amide of glutamine 19. Similar to a natural substrate, the nitrogen of the thioimidate was assumed to be bound in the oxyanion hole of papain. Hydrolysis of a thioimidate in solution is acid catalyzed suggesting that a protonated thioimidate is more reactive. The new activity was therefore installed by introducing an active-site residue that could provide a proton in the oxyanion hole vicinity. Consequently, the single mutation Gln19Glu caused a dramatic increase in the hydrolysis rate of the thioimidate to the amide at pH 5, as reflected by the more than 10^5-

fold increase in k_{cat}. The mutant had an unchanged amidase activity which led to immediate hydrolysis of the formed amide to the acid as the final product (Dufour et al 1995), Scheme 8.

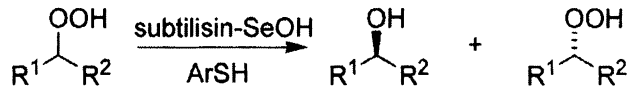
This is the product that would be obtained from a nitrilase. The difference between a nitrilase and the papain-Gln19Glu mutant is that no amide is generated in a nitrilase reaction. The papain mutant was active also in an aqueous organic reaction medium, containing up to 50% acetone, on aromatic nitriles that contained a peptide-like bond (Versari et al 2002). The reaction has recently been investigated by molecular dynamics simulations on the nanosecond scale confirming protonation of the thioimidate by Glu 19 as the key reason for the nitrile hydratase activity of the papain-Gln19Glu mutant (Reddy et al 2002).

3. CHEMICAL MODIFICATION STRATEGIES

Rational redesign through site-directed mutagenesis and evolution through random mutagenesis methods constitute powerful strategies for altering reaction specificity of enzymes. However, work on altering enzyme reaction specificity was made prior to the discovery of genetic methods through chemical modification. The pioneering work in the area of chemical modification for new enzyme activities was simultaneously made by Polgar and Bender (1966) and Neet and Koshland (1966). They made thiolsubtilisin by chemically converting the active-site serine residue to cysteine.

The group of Kaiser investigated the potential of semisynthetic enzymes further and introduced cofactors like flavines into the active site of papain (EC 3.4.22.2) to create a new dehydrogenase activity which was comparable in activity to a number of the natural occurring enzymes (Kaiser & Lawrence 1984).

Reetz suggests a completely different approach for engineering the reaction specificity of enzymes, namely a concept of directed evolution of hybrid catalysts (Reetz 2002). The idea is to use an enzyme with a group prone to chemical modification, such as the cysteine side chain, and produce libraries of mutant genes, express these enzymes followed by chemical modification and then screen for the desired unnatural activity. The process is then repeated in a directed evolutionary fashion. The similar strategy of using covalent chemical modification to alter enzyme properties, including reaction specificity, has been summarized in a review by DeSantis and Jones (1999).



Scheme 9. Kinetic resolution of hydroperoxides through enantioselective reduction catalyzed by the semisynthetic enzyme seleno-subtilisin in the presence of thiophenols (Häring et al 1999).

Another important example in this area is the conversion of the protease subtilisin (EC 3.4.21.62) into selenosubtilisin by chemically substituting the active-site serine hydroxyl for a selenol (Wu et al 1989). Selenosubtilisin was later shown to be a potent hydroperoxidase, catalyzing reduction of a wide variety of functionalized racemic hydroperoxides, with opposite enantioselectivity compared to the natural peroxidases horse radish peroxidase (EC 1.11.1.7) and chloroperoxidase (EC 1.11.1.10) (Häring et al 1999). Thus, this semisynthetic enzyme effectively complements the naturally available enzymes for enantioselective reduction of hydroperoxides. Selenosubtilisin was used for kinetic resolution of hydroperoxides leading to alcohol products and remaining hydroperoxide in high enantiomeric excess (Häring et al 1999), Scheme 9.

4. CONCLUSIONS

Numerous review articles have appeared in recent years addressing the issue of rational design versus directed evolution to obtain enzyme variants with improved properties. One conclusion that can be drawn from most of these review articles is, not surprisingly, that combined approaches of rational design and directed evolution have advantages over the single methods alone.

To install new enzymatic activity into existing enzymes is a challenging task. To do this in a predictive manner requires detailed understanding of catalytic mechanisms both of the natural reaction and of the expected new one, as well as detailed knowledge of the three-dimensional structure of the active site. Rational redesign of enzyme reaction specificity is therefore an area well suited for site-directed mutagenesis to create mutant enzymes at pre-selected positions. Maximizing activity, on the other hand, is better suited to directed evolution.

The redesign of enzyme reactions and creation of new chemistry in well-studied enzyme active sites have expanded the field of enzyme technology and the already large interest for applications within applied chemistry will most certainly increase. New alternative and selective synthetic methods in organic synthesis are highly needed and enzyme technology, representing an area of explosive growth, can solve many new problems where stereo-, regio-, or chemoselectivity is an issue.

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α-Amylases

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1. INTRODUCTION

Among starch-hydrolysing enzymes that are produced on an industrial scale, α-amylases (EC. 3.2.1.1) are of considerable commercial interest. α-Amylases randomly hydrolyse α-1,4 glycosidic linkages in starch or its hydrolysis products. Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylases, which include, *Bacillus licheniformis, B. coagulans, B. stearothermophilus, B. caldolyticus, B. brevis, B. acidocaldarius*, and *B. thermoamyloliquefaciens*. The extracellular enzymes produced by various *Bacillus* species play a vital role in the biotechnology industry (Priest 1977, 1984). By 2005, the annual worldwide sale of industrial enzymes is estimated to be around US$ 1.7 - 2 billion (Godfrey & West 1996), and a half of this would be produced by *Bacillus* species (Meima et al. 2003). The sale of amylolytic enzymes accounted for almost US$ 225 million world-wide (Walsh 2002). Today, a large number of microbial amylases are available commercially and these have almost completely replaced chemical hydrolysis of starch in starch processing industry (Vihinen & Mantsala 1989, Pandey et al. 2000, Van der Maarel et al. 2002). Industrial processes for starch hydrolysis to glucose rely on inorganic acids or enzyme catalysis. The use of enzymes is preferred as it offers a number of advantages including improved yields and favourable economics. Enzymatic hydrolysis allows greater control over amylolysis, the specificity of the reaction, and the stability of the generated products (Antranikian 1992, Pandey et al. 2000). The milder reaction conditions involve lower temperatures and near neutral pH, thus reducing unwanted reactions (Fogarty & Kelly 1980, Hamilton et al. 1999a, Veille & Zeikus 2001). Enzymatic methods are favoured because they also lower energy requirements and eliminate neutralization steps.

The history of amylases is as old as 1811 when the first starch-degrading enzyme was discovered by Kirchhoff, followed by several reports on digestive amylases and malt amylases. It was much later in 1930 that Ohlsson suggested the classification of starch digestive enzymes in malt as α- and β-amylases according to the anomeric forms of sugars produced by the enzyme reaction. Amylolytic enzymes that produce specific malto-oligosaccharides from starch in high yields have received a considerable attention. High maltose syrups have an increasing demand due to their potential uses in the food, chemical and pharmaceutical industries. A number of reviews have dealt with amylases and their applications (Vihinen & Mantsala 1989, Pandey et al. 2000, Van der Maarel et al. 2002, Gupta et al. 2003). However, there are very few exclusively on α-amylases (Pandey et al. 2000, MacGregor et al. 2001,Van der Maarel et al. 2002). This chapter deals with the production, characterization and potential applications of α-amylases from various microbial sources.

1.1. Starch

Starch, one of the most abundant polysaccharides, is produced by plants and is composed of two high-molecular weight compounds, amylose (15% to 25%) and amylopectin (75% to 85%). High-amylose starches have a low average degree of polymerization (Jacob & Rendleman 2000). Amylose consists of linear chain in which the glucose residues are linked between C-1 and C-4 in the alpha orientation. One end of the chain has a free, C-1 hydroxyl and is called the reducing end. Amylopectin, on the other hand, is a branched polymer containing α- 1,4 linked glucose with α-1,6 –linked branching points every 17 to 26 glucose residues; it is one of the largest molecules in nature and may have a molecular weight $>10^3$ kDa.

1.2. Starch hydrolyzing enzymes

Microorganisms produce a variety of enzymes for complete hydrolysis of starch. Amylolytic enzymes are categorized into endo-amylases [α-1,4-glucan-glucanohydrolase; EC 3.2.1.1]; exo-acting amylases [β-amylase (EC 3.2.1.2), glucoamylase (1,4-α-D-glucan glucanohydrolase), α-glucosidase (EC 3.2.1.20), cyclodextrin glycosyl-transferase (EC 2.4.1.19), maltogenic α-amylase (EC 3.2.1.133), maltooligosaccharide-forming amylase and maltohexaose-forming amylase (EC 3.2.1.98)]. Debranching enzymes exclusively hydrolyze α-1,6 glycosidic bonds. Isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41) and amylopullulanase cleave α-1,6-glucosidic bonds at the branching points of amylopectin.

1.3. α-Amylases

Endo-acting enzymes, such as α-amylase, hydrolyse linkages in the interior of the starch polymer in a random fashion, that leads to the formation of linear and branched oligosaccharides. The sugar reducing groups are liberated in the α-anomeric configuration. Most starch hydrolyzing enzymes belong to the α-amylase family (Janecek 1997), which contain a characteristic catalytic $(\beta/\alpha)_8$-barrel domain. α-Amylase family is also known as family 13 glycosyl hydrolases (Henrissat 1991). This group comprises the enzymes with the following features:

(i) They act on α-glycosidic bonds and hydrolyze this bond to produce α-anomeric mono-or oligosaccharides (hydrolysis), form α-1,4 or 1,6 glycosidic linkages (transglycosylation), or a combination of both activities.

(ii) They possess a $(\beta/\alpha)_8$ or TIM (Fig. 1) barrel structure containing the catalytic site residues.

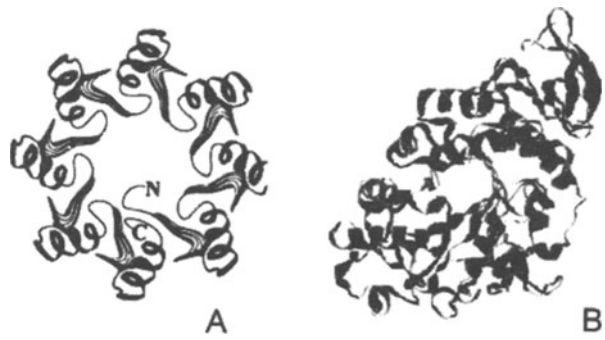

Fig. 1. Schematic representation of the $(\beta/\alpha)_8$ barrel (A)and 3D structure of the α-amylase of *Aspergillus oryzae* or Taka amylase (B), obtained from protein database.

(iii) They have four highly conserved regions in their primary sequence, which contain the amino acids that form the catalytic site, as well as some amino acids that are essential for the stability of the conserved TIM barrel topology (Kuriki & Imanaka 1999).

2. SOURCES OF α-AMYLASES

The ability to utilize starch as a carbon and energy source is widely prevalent among animals, plants and microorganisms. The microbial sources of α-amylases are shown in Tables 1a and 1b.

3. PRODUCTION OF α-AMYLASE

The production of α-amylase by submerged fermentation (SmF) as well as solid-state fermentation (SSF) has been thoroughly investigated. A variety of physicochemical factors affect synthesis and secretion of α-amylases (Francis et al. 2003). Most notable among these are the composition of the growth medium, pH, phosphate concentration, inoculum level and age, temperature, aeration, and carbon and nitrogen sources.

3.1. Physiochemical parameters

The role of various physico-chemical parameters, such as carbon and nitrogen sources, surface acting agents, phosphate, metal ions, temperature, pH and agitation has been studied. *Bacillus* spp. are able to synthesize and secrete large quantities of proteins into the extracellular fluid. Their growth requirements are simple, and physio-chemical parameters such as carbon, nitrogen, phosphate, metal ions, pH, temperature, aeration and agitation affected the enzyme production (Fogarty & Kelly 1979a, 1979b, Lonsane & Ramesh 1990).

Mostly α-amylases are inducible enzymes and are generally induced in the presence of starch or its hydrolytic product, maltose (Yabuki et al. 1977, Tonomura et al. 1961, Lachmund et al. 1993, Babu and Satyanarayana 1993a). Most reports available on the induction of α-amylase in different strains of *Aspergillus oryzae* suggest that the general inducer molecule is maltose. Apart from maltose, other carbon sources such as lactose, trehalose, α-methyl-D-glycoside also served as inducers of α-amylase in some strains (Yabuki et al. 1977). Amylase production by *Geobacillus thermoleovorans* is constitutive since the enzyme synthesis takes place not only in starch but also in other simple carbon sources (Narang & Satyanarayana 2001). Moreover, the amylase yield was similar in carbon sources such as starch, glucose, maltose and lactose, and therefore, this was not considered to reflect inducibility. α-Amylase production is also subjected to catabolite repression by glucose and other sugars as observed in *A. oryzae* (Morkeberg et al. 1995) and *B. coagulans* (Babu & Satyanarayana 1993a). Although carbon sources such as glucose and maltose have been utilized for the production of α-amylase, the use of starch remains prominent and ubiquitous. A number of other non-conventional substrates such as lactose (Kelly et al. 1997), casitone (Cheng et al. 1989b), fructose (Welker & Campbell 1963), oilseed cakes (Krishnan & Chandra 1982) and starch processing waste water (Jin et al. 1999) have also been used for the production of α-amylase. While the agro-residue, wheat bran has been extensively used for the economic production of α-amylase by SSF (Lonsane & Ramesh 1990, Babu & Satyanarayana 1994). Wheat bran was also used in liquid surface fermentation for the production of α-amylase by *A. fumigatus* (Kekos et al. 1987) and *Clavatia gigantea* (Domingues & Peralta 1993). High α-amylase production by *A. fumigatus* was also reported in α-methyl-D-glycoside (a synthetic analogue of glucose) as substrate (Goto et al. 1998).

α-Amylase production by *A. oryzae* DSM 63303 was not repressed by glucose; rather a minimal level of the enzyme was induced in its presence (Lachmund et al. 1993). Xylose and fructose have been classified as strongly repressive though they supported good growth (Arst & Bailey 1977); similar findings have also been recorded with the transformed *A. nidulans.*

A 2-fold increase in α-amylase secretion was reported in *G. thermoleovorans* in the presence of 0.03% cholic acid, probably due to its role in enhancing cell membrane permeability (Uma Maheswar Rao & Satyanarayana 2003a). In the thermophilic fungus *Thermomyces languinosus* (ATCC 200065), Triton X-100 had no observable effect, while Tween 80 effected nearly 2.7-fold increase in α-amylase production (Arnesen et al. 1998). α-Amylase secretion by *Aspergillus oryzae* and *A. nidulans,* at high biomass concentration, occurred due to slow mixing of the concentrated feed solution in the viscous fermentation medium (Agger et al. 2001).

3.2. Nitrogen sources

As regards to the use of nitrogen sources, organic nitrogen sources have been preferred for the production of α-amylase. Among nitrogen sources, tryptone (0.3%) supported a high amylase secretion in *G. thermoleovorans* (Malhotra et al. 2000). Tryptone and peptone have also been reported to support good α-amylase secretion in *B. sterothermophilus* (Chandra et al. 1980). The concentration of yeast extract was critical for obtaining maximum enzyme yields. Yeast extract was used in production of α-amylase by *Streptomyces* sp. (McMahon et al. 1999), *Bacillus* sp. IMD 435 (Hamilton et al. 1999a) and *Halomonas meridiana* (Coronado et al. 2000). Yeast extract was also used in conjunction with other nitrogen sources such as bactopeptone in *Bacillus* sp. IMD 434 (Hamilton et al. 1999a), with ammonium sulfate in *B. subtilis* (Dercova et al. 1992), with ammonium sulfate and casein for *Calvatia gigantea* (Kekos 1987) and with soybean flour and meat extract for *A. oryzae* (Imai et al. 1993). Organic nitrogen sources viz. beef extract, peptone and corn steep liquor supported high α-amylase titres in several bacterial strains (Emanuilova & Toda 1984; Krishnan & Chandra 1982, Hayashida et al. 1988, Cheng et al. 1989a). Soybean meal and casamino acids (Ueno et al. 1987) and corn steep liquor (Shah et al. 1990) have also been used for the economical and efficient production of α-amylase.

Amino acids in conjunction with vitamins been shown to affect α-amylase production in *G. thermoleovorans* (Narang & Satyanarayana 2001, Uma maheswar Rao & Satyanarayana 2003b). α-Amylase production by *B. amyloliquefaciens* ATCC 23350 increased tremendously by a factor of 300 in presence of glycine (Zhang et al. 1983). The effect of glycine was not exactly as a nitrogen source, rather it affected α-amylase production by controlling pH. β-Alanine, valine and D-methionine were found to be effective for the production of alkaline amylase by *Bacillus* sp. A-40-2 (Ikura & Horikoshi 1987). Asparagine also supported good enzyme yields (Kundu et al. 1973). Arginine influenced α-amylase production in *B. subtilis* (Lee & Parulekar 1993).

3.3. Role of phosphate

The presence of inorganic phosphate (0.1%) in the production medium caused a marked enhancement in α-amylase production in *G. thermoleovorans* and *Bacillus coagulans* (Malhotra et al. 2000; Babu & Satyanarayana 1994). In addition to its role as an important constituent of cellular biomolecules such as cAMP, nucleic acids, phospholipids and coenzymes, phosphate is known to play a regulatory role in the synthesis of primary and secondary metabolites in micro-organisms (Demain 1972, Weinberg 1974,

Mertz & Doolin 1973). A significant increase in the enzyme production and conidiation in *A. oryzae* above 0.2M phosphates was reported (Ueno et al. 1987). Similar findings were corroborated in *B. amyloliquefaciens,* where low levels of phosphate resulted in severely low cell density and no α-amylase production (Hillier et al. 1997). High phosphate concentrations were, however, inhibitory to the enzyme production in *B. amyloliquefactens* (Zhang 1983).

3.4. Role of other ions

K^+, Na^+, Fe^{2+}, Mn^{2+}, Mo^{2+}, Cl^-, SO_4^{2-} had no observable effect, while Ca^{2+} was inhibitory to amylase production by *Bacillus coagulans* (Babu & Satyanarayana 1994). Ca^{2+} was not required for α-amylase production in *G. thermoleovorans* (Narang & Satyanarayana 2001). The enzyme production was reduced to 50% when Mg^{2+} was omitted from the medium. Na^+ and Mg^{2+} showed coordinated stimulation in enzyme production by *Bacillus* sp. CRP strain (Wu et al. 1999). Addition of zeolites, to control ammonium ions in *B. amyloliquefaciens*, enhanced yield of α-amylase (Pazlarova & Votruba 1996). Inverse relationship between α-amylase production and growth rate was observed for *Streptomyces* sp. in the presence / absence of Co^{2+} (McMahon et al. 1999). The presence of Co^{2+} enhanced the final biomass levels by 13 fold with the concomitant reduction in the enzyme yield.

3.5. pH

Among physical parameters, pH of the growth medium plays an important role by causing morphological changes in the organism and in enzyme secretion. The pH changes observed during the growth of the organism also affect the product stability in the medium. Most of the *Bacillus* strains, used commercially for the production of α-amylases, optimally grow and secrete α-amylase in the pH ranging between 6.0 and 7.0. This is also true in case of strains used in the production of the enzyme by SSF. In most cases the pH used is not specified excepting pH 3.2-4.2 in the case of *A oryzae* DAE 1679 (Jin et al. 1999), 7.0-8.0 in *A. oryzae* EI 212 (Kundu et al. 1973) and pH 6.8 for *B. amyloliquefaciens* MIR-41 (McMahon et al. 1997). In fungal processes, the buffering capacity of some media constituents some times eliminates the need for pH control (Castro et al. 1992). The pH values also serve as valuable indicator of the initiation and end of enzyme synthesis (Chahal 1983). It is reported that *A. oryzae* 557 accumulated

α-amylase in the mycelia when grown in phosphate or sulfate deficient medium, and released when the mycelia were placed in a medium with alkaline pH above 7.2 [Yabuki et al. 1977].

3.6. Temperature

α-Amylase has been produced at a much wider range of temperatures among the bacteria. Continuous production of amylase from *B. amyloliquefaciens* at 36°C has been reported (McMahon et al. 1997). Elevated temperatures such as 105, 95 and 70°C have been used for amylase production by extreme/ hyper thermophiles *Desulfurococcus mucosus* (Canganella et al. 1994), *Thermococcus celer* (Canganella et al. 1994) and *Geobacillus thermoleovorans* (Malhotra et al. 2000), respectively. The influence of temperature on amylase production is related to the growth of the organisms (Table 1a & 1b). Optimum yields of α-amylase were achieved at 30°C-37°C for *A. oryzae* (Ueno et al. 1987; Kundu et al. 1973). α-Amylase production was also reported at 70°C by thermophilic bacterium *Thermomonospora fusca* (Friedrich et al. 1989) and at 50°C by a thermophilic mould *Thermomyces lanuginosus* (Mishra & Maheshwari 1996).

3.7. Agitation

Agitation intensity influences the mixing and oxygen transfer rate in many microbial fermentations, and thus, influences the morphology and product formation (Humphrey 1998). Agitation rates in the range of 100 to 250 rpm are generally reported for the production of α-amylases (Antranikian et al. 1987). The higher agitation might hinder the uptake of nutrients from the outer environment in *Bacillus thermoamyloliquefaciens* KP107I (Suzuki et al. 1987). In fungi, higher agitation is sometimes detrimental to mycelial growth leading to decline in enzyme production. The variations in mycelial morphology as a

Table 1a. Culture conditions for the production of microbial α-amylases

Organism	Growth Temp. (°C)	Temp. Optimum (°C)	pH-Optimum	Reference
Bacteria				
Acinetobacter sp.	30	50-55	7.0	Onishi & Hidaka 1978
Bacillus acidocaldarius A- 2	50	70	3.5	Kanno 1986
B. alcalophilus subsp.*halodurans*	37	-	10.5.	Yamamoto 1972
B. amyloliquefaciens	37	50-70	5.5	Granum 1979
B. cereus	30	55	6.0	Yoshigi et al 1985a & b
B. coagulans B 49	30	60	7.0	Babu & Satyanarayana 1993 a
B. coagulans	35	45-55	6.5-8.0	Campbell 1955
B. licheniformis	30	90	7.0-9.0	Morgan & Priest 1981
B. macerans	40	-	6.0	DePinto 1968
B. subtilis	37	55	6.5	Mitricia & Granum 1979,
Halobacterium halobium	37	55	6.5	Onishi 1972
Bacteroides amylophilus	37	43	6.3	Weber et al. 1990,
Micrococcus halobius	30	50-55	6.0-7.0	Onishi 1972
Streptococcus sp.	40	48	5.5-6.5	Hobson & Macpherson 1952
Fungi				
Aspergillus niger	30	50	5.0	Bhumibhamon 1983
A. oryzae	30	50-55	4.5-5.0	Kundu & Das 1970
Fusarium oxysporum	27	25	4.0	Chary & Reddy 1985
Humicola insolens	45	50	6.0	Ogundero 1979
H. lanuginosa	45	50	6.0-7.0	Mishra & Maheshwari 1996
Mucor pusillus	45	50	6.0	Ogundero 1979
Trichoderma viride	29	-	5.0-55	Ebertova 1963
Yeasts				
Candida Japonica	30	55	5.0-6.0	Ebertova 1963
Endomycopsis fibuligera	21	30-40	5.0-6.0	Sills et al. 1983
Lipomyces starkeyi	28	50	3.0-4.0	Kelly et al. 1985
S. castellii	28	60	6.0	Oteng-Gyang et al. 1981
Pichia polymorpha	28	40	4.0	Moulin et al. 1982
Sachwanniomyces alluvius	30	40	6.5	Lusena et al. 1985

Table 1b. Culture conditions for the production of α-amylases by thermophilic bacteria

Organism	Growth Temp. (°C)	Temp. Optimum (°C)	pH-Optimum	Reference
α-Amylases				
Geobacillus thermoleovorans	55	70	7	Malthora et al 2000
Thermococcus celer	80-100	95	6.0	Canganella et al 1994
Desulfurococcus mucosus	85-110	105	6.0	Canganella et al 1994
B. caldolyticus	72	70	5.5	Emanuilova & Toda 1984
B. stearothermophilus	55	70-80	5.0-6.0	Pfueller & Elliott 1969
Thermomonospora curvata	53	65	5.5-6.5	Glymph & Stutzenberger 1977
C. thermosaccharolyticum	60	70-75	5.5	Koch et al 1987
C. thermohydrosulfuricum	60	85-90	5.5	Plant et al. 1987
Strain TY	90-105	100	6.0	Canganella et al 1994
Dictyoglomus sp. Rt 46-B1	68	-	-	Plant et al 1987
Fervidobacterium sp.	70	90	5.5	Plant et al 1987
Pyrococcus woessi	100	100	5.5	Koch et al 1991, Brown et al 1990
P. furiosus	100	100	5.0	Ladermann et al 1993a, Koch et al 1990, Brown et al 1993
Thermoanaerobacter ethanolicus	65	90	5.5	Koch et al 1987
T. finnii	65	90	5.5	Koch et al 1987
Thermobacteriodes acetoethylicus	65	90	5.5	Koch et al 1987

consequence of changes in agitation did not affect enzyme production at a constant specific growth rate (Cui et al. 1997). Agitation intensities between 100-300 rpm have normally been employed for the production of amylase by various microorganisms.

3.8. α-Amylase productions in fermenters

The effect of environmental conditions on the regulation of extracellular enzymes in batch cultures is well documented (Amanullah et al. 1999). α–Amylase production and biomass of *B. flavothermus* peaked twice and highest production was attained after 24h in 20 L fermenter (Kelly et al. 1997). The enzyme synthesis was more of the growth-associated than nongrowth -associated type (Emanuilova & Toda 1984). Similar findings were cited in another investigation with *B. amyloliquefaciens* (Hillier et al. 1997). A 70% enhancement in the production of α-amylase was achieved when *G. thermoleovorans* was cultivated in a laboratory fermenter (Uma Maheswar Rao & Satyanarayana 2003b). The production of α-amylase by *B. subtilis* TN106 (pAT5) was enhanced substantially by extending the batch cultivation with fed-batch operation (Lee & Parulekar 1993, Baig et al. 1984). The bulk enzyme activity was nearly 54% greater in a two-stage fed-batch operation at a feed rate of 31.65 mL h^{-1} of medium than that attained in the single stage batch culture. At a feed rate of 1 g h^{-1}, the yields of α-amylase were twice that obtained in batch cultures. When the fed-batch cultivations were performed in a pilot scale rotary draft tube fermentor at a feed rate of 24 g h^{-1}, the biomass and α-amylase yields were higher than those obtained in the laboratory scale jar fermentor (Imai et al. 1993).

A model was proposed to simulate steady-state values for biomass yield, residual sugar concentration and specific rate of α-amylase production, which simulated the experimental data very well (Spohr et al. 1997). Furthermore, it was found in chemostat experiments that the specific rate of α-amylase production decreased by upto 70% with increasing biomass concentration at a given dilution rate. The shifts in the dilution rate in continuous culture could be used to obtain different proportions of the enzymes by the same strain (McMahon et al. 1997). A high production of α-amylase occurred in continuous culture at a dilution rate of 0.15 h^{-1}, and it was low at the dilution rates above1.2 h^{-1}. In contrast, the switching of growth from batch to continuous cultivation resulted in a change of α-amylase producing strain of *Bacillus* sp. to a nonamylase-producing variant (Hillier et al. 1997). A decline in enzyme production was accompanied by morphological and metabolic variations during continuous cultivation (Agger et al. 1998, Heineken & Conner 1972).

The industrial exploitation of SSF for enzyme production has been confined to process involving bacteria and fungi (Lonsane & Ramesh 1990). The use of SSF techniques in α-amylase production and its specific advantages over other methods have been discussed extensively (Babu & Satyanarayana 1994, Haq et al. 2003).

3.9. Aqueous two-phase system

A two-phase system can be obtained by simply mixing incompatible polymers in an aqueous solution. Specifically, a mixture of polyethylene glycol (PEG) and dextran becomes turbid above certain polymer concentrations and subsequently separates into two distinct phases when left undisturbed. While both phases have a high water content of typically over 90%, the top phase is rich in PEG and bottom phase is rich in dextran (Anderson et al. 1984, 1985, Brooks et al. 1985). As compared to conventional extractive systems based on organic solvents, an aqueous two-phase system has a low liquid-liquid interfacial tension and is highly biocompatible and nontoxic. For the last 30 years, there have been several developments in aqueous two-phase systems. Aqueous two-phase systems have been used to maximize α-amylase yields (Anderson et al. 1984,1985, Gupta et al. 1999). In single-phase batch fermentation, a lower maximum cell density, longer stationary phase, and generally higher α-amylase titres were attained in the presence of PEG or dextran (Park & Wang 1991). The rate of α-amylase synthesis decreased at high concentrations of dextran but not PEG in *B. amyloliquefaciens* (Park & Wang 1991).

3.10. Statistical optimization

Optimization through factorial design and response surface methodology (RSM) is a common practice in biotechnology for the optimization of media components and culture conditions (Rao et al. 1993, Chen 1996). In order to obtain optimum yield of an enzyme, development of a suitable medium and cultural conditions is obligatory (Dey et al. 2002). Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components (Uma Maheswar Rao & Satyanarayana 2003b). A 1.25 –2.0-fold increase in α-amylase production was reported in *Bacillus circulans* GRS 313 (Dey et al. 2001), *Aspergillus oryzae* (Gigras et al. 2002) and *Nocardiopsis* sp. (Stamford et al. 2001). A 70 % enhancement in enzyme production was reported in *Geobacillus thermoleovorans* due to optimization using statistical methods (Uma Maheswar Rao & Satyanarayana 2003b).

4. ASSAY OF α-AMYLASES

4.1. Determination of α-amylase activity

Microbial α-amylases are generally assayed using soluble starch or modified starch as the substrate. α-Amylase catalyses the hydrolysis of α-1, 4 glycosidic linkages in starch to produce glucose, dextrins and limit dextrins. The reaction is monitored by an increase in the reducing sugar levels or decrease in the iodine color of the treated substrate. Various methods are available for the determination of α-amylase activity (Priest 1977), which are based on decrease in starch-iodine color intensity, increase in reducing sugars, degradation of color-complexed substrate and decrease in viscosity of the starch suspension.

4. 2. Dinitrosalicylic acid (DNSA) method

This method determines the increase in the reducing sugars as a result of amylase action on starch and was originally described by Bernfeld (1955). The major defect in this assay is slow loss in the amount of color produced and destruction of glucose by various constituents of the DNSA reagent.

To overcome these limitations in the DNSA reagent, a modified method for the estimation of reducing sugars was developed (Miller 1959). In the modified reagent, the Rochelle salts were excluded and 0.05% sodium sulfite was added to prevent the oxidation of the reagent. Since then the modified method has been used extensively to measure reducing sugars without any further modifications in the procedure.

4.3. Determination of dextrinizing activity

The dextrinizing activity of α-amylases employs soluble starch as substrate and after terminating the reaction with dilute HC1, and adding 0.1mL of iodine solution. The decrease in optional density at 620 nm is then measured against a substrate control. Ten percent decline in OD is considered as one unit of enzyme (Fuwa 1954, Babu & Satyanarayana 1994). Recently, a modified dextrinizing method was suggested for determining the dextrinzing activity (Nguyen et al. 2002).

The major limitation of this assay is interference of various media components such as Luria broth, tryptone, peptone, corn steep liquor, etc. and thiol compounds with starch iodine complex. Copper sulfate and hydrogen peroxide protect the starch-iodine color in case of interference by these media components (Manonmani & Kunhi 1999a). Further, zinc sulfate was found to be the best for counteracting the interference of various metal ions also. Various workers (Hansen 1984, Carlsen et al. 1994) have successfully used the original assay procedure in combination with flow injection analysis (FIA). The flow system comprised an injection valve, a peristaltic pump, a photometer with a flow cell and 570 nm filter and a pen recorder. The samples were allowed to react with starch in a coil before iodine is added. The absorbance is then read at 570 nm. This method has many advantages such as high sampling rates, fast response, flexibility and simple apparatus.

4.4. Decrease in starch-iodine color intensity

Starch forms a deep blue complex with iodine (Hollo & Szeitli 1968) and with progressive hydrolysis of the starch, it changes to red dish brown. Several procedures have been described by various groups for the quantitative determination of amylase based on this property. This method determines the dextrinizing activity of α-amylase in terms of decrease in the iodine color reaction.

4.5. SKB (Sandstedt Kneen and Blish) method

SKB method (Sandstedt et al. 1939) is one of the most widely adopted methods for determination of amylases used in the baking industry. The potency of most of the commercial amylases is described in terms of SKB units. This method is used generally to express the diastatic strength of the malt and not for expressing α-amylase activity alone (Kulp 1993).

4.6. Indian pharmacopoeia method

As described in the Indian Pharmacopoeia, this method is used to calculate α-amylase activity in terms of grams of starch digested by a given volume of enzyme (Indian Strandard). This procedure involves incubation of the enzyme preparation in a range of dilution in buffered starch substrate at 40° C for one hour. The solutions are then treated with iodine solution. The tube, which does not show any blue color, is then used to calculate activity in terms of grams of starch digested. This method is usually employed for estimating the α-amylase activity in cereals. Other assay methods for α-amylase have been described recently (Gupta et al. 2003).

5. PURIFICATION AND CHARACTERIZATION OF MICROBIAL α-AMYLASES

Isolation of a protein from the biological environment requires a series of purification steps, each step removing some of the impurities and bringing the product closer to the final specification. The choice of procedure for enzyme purification depends on their location. The strategies used for purification of α-amylases are enormous. Initial processes include crude fractionation, clarification, concentration of crude enzyme using processes such as centrifugation, ultrafiltration (Bohdziewicz 1996) and salt/solvent precipitation (Babu & Satyanarayana 1993b). The concentrate is then further purified using high-resolution techniques based on chromatographic and electrophoretic separations. It is designed to remove aggregates, degradation products and to prepare a solution suitable for the final formulation of the purified enzyme. The commercial use of α-amylases generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. Some purified microbial α-amylases and their characteristics are listed in Tables 2a and 2b.

5.1. Substrate specificity

As it holds true for other enzymes, the substrate specificity of α-amylase also varies from microorganism to microorganism. In general, α-amylases display highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen and maltotriose (Antranikian 1992, Nirmala & Muralikrishna 2003).

5.2. pH optima and stability

The pH optima of α-amylases varied from 2.0 to 12.0 (Vihinen & Mantsala 1989). α-Amylases from most bacteria and fungi have their pH optima in the acidic to neutral range (Pandey et al. 2000). α-Amylase from *Alicyclobacillus acidocaldarius* showed an acidic pH optima of 3.0 (Bohdziewicz 1996), while alkaline amylase of *Bacillus* sp. exhibited optima at pH 9-10.5. (Schwermann et al. 1994). Extremely alkalophilic α-amylase with pH optima of 11.0-12.0 has been reported from *Bacillus sp.* GM8901 (Shinke et al. 1996). In *B. stearothermophilus* DONK BS-1, pH optimum was dependent on temperature (Kim et al. 1995), and on Ca^{2+} in *B. stearothermophilus* (Ogasahara et al. 1970).

Table 2a. Characteristics of bacterial α-amylases

Strain	Tem. (Opt)	pH (Opt)	K_m (mg/ml)	Protein (Da)	Inhibitors	Reference
B. coagulans	70	6-7	1.53	66,000	Mg^{++}, Fe^{++},Hg^{++}	Babu & Satyanarayana 1993
B. stearothermophilus	40-45	5.4-6.1	-	51,600	-	Pfueller & Elliot
-do-	55-70	4.6-5.1	1.0	53,000	EDTA	Manning & Campbell 1961
-do-	70	7.4	0.0485	-	-	Isono 1970
B. subtilis	60-65	6.8	-	55,000	-	Yamane et al. 1973
B. subtilis	60-65	6.0	-	68,000	-	Hayashida et al 1988
B. amyloliquefaciens	65	5-9	2.63	-	-	Welker & Campbell 1963
B. amyloliquefaciens	65	5-9	2.63	-	-	Welker &Campbell 1967
B. licheniformis	90	7-9	1.27	28,000	Hg^{++},Ag^{++}, Ni^{++}, Co^{++}, Cu^{++}, Fe^{++}	Krishnan & Chandra 1982, 1983
B. circulans	60	8.0	0.65	76,000	Hg^{++}, Zn^{++}, Cu^{++}, Fe^{++}	Takasaki et al 1982
B. coagulans	-	6.5-8.0	-	-	-	Campbell 1955
B. cereus	55	6.0	-	55,000	Hg^{++}, Ag^{++}, Cu^{++}, Fe^{++}	Yoshigi et al. 1985a
B. thermoamyloliquefaciens	(i) 70	5.6	0.21	78,000	Hg^{++}	Suzuki et al. 1987
	(ii) 63	6.2	0.25	67,000	Hg^{++}, Cu^{++}, Zn^{++}·Pb^{++}	
B. brevis	80	5-9	0.8	58,000	Fe^{++}	Tsvetkov & Emanuilova 1989,
B. megaterium	70	-	-	59,000	-	Brumm et al 1991
B. acidocaldarius	70	3.5	0.16	66,000	Cu^{++},Fe^{++}, Zn^{++}	Kanno 1986
Streptococcus bovis JBI	50	5.0-6.0/5.5-8.5	0.88	77,000	Hg^{++}, p-chloromercuribenzoic acid	Freer 1993
Micromonospora melanosporea	45-50	7.0	-	12,500	-	Robyt & Ackerman 1971
Thermomonospora curvata	65	6.0	-	60,900	-	Collins et al 1993
Escherichia coli	50-70	6.5-7.0	-	48,000	Hg^{++},Fe^{+++}, Al^{+++}	Marco et al 1996
Lactobacillus plantarum	65	3.0-8.0	2.38 g/ml	50,000	N- bromosuccinmide, iodine, acetic acid, Hg^{++}, dimethyl aminobenzaldehyde	Girand et al 1993
Lactobacillus kononenkoae CBS	70	5.0	0.8g/ml	76,000	DTT,Cu^{++}, Ag^{++}	Prieto et al 1995

Contd...

Strain	Tem. (Opt)	pH (Opt)	K_m (mg/ml)	Protein (Da)	Inhibitors	Reference
B. stearothermophilus	55-70	4.6-5.1	-	-	EDTA	Manning & Campbell 1961
B. licheniformis	85-90	6.5-8.5	-	56,000	-	Ramesh & Lonase 1990b
T. curvata	65	5.5-6.0	0.3	62,000	Bovine serium albumin	Glymph & Stutzenberger 1977
Bacillus sp. IMD 434	55	4.0-9.0	1.9 m.m	69,200	N-bromo-succinimide, p-hydroxymer-curibenzoic acid	Hamilton et al 1999
B. subtilis 65	60-65	6.0-9.0	3.8	68,000	Cu^{++},Fe^{++}, Mn^{++},Hg^{++}, Zn^{++},Ca^{++}, Pb^{++},Al^{+++}, Cd^{++},Ag^{++}, EDTA	Hayashida et al 1988
Pyrococcus furiosus	115	5.6		48,000	DTT	Savchenko et al 2002

Table 2b. Characteristics of fungal α-amylases

Strain	Tem. (Opt)	pH (Opt)	K_m (mg/ml)	Protein (Da)	Inhibitors	Reference
Thermomyces lanuginosus	80	4.0	0.68	61,000	Zn^{++}	Nguyen et al 2002
Paecilomyces sp.	45	4.0	-	69,000	-	Zenin & Park 1983
Cryptococcus S-2	60	6.0	-	66,000	-	Iefuji et al 1996
Aspergillus usamii	65	4.0	-	54,000	-	Suganuma et al. 1996
A. oryzae M13	50	4.0	0.13%	52,000	-	Yabuki et al 1977
A. flavus LINK	60	6.0	0.5g/ml	52,500	Ag^{++},Hg^{++}	Khoo et al 1994
A. awamori ATCC 22342	55	5	1.0	54,000	Ag^{++},Cu^{++}, $Fe^{+++}Hg^{++}$	Bhella & Altosaar 1985
A. chevalieri NSPRI	60	5.5	0.19	68,000	EDTA, DNP	Olutiola 1982
A. hennebergi	50	5.5	-	50,000	-	Alazard & Baldensperger 1982
A. niger ATCC 13469	50	5.0	-	-	-	Bhumibhamon 1983
A. fumigatus	55	6.0	-	-	-	Domingues & Peralta 1993
A. oryzae	50	5.0	0.13%	-		Yabuki et al 1977
A. foetidus ATCC 10254	45	5.0	2.19	41,500	-	Michelena & Castillo 1984
Trichoderma viride	60	5.5	-	-	-	Schellart et al. 1976
Thermomyces lanuginosus IISc91	55	5.6	2.5	42,000	-	Mishra & Maheswari 1996

5.3. Temperature optima and stability

The temperature optimum for the activity of α-amylase is related to temperature optima for the growth of the microorganism (Vihinen & Mantsala 1989). The lowest temperature optimum 20-30 °C has been reported in *F. oxysporum* amylase (Robyt & Ackerman 1971) and the highest of 100°C and 130°C for amylases of archaebacteria, *Pyrococcus furiosus* and *P. woesei* (Chary & Reddy 1985, Fogarty & Kelly 1980, Giri et al. 1990). Temperature optima of enzymes from *Micrococcus varians* were Ca^{2+} dependent (Ray et al. 1995) and the one from *Halomonas meridiana* was sodium chloride dependent (Coronado et al. 2000).

Thermostabilities have not been estimated in many studies. Thermostability as high as 3 hours at 100°C has been reported for *Geobacillus thermoleovorans* (Malhotra et al. 2000). Many factors that affect thermostability include-presence of calcium, substrate and other stabilizers (Vihinen & Mantsala 1989). The stabilizing effect of starch was observed in α-amylases from *B. licheniformis* CUMC 305 (Saito 1973), *Lipomyces kononenkoae*, (Kobayashi et al. 1986) and *Bacillus sp.* WN 11 (Prieto et al. 1995). Thermal stabilization of the enzyme in the presence of Ca^{2+} has also been reported (Prieto 1995, McKelvy 1969).

5.4. Molecular weight

Molecular weights of α-amylases vary widely. The lowest value, 10 KDa was reported for α-amylase of *B. caldolyticus* (Grootegoed et al. 1973) and the highest of 78 KDa for that of *B. thermoamyloliquefaciens* (Suzuki et al. 1987). Molecular weights of microbial α-amylases are usually 50 to 60 KDa as shown directly by analysis of cloned α-amylase genes and deduced amino acid sequences (Vihinen & Mantsala 1989). Carbohydrate moieties raise the molecular weight of some α-amylases. Glycoprotein α-amylases have been detected in *A. oryzae* (McKelvy & Lee 1969, Eriksen et al. 1998), *Lipomyces kononenkoae* (Kobayashi et al. 1986), *B. stearothermophilus* (Srivastava 1984) and *B. subtilis* strains (Yamane et al. 1973; Matsuzaki et al. 1974b). Glycosylation of bacterial proteins is rare. Carbohydrate content as high as 56% was recorded in *Saccharomyces castellii* (Sills et al. 1984), and it was about 10% for other α-amylases (Vihinen & Mantsala 1989).

5.5. Inhibitors

Many metal cations, especially heavy metal ions, sulfhydryl group reagents, N-bromosuccinimide, *p*-hydroxy mercuribenzoic acid, iodocetate, BSA, EDTA and EGTA are known to inhibit α-amylases (Hamilton et al. 1999b)

5.6. Calcium and stability of α-amylase

The α-amylase is a metalloenzyme, which contains at least one Ca^{2+} ion (Vallee et al. 1959). The affinity of Ca^{2+} to α-amylase is much stronger than that of the other ions. The amount of bound calcium varies from one to ten. Crystalline Taka-amylase A (TAA) contains ten Ca^{2+} ions but only one is tightly bound (Oikawa & Maeda 1957). In other systems usually one Ca^{2+} ion is enough to stabilize the enzyme. Ca^{2+} can be removed from amylases by dialysis against EDTA or by electrodialysis. Calcium-free enzymes can be reactivated by adding Ca^{2+}. Some studies have been carried out on the ability of other ions such as strontium to replace Ca^{2+} in *B. caldolyticus* amylase (Heinen & Lauwers 1975). Calcium in Taka-amylase A has been substituted by strontium and magnesium in successive crystallizations in the absence of Ca^{2+} and in excess of Sr^{2+} and Mg^{2+} (Oikawa 1959). EDTA inactivated TAA can be reactivated by Sr^{2+}, Mg^{2+} and Ba^{2+} (Oikawa 1959). Some *Bacillus* spp. produces chelator resistant amylases (Burhan et al. 2003). In the presence of Ca^{2+}, α-amylases are much more thermostable than without it (Vihinen

& Mantsala 1989, Robyt & French 1963, Savchenko et al. 2002). α-Amylase from *A oryzae* EI 212 was inactivated in the presence of Ca^{2+}, but retained activity after EDTA treatment (Kundu & Das 1970). There are also reports where Ca^{2+} did not have any effect on the enzyme activity (Laderman et al. 1993, Malhtora et al. 2000), and inhibition by Ca^{2+} in *B. coagulans* (Babu & Satyanarayana 1993).

5.7. Catalytic mechanism of α-amylase

The α-glycosidic bond is very stable having a spontaneous rate of hydrolysis of approximately $2 \times 10^{-15} \times s^{-1}$ at room temperature (Wolfenden et al. 1998). Members of the α-amylase family enhance this rate of hydrolysis to $3s^{-1}$ (Van der Veen et al. 2000b), and thereby increasing the rate by 10^{15} fold. The generally accepted catalytic mechanism of the α-amylase family is that of the α-retaining double displacement. The mechanism involves two catalytic residues in the active site, a glutamic acid as acid/base catalyst and an aspartate as the nucleophile, and it involves five steps (Koshland 1953):

(i) After the substrate is bound in the active site, the glutamic acid in the acid form donates a proton to the glycosidic bond oxygen, i.e. the oxygen between two glucose molecules at the subsite-1 and +1 and the nucleophilic aspartate attacks the C1 of glucose at subsite-1

(ii) An oxocarbonium ion-like transition state is formed followed by the formation of a covalent intermediate

(iii) The protonated glucose molelcule at subsite +1 leaves the active site while a water molecule or a new glucose molecule moves into the active site and attacks the covalent bond between the glucose molecule at subsite −1 and the aspartate.

(iv) An oxocarbonium ion-like transition state is formed again

(v) The base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1, the oxygen of the incoming water or the newly entered glucose molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subiste-1 and the aspartate forming a new hydroxyl group at the C1 position of the glucose at subsite −1 (hydrolysis) or a new glycosidic bond between the glucose at subsite −1 and +1 (transglycosylation).

Double displacement mechanism proposed by (Koshland 1953) is given in Fig. 2. Neutral α-amylase from *A. oryzae* consisted of single polypeptide chain of 478 amino-acid residues and its aminoacid

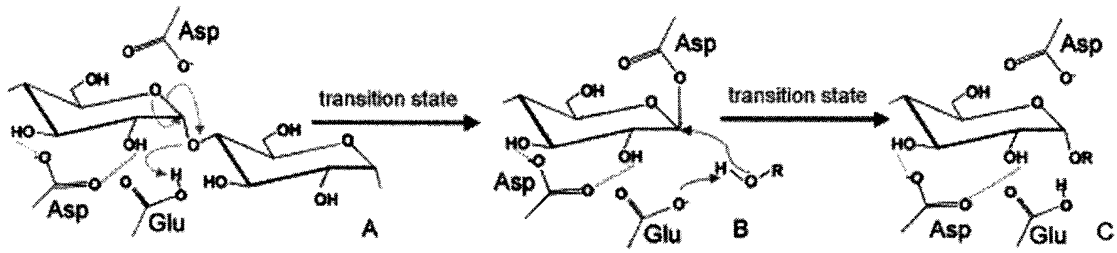

Fig. 2. Double displacement and the formation of a covalent intermediate by which retaining glycosylhydrolases act (Van der Maarel et al 2002)

sequence was determined (Toda et al. 1989). The enzyme of *A. oryzae* contains three main domains, and the tertiary structure is stabilized by four disulfide bonds (Carlsen et al. 1996).

5.8. Calcium and sodium ions

All known α-amylases contain a conserved calcium ion, which is located at the interface between domains A and B (Boel et al. 1990, Machius et al. 1995, Machius et al. 1998), and which is known to be essential for having a stable, enzyme activity (Vallee et al. 1959). The calcium ion is bound very tightly, as is shown by the dissociation constant, in pig pancreatic α-amylase (PPA) and *Alteromonas haloplanktis* α-amylase (AHA), which have been shown to be 44 nM (Feller et al. 1994) and 0.005 nM (Levitsky & Steer 1974), respectively. It has been suggested that the role of the conserved calcium ion is mainly structural (Buisson et al. 1987, Machius et al. 1998, Larson et al. 1994), since it is too far away from the active site to participate directly in catalysis. One or more additional calcium ions have been found in several structures (Boel et al. 1990, Machius 1998, Kadziola et al. 1998), and a particularly interesting case is the linear Ca-Na-Ca arrangement found in *Bacillus licheniformis* α-amylase (BLA) (Machius et al. 1998, Brozozowski et al. 2000). X-ray structures are available for both the calcium-depleted wild type and for a three-fold mutant of this enzyme (Machius et al. 1995, 1998). This has allowed Machius et al. (1998) to propose a disorder → order transition that should supposedly occur when Ca^{2+} is bound to the calcium-depleted form of the enzyme (Machius et al. 1995). It is unknown, whether the calcium-depleted α-amylase can be reactivated by the addition of calcium, thus making the disorder → order transition theory hypothetical.

5.9. Chloride ions

Several α-amylases contain a chloride ion in the active site, which has been shown to enhance the catalytic efficiency of the enzyme. Presumably by elevating the pKa of the hydrogen-donating residue in the active site (Levitsky & Steer 1974, Feller et al. 1996). Chloride ions have been found mainly in mammalian α-amylases (Ramasubbu et al. 1996, Brayer et al. 1995, Larson et al. 1994), although a chloride ion has also been reported in a psychrophilic α-amylase (AHA) from the *Alteromonas haloplanktis* bacterium (Aghajari et al. 1998). It has been observed that the affinity for the conserved calcium ion increases dramatically upon chloride binding (Levitsky & Steer 1974), and it is therefore, conceivable that chloride ion binding also induces conformational changes around the active site. A puzzling feature of chloride containing α-amylase is a serine protease like Glu-His-Ser triad in the interface between domains A and C. Aghajari et al. (1998) proposed that this triad is capable of performing an autoproteolytic cleavage. The experimental evidence for an autoproteolytic event is mostly indirect (Aghajari et al. 1998), and the hypothesis still awaits experimental confirmation.

5.10. Active site cleft

The active site cleft is located in the interface between domain A and domain B, and is found at the C-terminal end of the β-strands in the TIM barrel. X-ray structures of α-amylases complexes with the inhibitory pseudo tetra-saccharide, acarbose have shown that the substrate binding cleft can accommodate four to ten glucose units (depending on species). Each glucose unit is bound by certain of the amino acid residues to constitute the binding subsite for that glucose unit. Subsite nomenclature has been defined by Davies et al. (1997), i.e., subsites are numbered according to location of the scissile bond, with negative subsite numbers on the non-reducing side of the scissile bond. In α-amylases, there are two or three subsites present on the reducing end of the scissile bond (subsites +1, +2 and +3), whereas the

number of subsites on the non-reducing side of scissile bond varies between two and seven (MacGregor 1988, Brzozowski et al. 2000).

5.11. Sequence

In the CAZY database, the α-amylases are grouped with different kinds of glyucosylhydrolases in Family 13. In order to identify a sequence in this very large group as an α-amylase sequence, one ultimately needs experimental results. A more convenient approach, however, is to align the protein sequence in question to a known α-amylase sequence and look for conserved sequence patterns. α-Amylase sequences contain at least four such conserved patterns (numbered I-IV), which are found in the TIM-barrel on β-strands 3,4 and 5 and in the loop connecting β-strand 7 to a-helix 7. The residues that form region I are found in the C-terminal end of the third β-strand of the TIM barrel (b3). This region contains the three fairly conserved amino acids Asp 100, Asn104 and His105 (BLA numbering). Furthermore, there is a preference for Val at position 102, which is not readily explainable. Asp100 is important for the active site integrity as it hydrogen bonds to Arg229, which is a fully conserved residue within hydrogen bond distance of both the catalytic nucelophile Asp231 and the proton donor Glu26. Asn104, on the other hand, does not participate directly in the stabilization of the active site structure, but coordinates the conserved calcium ion between the A and B domains (Boel et al. 1990, Machius et al. 1995, Machius et al. 1998). His105 stabilizes the interaction between the C-terminal of b3 and the rest of TIM barrel by hydrogen bonding to Asn104 and to the backbone oxygen of Tyr56 (BLA numbering), which is situated in the loop connecting β2 to α2. Region II is located in b4 and contains the catalytic nucleophile Asp231 and the invariant residue Arg229. These two residues are found in all α-amylases and are believed to be indispensable for catalytic activity (Svensson 1994). Lya234 and His235 are also found in this region, which form part of subsite +2 and are thus supposed to bind the reducing end of the glucose chain in the substrate-binding cleft (Svensson & Sogaard 1994, Svensson 1994).

5.12. Gene structure, cloning and sequencing

Genetic engineering has been used extensively for cloning α-amylase genes from amylase-producing strains. A great deal of work has been done on the cloning of α-amylase genes in different microbes, mostly in *E. coli* or *Saccharomyces cerevisiae* (Table 3). α-Amylase was one of the first proteins adopted for molecular biological studies for several reasons: (1) easy screening assays exist (2) amylase negative strains are available and (3) the genetics, protein production, and fermentation technology of α-amylase in *B. subtilis* is well known. The *Bacillus* strucutural gene *amyE*, its promoter, and a regulatory element, *amyR*, have been cloned from several strains. Lieble et al. (1987) described the gene structure of the α-amylase from *Thermotoga maritima* MSB8; it is a chromosomal α-amylase gene, designated *amy*A, and was predicted to code for a 553 amino acid preprotien with significant amino acid sequence. The *T. maritima* α-amylase appeared to be the first known example of lipoprotein α-amylase that appeared to be the first known example of a lipoprotein α-amylase. Following the signal peptide, 25-residue putative linker sequence rich in serine and threonine residues, putative linker sequence rich in serine and threonine residues were found. Suganuma et al. (1996) studied the N-terminal sequence of the amino acids of the α-amylase of *Aspergillus usanii* and the amylase gene was expressed in *E. coli*. The sequence of the first 20 amino acids was identical to the α-amylase from *A. niger*. Kim et al. (1992) described a gene encoding a new α-amylase of *Bacillus licheniformis*, which was cloned and expressed in *E. coli*. The genomic DNA of *B. licheniformis* was double-digested with EcoR1 and BamH1 and ligated in the pBR322. The transformed *E. coli* carried the recombinant plamid pIJ322 containing a 3.5

Table 3. Cloning of α-amylase genes

Donor	Plasmid	Vector	Host	Reference
Aeromonas hydrophila MP636	pJP3101 pUC12J		*Escherichia coli*	Gobius & Pemberton 1988
Bacillus sp. 707	pTUE306 pTUB812	pBR322 pUB110	*E. coli B. subtilis*	Tsukamoto et al 1988
B. amyloliquefaciens E 18	pKTH10	pUB110	*B. subtilis*	Takkinen et al 1983
B. stearothermophilus	pUC13BS pBS42BS	pUC13 pBS43	*E. coli* *B. subtilis*	Gray et al 1986
B. stearothermophilus CU21	pAT5	pTB53	*B. subtilis*	Aiba et al 1983
B. subtilis 168	pBR322		*E. coli*	Yang et al 1983
Saccharomycopsis fibuligera HUT 7212	pSf2	pY11	*S. cerevisiae*	Yamashita et al 1985
Streptomyces hygroscopicus	pPOD1039	pIJ702	*S. lividans*	McKillop et al 1986
S. limosus ATCC 19778	pSYC1318	pIJ941	*S. lividans*	Long et al 1987

kb fragment of *B. licheniformis* DNA. The purified enzyme encoded by pIJ322 was capable of hydrolyzing pullulan and cyclodextrin as well as starch. Iefuji et al. (1996) described the cloning and sequencing of a raw starch -digesting and thermostable α-amylase from *Cryptococcus* sp. S2. An open reading frame of the cDNA specified 611 amino acids. Including a putative signal peptide of 20 amino acids. The N-terminal region of the enzyme (from the N-terminus to position 496) shared 49.7% similarity with that of *A. oryzae* (Marco et al. 1996).

5.13. Enzyme engineering for improved thermostability

The interesting so-called proline rule for thermo-stabilization of proteins proposed by Suzuki and co-workers (Suzuki et al. 1987, Suzuki 1989) has been demonstrated on the enzyme from the α-amylase family, oligo-1,6-glucosidase (Watanabe et al. 1994). An increasing number of prolines (19 in *Bacillus cereus*, 24 in *Bacillus coagulans* and 32 in *Bacillus thermoglucosidasius* enzymes) was observed with the rise in thermostabilities of the oligo-1,6-glucosidase. The prolines at the second sites of β-turns and in the first turn of α-helices have been found to contribute to the thermostability (Watanabe et al. 1994). This appears to have a clear evolutionary implication for how the proteins have achieved their naturally enhanced thermostability (Janecek 1997, Turner 2003).

The conditions prevailing in the industrial applications in which enzymes are used are rather extreme, especially with respect to temperature and pH. Therefore, there is a continuing demand to improve the stability of the enzymes and thus meet the requirements set by specific applications. An α-amylase from *Pyrococcus woesei* (Antranikian et al. 1990) had better thermostability than the currently available commercial enzymes; however, none has been introduced into the market yet. One of the reasons being that besides thermostability and activity, other factors such as activity at high concentrations of starch (more than 30% dry solids) and the protein yields of the industrial fermentation are important criteria for commercialization (Schafer et al. 2000, Nielsen & Borchert 2000). Most, if not all, α-amylase family

enzymes found by screening new and exotic strains do not meet these criteria. The other well-known approach to find new and potentially interesting enzymes is to use the nucleotide or amino acid sequence of the conserved domains in designing degenerated PCR primers. These primers can then be used to screen microbial genomes for the presence of genes putatively encoding the enzyme of interest. This approach has been used successfully by Tsutsumi et al. (1999) to find and express a novel thermostable isoamylase enzyme from two *Sulfolobus* spp. and *Rhodothermus marinus*.

6. INDUSTRIAL APPLICATIONS OF α-AMYLASES

Today, amylases have the major world market share of enzymes (Aehle & Misset 1999). Several different amylase preparations are available with various enzyme manufacturers for specific use in varied industries. A comprehensive account on commercial applications of α-amylases has been provided by Godfrey and West (1996). Bacterial amylase, however, is generally preferred over fungal amylase due to several characteristic advantages that it offers (Hyun et al. 1985, Babu & Satyanarayana 1994, Malhotra et al. 2000). Mostly thermostable α-amylases are generally preferred as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving (Sonnleitner & Fiechter 1983, Pandey et al. 2000). Thermophilic α-amylases are used in starch, detergent, paper, textile, baking and other industries.

7. CONCLUSIONS

During the last three decades, α-amylases have been exploited by the starch processing industry as a replacement for acid hydrolysis in the production of starch hydrolysates. This enzyme is also used for removal of starch in beer, fruit juices, or from clothes and porcelain. A new and recent application of maltogenic amylase is as an anti-staling agent to prevent the retrogradation of starch in bakery products. In the light of modern biotechnology, α-amylases are now gaining importance in biopharmaceutical applications. Their application in food and starch based industries is the major market, and further the demand for α-amylases would always be evergreen in these sectors.

8. PERSPECTIVES

Despite the fact that α-amylases have been used in a wide variety of technical applications for several years, there have not been many new developments. The available enzymes are good and have fulfilled until recently the needs of the customers. The interest in new and improved α-amylase is growing, and consequently the research is intensified as well. Genetic engineering approaches are required to develop the enzyme with desired characteristics for specific purpose. Further, there is a need for Ca^{2+}-independent and hyper-thermostable α-amylases, which are functional above 100° C at acidic pH for the improved starch saccharification.

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Glucoamylase

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1. INTRODUCTION

Starch from cultivated plants is one of the most important storage forms of polysaccharides in nature and is the most ubiquitous and accessible energy source on the planet. It is found in crops such as corn, wheat, rye, potato, cassava, rice, barley, etc. Natural starch is insoluble in water and is present in plants in the form of microscopic granules. It is a mixture of 15-20% amylose (α-1,4- linked glucose polymer) and 75-80% amylopectin (α-1,4- linked glucose polymer branched by α-1,6- linkages). If starch is heated in water, the hydrogen bond, which binds the granules weakens and this results in swelling and gelatinisation. Generally, amylose consists of about 1000 glucose units and amylopectin consists of shorter α-1, 4– linked linear chains of 10-60 glucose units, and α- 1,6- linked side chains with 15-45 glucose units.

1.1. Amylases

The amylase family of enzymes plays an important role in the enzyme industry due to its wide range of application. Amylases are used in a number of industrial processes such as food fermentation, textiles, paper industries, etc. In the history of enzyme technology, the first enzyme to be produced industrially was a fungal amylase, *viz.* takadiastase, in 1894, which has been used for the treatment of digestive disorders.

Three main classes of amylases act on starch, which include α–amylase (EC 3.2.1.1), β- amylase (EC 3.2.1.2, exo-amylase) and glucoamylase (GA, EC 3.2.1.3). α–amylase splits randomly α-1, 4- glucosidic bonds between adjacent glucose units in linear amylose chains, while glucoamylase hydrolyses single glucosidic residues from the non-reducing ends of amylose and amylopectin in a stepwise manner. GA also hydrolyses the 1, 6 α-linkages in the branching points of amylopectins, although at a slower rate than the α-linkages (Najafpour & Shan 2003, Gupta et al. 2003, Sauer et al. 2000, Pandey et al. 2000a, b, Ellaiah et al. 2002, Pandey 1995, Crabb & Mitchinson 1977, Goto et al. 1998). Starch saccharification is a multi-step process involving several enzymes such as α-amylase, β-amylase, GA, pullulanase and isoamylase. Liquefaction is the first step, where α-amylase disrupts the granular structure of starch and also causes thinning by breaking down amylose and amylopectin. This usually occurs at pH 6.0-6.5 at 105°C for 5-10 min, and then at 95°C for 2h. This is followed by saccharification of starch by GA at pH 6.0 and 60°C. β-amylase is used in the saccharification step only if maltose is needed as a product. Pullulanase is required to obtain a higher dextrose/maltose concentration. Amylases have replaced the use of acid hydrolysis of starch as they offer potential benefits.

2. GLUCOAMYLASE

GA degrades starch to glucose in theoretically 100% yield. The reaction rate decreases with the decreasing chain length of the dextrin substrate. GA is capable of catalysing a reverse of the normal hydrolysis reaction to produce mainly maltose and isomaltose. This reaction is important in industrial processes in which high concentrations of up to 40% sugars can occur, which favour some maltose formation.

GA finds application in the food and fermentation industries for the saccharification of starch, brewing, distilling, etc (Ellaiah et al. 2002, Myoung-Hee et al. 2002, Najafpour & Shan 2003, Pandey 1995). Fungal GA is widely used in the manufacture of glucose and fructose syrups, and in the production of sweeteners. Enzymatic process to produce high-glucose syrups is widely used in food industry, and alternatively, glucose may be an important substrate for fermentative processes to produce other products such as ethanol, amino acids or organic acids (Polakovic & Bryjak 2004). GA also helps in reducing the dough viscosity to improve the texture and appearance of bread. Further, they are used in the manufacture of pharmacologically active digestive aids.

2.1. Classification of GA

GA has been classified by Fleming (1968) into two groups, one which converts starch and β-limit dextrins completely into glucose, and the other which converts 80% starch and 40% β-limit dextrins into glucose. However, both the groups completely hydrolyse panose and α-limit dextrins to glucose. Hydrolysis rate by the GA depends on the molecular size, structure, position and nature of bonds present in the sequence of substrate (Kulp 1975). Pretreatment of substrate also helps in better hydrolysis rate and improved product recovery. The enzyme acts on substrates such as starch, amylose, amylopectin, dextrins, maltodextrins, maltose, isomaltose, dextran, panosan and oligo-, di- and polysaccharides, etc.

3. SOURCES

GA occurs in plants (Takahasi et al. 1971), animals (Azad & Lebenthal 1990) and microorganisms, including bacteria, fungi and yeasts. (Aleshin et al. 2003, Najafpour & Shan 2003, Ellaiah et al. 2002, Pandey et al. 2000a, Chiarello et al. 1997, Pandey 1995). Table 1 shows some microorganisms reported as the sources of GA.

3.1. Fungal source

GA is mostly produced by molds, mainly *Aspergillus* sp, *Rhizopus* sp and *Endomyces* sp; production is generally extracellular and enzyme can be recovered from culture filtrates (Saha & Zeikus 1989, Pandey et al. 2000a, Pandey 1995). Most of the industrial processes use GA obtained from *Aspergillus awamori* or *A. niger* (Koutinas et al. 2003, Polakovic & Bryjak 2003, Christensen et al. 2002, MacKenzie et al. 2000, Pandey et al. 2000a, Selvakumar et al. 1996a). Species of *Rhizopus* such as *Rhizopus* sp A11, *R. oryzae, R. niveus, R. delemar, R. javanicus* are also potent producers of GA (Yu & Chang 1991, Fujio & Morita 1996, Pazur et al. 1990, Kusunoki et al. 1982, Saha & Ueda 1983). A few species of *Penicillium* (Gombert et al. 1999) and *Mucor* (Yamasaki et al. 1977a) have also been reported to produce GA. *Monascus* sp, which generally produces pigment, can also produce GA (Yongsmith et al. 2000). Few species of *Trichoderma* have also been reported to produce GA, namely, *T. viride* (Schellart et al. 1976), *T. reesei* (Fagerstrom & Kalkkinen 1995), etc.

Few thermophilic fungi have also been reported as the sources of GA. Some examples are *Thermomyces lanuginosus* (Nguyen et al. 2000, 2002), *Scytalidium thermophilum* (Cereia et al. 2000), *Thermomucor*

Table 1. Sources of GA

Strains	References
Bacillus stearothermophillus	Srivastava 1984
Flavobacterium sp.	Bender 1981
Clostridium thermosaccharolyticum	Specka et al 1991
C thermohydrosulfuricum	Hyun & Zeikus 1985
Lactobacillus amylovorus	James & Lee et al 1995
Candida antarctica	Mot & Verachert 1987
Pichia subpelliculosa	Kumar & Satyanarayana 2001
Saccharomyces diastaticus	Furuta et al 1997
Saccharomycopsis fibuligera	Futatsugi et al 1993
Streptosporangium sp.	Stamford et al 2002
Thermoanaerobacterium thermosaccharolyticum	Aleshin et al 2003
Aspergillus sp. A3	Ellaiah et al 2002
A. awamori	Koutinas et al 2003
A. fumigatus	da Silva & Peralta 1998
A. niger	Pandey 1990, Pandey & Radhakrishnan 1993, MacKenzie et al 2000, Christensen et al 2002, Polakovic & Bryjak 2004
A. oryzae	Miah & Ueda 1977
A. saitri	Takahashi et al 1981
A. terreus	Ghosh et al 1991
Humicola grisea	Campos & Felix 1995
Monascus sp. KB9	Yongsmith et al 2000
Mucor rouxianus	Yamasaki et al 1977a
M. javanicus	Yamasaki & Kano 1991
Penicillium restrictum	Gombert et al 1999
Rhizopus sp. MKU 40	Fujio & Morito 2000
R. oryzae	Yu & Chang 1991
R. delemer	Kusunoki et al 1982, Pazur & Okada 1967
R. javanicus	Sana & Ueda 1983
R. niveus	Pazur et al 1990
Sclerotinia sclerotiorum	Martel et al 2002
Sclerotium rolfsii	Kelkar & Deshpande 1993
Streptomyces rimosus	Yang &Wang 1999
Streptosporangium sp.	Stamford et al 2002
Talaromyces flavus	Hang et al 1993
Thermomucor indicae-seudaticae	Kaur & Satyanarayana 2001
Thermomyces lanuginosus	Nguyen et al 2000, Nguyen et al 2002
Thermotoga maritima	Myoung-Hee et al 2002
Trichoderma viride	Schellart et al 1976
T. reesei	Fagerstrom & Kalkkinen 1995

indicae-seudaticae (Kaur & Satyanarayana 2001), *Humicola grisea* (Campos & Felix 1995), *Talaromyces flavus* (Hang & Woodams 1993) and *Streptosporangium* sp (Stamford et al. 2002).

3.2. Bacterial sources

A few aerobic bacteria such as *Bacillus stearothermophillus, Flavobacterium* sp, *Lactobacillus amylovorus, Halobacterium sodamense, Sclerotinia sclerotiorum* and *Sclerotium rolfsii* (James & Lee 1999, Srivastava 1984, Bender 1981, Martel et al. 2002, Kelkar & Deshpande 1993) are known as the sources of GA. Some anaerobic bacteria such as *Clostridium thermosaccharolyticum* (Specka et al. 1991) and *C. thermohydrosulfuricum* (Hyun & Zeikus 1985) have also been reported as the source of GA.

3.3. Yeast sources

Some species of yeast such as *Candida antartica, Saccharomycopsis fibuligera, Pichia subpelliculosa, Saccharomyces diasticus* have been described as the source of GA (Mot & Verachert 1987, Futatsugi et al. 1993, Kumar & Satyanarayana 2001, Furuta et al. 1997, Sevcik et al. 1998).

4. PRODUCTION OF GA

Traditionally, GA has been industrially produced by submerged fermentation (SmF); however, in recent years, solid-state fermentation (SSF) has also been considered promising for GA production (Pandey 1992a, b, c, 1994, 2003, Pandey et al. 1999, 2000c, 2001). For this, agro-industrial residues are generally considered good substrates (Ellaiah et al. 2002, Pandey et al. 1999, Pandey & Soccol 2000, Pandey et al. 2000d, e, f). Some examples include wheat bran, rice bran, rice husk, gram flour, wheat flour, corn flour, tea waste, copra waste, etc (Pandey 1990, 1991a, 1992b, 1992c, 1994, Pandey & Radhakrishnan 1992, 1993, Pandey et al. 1994a, b, 1995a, Selvakumar et al. 1998). In SSF, several parameters such as particle size, moisture content and water activity of the substrate have been shown to be important factors affecting the enzyme production (Pandey 1991a, 1992c, Pandey et al. 1994a). Particle size of the substrate has strong influence on growth rate and enzyme producing activity of the organism due to the influence of surface area on hydrolysis/growth rates. Particle size determines the void space, which is occupied by oxygen. Smaller particles result in a high degree of solubilisation whereas larger particles provide less surface area. Pandey (1991a) reported maximum GA with substrate (wheat bran) particles of 425-500 microns. Lowest GA activity was attained when the substrates contained particles bigger than 1.4 mm and smaller than 180 microns. Hence, a compromise has to be made in selecting a suitable particle size, which would enhance mass transfer. Water activity of the medium is also a fundamental parameter for mass transfer of the water and solutes across the cell membrane. When the initial water activity values of substrate were higher, the GA yields were also found to be higher (Pandey et al. 1994a).

SSF for GA production is generally carried out at mesophilic temperature (~ 30°C) and depending upon the nature of the substrate, the moisture content varies, e.g. starchy substrates may have about 50-70% moisture, which, however, could be higher too (depending up on the composition of the substrates for starch, cellulose, etc). Sometimes, the substrates may require supplementation with another carbon source (easily accessible source), which could enhance culture's activity. This could be simple sugars such as glucose, maltose, sucrose, etc or polymeric compounds such as starch from some other source. For example, supplementation of corn starch to wheat bran medium generally enhances GA production by fungal cultures (Pandey 1990). Similarly, supplementation of the substrate with external

nitrogen sources (organic or inorganic nature) has been practised successfully to increase the yields of GA in SSF. N-sources too could be simple compounds such as ammonium or nitrate salts, or urea, or complex sources such as corn steep liquor. The latter has often been found useful for GA production in SSF.

Addition of fructose, ammonium sulphate, urea and yeast extract in the substrate such as wheat bran has beneficial effect on GA production in SSF by *Aspergillus* sp and *A. niger* (Ellaiah et al. 2002, Bertolin et al. 2002, Morita & Fujio 2000, Pandey et al. 1995b). GA production by *A. awamori* showed 100% increase when ammonium sulphate was replaced by urea, with C/N 4.8; however, C/P ratios in the range 5.1 to 28.7 did not influence GA production (Bertolin et al. 2002).

An important aspect related to the production of GA in SSF has been use of different kinds of laboratory model bioreactors such as Erlenmeyer flasks, roux bottles, trays and glass columns to evaluate their performance and the influence of the bioreactor design on GA production (Pandey 1991b, 1992a, 1994, Pandey & Radhakrishnan 1992, Ashakumary et al. 1994, Pandey et al. 1996). The enzyme production with *A. niger* showed to be faster in trays (36 h) comparing to flasks (96 h) (Pandey 1990).

As has been mentioned earlier, traditionally SmF has been used for the production of GA. In SmF, however, nutritional requirements are generally more complex than in SSF. Composition of the media greatly influences the production of GA. Generally some kind of starch is needed as carbon source and fermentation is carried out under acidic pH (~ 4.5) with continuous aeration. *Rhizopus* sp. A-11 produced GA to a high concentration in a basal liquid medium supplemented with zinc and calcium ions (Fujio & Morito (1996). Nguyen et al. (2000) reported that the medium pH, salts such as K_2HPO_4, KH_2PO_4, and the nature of nitrogen and carbon sources greatly affected GA production by *Thermomyces lanuginosus*. Thermostable glucoamylase was produced by *Streptosporangium* sp. cultured on starch-czapek medium. Maximum glucoamylase activity was obtained at pH 4.5 and 70°C. Queiroz et al. (1997) conducted a study on GA production by *A. awamori* in order to define the rheological parameter consistency index (K) from the Power law and another between either the specific growth rate or the specific GA production rate. Aeration is an important factor to be considered for GA synthesis (Kostka & Kaczkowski 1989).

Design of a fermentation media for industrial production of GA also depends upon the kinetic parameters of a fungal culture. Kinetic parameters of a fungal culture may vary significantly according to culture conditions, such as temperature, oxygen, pH, etc (Koutinas et al. 2003). The specific glucose consumption rate (δ) was used as an indicator of GA and for feed rate control. The statistical regression model was applied for the construction of a feeding strategy for the culture. GA was 34% higher than that obtained by a constant feed rate (Imai et al. 1994).

4.1 Genetic engineering in relation to GA production

The applications of recombinant DNA technology and genetic engineering have greatly contributed to the synthesis of novel gene products. The majority of microbial enzymes of current commercial interest are being produced by gene manipulation. Various genetic engineering approaches have been adapted to improve the GA producing strain to obtain enhanced production, thermostable GA, selectivity in producing glucose, etc. Site-directed mutagenesis of selected amino acid residues has been one of the extensively studied tools. Combining favorable mutations has decreased the ability of the enzyme to produce isomaltose, the main byproduct in GA catalyzed production of glucose by almost half, has increased

enzyme thermostability many-fold, and also increased enzyme activity by 15 %. In addition, catalytic efficiencies for maltose hydrolysis over isomaltose hydrolysis have also been improved by mutational studies. Reilly (1999) has reviewed the improvements that have been made in the properties of GA by protein engineering. Some of the studies involving heterologous gene expression are given here.

The cDNA of *R. oryzae* GA was placed under the *Candida boidinii* alcohol oxidase (*AOD1*) promoter. Secreted GA from transformant *C. boidinii* was purified and compared with the enzyme produced in *S. cerevisiae*. The enzyme produced in *C. boidinii* was found to have higher molecular weight than that produced in *S. cerevisiae*, which was due to the difference of the *N*-linked glycosylated sugar structure of the produced proteins (Sakai et al. 1996). Ma et al. (2000) reported the construction of recombinant yeast producing GA and isoamylase by multiple integration of *Pseudomonas amylodermosa* iso gene into the integrant G23-8 chromosome. With this recombinant yeast, 95% utilization of soluble starch was obtained. The combined use of growth rate independent and dependent promoters to improve production of recombinant proteins in fed-batch culture systems is possible. Gordon et al. (2001) illustrated this phenomenon by using *Fusarium venenatum* JeRS 325, a strain which produced recombinant GA under control of a growth rate independent promoter. This strain was transformed with a plasmid carrying the *A. niger* GA gene under control of its own growth rate correlated promoter. The double transformant produced GA as much as JeRS 325 in fed-batch cultures. Nakamura et al. (1999) investigated the breeding of recombinant yeast, *S. cerevisiae* SR93 having GA activity. The disruption of the MAT locus, which produced a represser protein, enhanced the expression of GA gene and GA activity per unit cell concentration increased about 1.6-fold due to the disruption of the MAT locus. The specific growth rate and GA synthesis rate was much higher than *S.cerevisiae* SR93.

The gene encoding a thermostable GA from *Talaromyces emersonii* was cloned and, subsequently, heterologously expressed in *A. niger*. This GA gene encoded a 618 amino acid long protein with a calculated molecular weight of 62,827 Da. *T. emersonii* GA fall into glucoside hydrolase family 15, showing approximately 60% sequence similarity to GA from *A. niger*. The expressed enzyme shows high specific activity towards maltose, isomaltose, and maltoheptaose, having 3–6-fold elevated k_{cat} compared to GA from *A. niger*. *T. emersonii* GA showed significantly improved thermostability with a half life of 48 h at 65°C in 30% (w/v) glucose, compared to 10 h for GA from *A. niger* (Nielsen et al. 2002). Heimo et al. (1997) reported the expression of *A. awamori* GA catalytical domain (GAc) in *Pichia pastoris*, which produced GAc to the level of 0.4 g per liter medium. In another study, Wallis et al. (1999) investigated the overexpression and secretion of a homologous model glycoprotein, GA (GAM-1) on glycosylation in a single gene copy wild-type parent and multiple gene copy transformants of *A. niger*. Overexpressing strain of *A. niger* that contained multiple copies (20 and 80) of the *glaA* (GAM-1) gene secreted 5–10-fold more of this protein.

5. PURIFICATION AND CHARACTERIZATION

GA, like other hydrolases is initially purified by precipitation (salt or solvent) or may be subjected to ultrafiltration. Further, separation from a crude solution requires the use of high capacity and resolution technique such as chromatographic or electrophoretic methods, which have commonly been employed. However, non-chromatographic separation technique such as macroaffinity ligand-facilitated three- phase partitioning (MLFTPP) can also be used. For example, GA from *A. niger* was purified using alginates by MLFTPP in which a crude preparation of the enzyme was mixed with alginate. On addition of appropriate

amounts of ammonium sulfate and *t*-butanol, the alginate bound enzyme appeared as an interfacial precipitate between the lower aqueous and the upper *t*-butanol phase. Enzyme activity from this interfacial precipitate was recovered using 1M maltose. GA purification 20-fold with 83% activity recovery (Mondal et al. 2003).

Purification of GA from *Acremonium* sp. was carried out by concentrating the crude enzyme using ultrafiltration followed by applying the solution in anion exchange column on DEAE-Toyopearl 650S and gel filtration column on Sephadex G-150. The final preparation was subjected to native –PAGE. A single band was observed indicating the homogeneity with respect to GA (Marlida et al. 2000). Extraction of GA produced by *A. awamori* NRRL 3112 was studied by Minami & Kilikian (1998) in two steps in PEG and potassium phosphate systems at pH values 6, 7 and 9. The optimal partitioning conditions for GA separation were obtained in PEG 4000 (first step), PEG 1500 (second step) at pH 7 and resulted in a three-fold increase in GA purification. Campos & Felix (1995) purified GA from *Humicola grisea*. They concentrated the enzyme supernatant by ultrafiltration (Amicon PM 10 membrane) and subjected it to ultragel AcA 44 (LKB-IBF) column chromatography. The active fractions were then loaded onto a SP-sephadex column. This was further chromatographed on a phenyl-sepharose column equilibrated with 0.01M sodium phosphate buffer. Finally, the enzyme was purified 13-fold and SDS PAGE resulted in a single band formation of 72kDa, confirming the homogeneity. Belshaw & Williamson (1990) purified GA from *A. niger* by chromatographing on anion -Mono Q (HR10/10) column and further purifying it by gel filtration (Sephacryl S200).

Thermostable GA from *Streptosporangium* sp was purified by fractionated precipitation with ammonium sulphate followed by gel filtration on Biogel-P2 column chromatography, which yielded 2.7 fold increase in the activity (Stamford et al. 2002). GA from *Thermomyces lanuginosus* ATCC 34626 was purified by sepharose CL 6B DEAE and superose 12 column and Q Sepharose fast flow column (Nguyen et al. 2002). GA produced by another thermophilic fungus *Scytalidium thermophilum* was purified 6.8-fold by ion exchange chromatography. The products of hydrolysis of starch, detected by thin layer chromatography showed only glucose after 30 min, indicating GA activity (Cereia et al. 2000).

5.1. Physicochemical and biochemical properties of GA

GA is a starch-saccharifying enzyme, which generally has optimum pH between 4.5-5.0, temperature between 40-60°C and molecular weight ranging between 28-120kDa depending upon the source (Table 2). Properties such as Michaelis constant (K_m), V_{max}, thermostability, isoelectric point, etc also differ from one another. Some of these are discussed below.

5.1.1. Temperature

GA from various microbial sources have optimum temperature in the range 50-55°C, which include GA from *A. niger* (Pandey 1990, Selvakumar et al. 1996b), *A. oryzae* (Miah & Ueda 1977), *Monascus kaoliang* (Iijuka & Minek 1977), *Mucor rouxinos* (Yamasaki et al. 1977a), *Penicillium oxalicum* (Yamasaki et al. 1977b), etc. However, GA from *R. delemar* (Pazur & Okada 1967) and *A. oryzae* (Miah & Ueda 1977) had an optimum temperature of 40°C.

GA from thermophilic fungi have a temperature optimum ranging from 60-75°C. Few of them include *Thermomyces lanuginosus* (Odibo & Ulbrich-Hofmann 2001, Nguyen et al. 2002), *Streptosporangium* sp. (Stamford et al. 2002), *Clostridium thermosaccharolyticm* (Specka et al. 1991) whose temperature optima were 70°C. However, GA from *C. thermohydrosulfuricum* had an optimum temperature of 75°C.

Table 2 Properties of some GA (adapted from Pandey 1995)

Source	Mol. wt. (Da)	Opt. pH	Opt. Temp. (°C)	Ref.
Aspergillus fumigatus	42,000	4.5-5.5	65	Da silva & Peralta 1998
A. niger	90,000	4.5	50	Takahasi et al 1985,
A. niger	-	4.0	60	Pandey 1990
A. oryzae	76,000	4.5	60	Miah & Ueda 1977
	38,000	4.5	50	
	38,000	4.5	40	
A. saitri	90,000	4.5	-	Takhashi et al 1981
A. terreus	70,000	5.0	60	Ghosh et al 1991
Clostridium thermosaccharolyticum	75,000	5.0	70	Specka et al 1991
C. thermohydrosulfuricum	-	4-6	75	Hyun & Zeikus 1985
Humicola grisea	74.000	6	60	Campos & Felix 1995
Monascus kaoligang	48,000	4.5	50	Iijuka & Minek1977
Mucor rouxianus	59,000.	4.6	55	Yamasaki et al 1977a
Penicillium oxalicum	84,000	5.0	55-60	Yamashaki et al 1977
	86,000	4.5	60	
Rhizopus sp.	74,000	4.5-5.0	-	Takahasi et al 1985
R. delemer	1,00,000	4.5	40	Pazur & Okada 1967
Scytalidium thermophilum	86,000	6.5	60	Cereia et al 2000
Thermomyces lanuginosus	75,000	4.4-5.6	70	Nguyen et al 2002

GA from some other sources such as *Humicola grisea* (Campos & Felix 1995), *Acremonium* sp. (Maze et al. 1996, Marlida et al. 2002) and *A. terreus* (Ghosh et al. 1991) had 60°C as optimum temperature.

5.1.2. pH

Most of the GA from different sources generally have optimum pH between 4.5-5.0. These include *A. niger* (Pandey 1990) *A. oryzae* (Miah & Ueda 1977), *A. fumigatus* (da Silva & Peralta 1998), *Monascus kaoliang* (Iijuka & Minek 1977), *Mucor rouxinos* (Yamasaki et al. 1977a), *A. terreus* (Ghosh et al. 1991), *Penicillium oxalicum* (Yamasaki et al. 1977b), etc. However, there are reports where GA with high acidity tolerance or near to neutrality have been optimally active, e.g. GA from *Streptosporangium* sp (Stamford et al. 2002) pH 5.5, *Thermomyces lanuginosus* (Odibo & Ulbrich-Hofmann 2001, Nguyen et al. 2002), and *Humicola grisea* (Campos & Felix 1995) pH 6.0. Optimum pH for *Scytalidium thermophilum* was 6.5 (Cereia et al. 2000).

5.1.3. Molecular weight

Depending upon the source, the molecular weight differs ranging from 28-120 kDa. Molecular weight of GA from *A. fumigatus* was 42 kDa (da Silva & Peralta 1998). GA produced by *Monascus kaoliang* (Iijuka & Minek 1977), *Mucor rouxinos* (Yamasaki et al. 1977a), *Penicillium oxalicum* (Yamasaki et al. 1977b) had 48, 59, 84 kDa, respectively. GA from *Acremonium* sp YT-78 isolated from soil also had a molecular weight of 74 kDa (Maze et al. 1996); however, another GA from *Acremonium* sp. isolated from forest trees was only 39 kDa (Marlida et al. 2002). GA produced by the thermophilic fungus *Scytalidium thermophilum* had a molecular weight of 86 kDa (Cereia et al. 2000).

5.1.4. Other properties

GA produced by *Thermomyces lanuginosus* had isoelectric point as 4.0 (Odibo & Ulbrich-Hofmann 2001) and had highest affinity for soluble starch with K_m values of 0.80 mg/ml. The activities of glucoamylase increased in the presence of Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+} and Fe^{2+}, but were inhibited by guanidine-HCl, urea and di-sodium EDTA. *Acremonium* sp GA was strongly inhibited by EDTA. The enzyme catalysed hydrolysis of amylose and amylopectin and showed apparent K_m values of 10.0 and 3.8 mg/ml and V_{max} of 195 μmol/ml/min and 391 mol/ml/min, respectively (Marlida et al. 2000). Another GA from the culture filtrate of *Acremonium* sp. YT-78 isolated from soil had a isoelectric point 5.1.

Thermostable extracellular GA from *Humicola grisea* showed isoelectric point as 8.4. The K_m of soluble starch hydrolysis at 50°C and pH 6.0 was 0.14mg/ml (Campos & Felix 1995). pI value of *Thermomyces lanuginosus* was 4.1-4.3. The K_m values on maltose, maltotriose, maltotetrose, maltopentose and soluble starch were 6.5, 3.5, 2.1, 1.1mM and 0.8mg/ml, respectively (Nguyen et al. 2002). The pI of GA produced by *Scytalidium thermophilum* was 8.4. The enzyme had a half-life of 22 min at 55 °C with starch as substrate. The enzyme contained approximately 25.5% carbohydrate. K_m and V_{max} values for starch and maltose were 0.28 mg/ml, 67.2 U/mg protein and 1.40 mg/ml, 5.61 U/mg protein, respectively (Cereia et al. 2000).

5.2. Action mechanism and structure

Although biochemical and cellular function of prokaryotic GA have been reported, relatively little is known about them. Two domains were detected at the prokaryotic GA from *Clostridium* sp and comparison among them was made. Similarities in structure and function between glucoamylases, maltose phosphorylase and glycoaminoglycan lyases suggest their evolution from a common domain ancestor (Aleshin et al. 2003, Bertoldo & Antranikian 2002). Two main domain, named α and β, were identified; α-domain being the active site entirely and the β-domain could stabilize a closed productive conformation of the active site. The crystal structures of maltose phosphorylase and glycoaminoglycan lyases suggest large movements of their domains over the course of catalytic cycle; in procaryotic GA the active site may open transiently to facilitate ligands exchange (Aleshin et al. 2003, Sauer et al. 2000). The evolution of eukaryotic GA may have been due to the loss of β-domain and change in the mechanism of ligand exchange. Most prokaryotic GA come from thermophilic organisms, where the β-domain does stabilizing influence, and hence, the thermostability of GA may be due to the β-domain. The loss of β-domain in eukaryotic GA may have originated from differences in secretion mechanisms between eukaryotics and prokaryotics and at the extensive glycosylation of secreted eukaryotic proteins (Aleshin et al. 2003).

6. ASSAY OF GA

GA activity is routinely assayed by the determination of reducing sugars liberated in the reaction mixture containing starch as substrate. The enzyme sample is allowed to react with 4% soluble starch solution at 60°C and pH 4.5. One unit (U) is defined as the amount of enzyme that releases 1 mg of glucose per h under the assay conditions (Freire & Sant'Anna 1990). The release of glucose from starch (0.5% w/v in 0.2 M sodium acetate buffer, pH 5.0) or maltose (0.125 M in the same buffer) at 30°C using the hexokinase procedure (Sigma) has been described by Withers et al (1998). One unit of GA released 1 μ mol of glucose min^{-1} at this temperature. In another method, the reaction mixture contained 0.5 ml of the enzyme solution and 0.5 ml of boiled 1% soluble starch in 0.05 M (final concentration) acetate

buffer pH 4.7 and incubated at 55°C for 20 min. The reducing sugar liberated was determined by the method of Somogyi (1952). One unit of the GA was defined as the amount of enzyme required to liberate reducing sugar equivalent to 1 μg of glucose/ml in 1 min at 55°C.

7. CONCLUSIONS AND PERSPECTIVES

GA production has been traditionally carried out in SmF under industrial sector but SSF too has gained attention from several sectors and is used for industrial level, although to a limited extent. Use of genetic engineering tools and strain improvement programmes have resulted hyper-producing strains. GA from some sources have capacity to digest raw starch. Site-directed mutagenesis has led to the development of GA with specific properties such as thermostability, capacity to produce glucose only (during the hydrolysis of starch), etc. There have been much development on media formulation with great emphasis on use of naturally occurring cheap material as the source of nitrogen and nutrients. This has successfully led much reduction in production cost and one-way process on production has been very well established. One another important aspect has been development of large-size bioreactors with suitable controls for aeration and other process controls. Much success has been achieved in reducing the cost of down-stream processing. However, in spite of tremendous successes in GA production with continuous increase in the productivity and substantially low sale price, the potential to improve the enzyme productivities and characteristics still exist. Development of strains with dual enzymes, *viz* alpha amylase and GA could be another area to explore. SSF has shown its potential for GA production but it remains yet to be exploited in a major way commercially. Efforts should also continue to explore site directed mutagenesis and other enzyme engineering approaches for obtaining GA with specific improved properties.

Acknowledgements

CRS thanks CNPq for a scholarship under Scientific Productivity scheme. PJR thanks CSIR, New Delhi for the award of a Junior Research Fellowship.

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Glucose Isomerase

Vasanti Deshpande and Mala Rao

1. INTRODUCTION

D-Glucose/Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) (GI) occupies a pivotal position with respect to its applications in both physiological and commercial fields. It is one of the three highest tonnage value enzymes, amylase and protease being the other two. According to Wiseman (1975), GI is the most important of all industrial enzymes of the future. It catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively (Fig. 1). Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant material and also aids in the bioconversion of hemicellulose to ethanol. Isomerization of glucose to fructose is of commercial importance in the production of high fructose corn syrup (HFCS). Sucrose derived from sugar beet (40%) and sugarcane (60%) was the main sweetener in the world until 1976. The production of HFCS by using glucose isomerase was developed first in Japan and later in the United States. GI gained commercial importance in the United States because of the lack of supply of sucrose after the Cuban revolution in 1958, and it continues to be one of the most important industrial enzymes to this day.

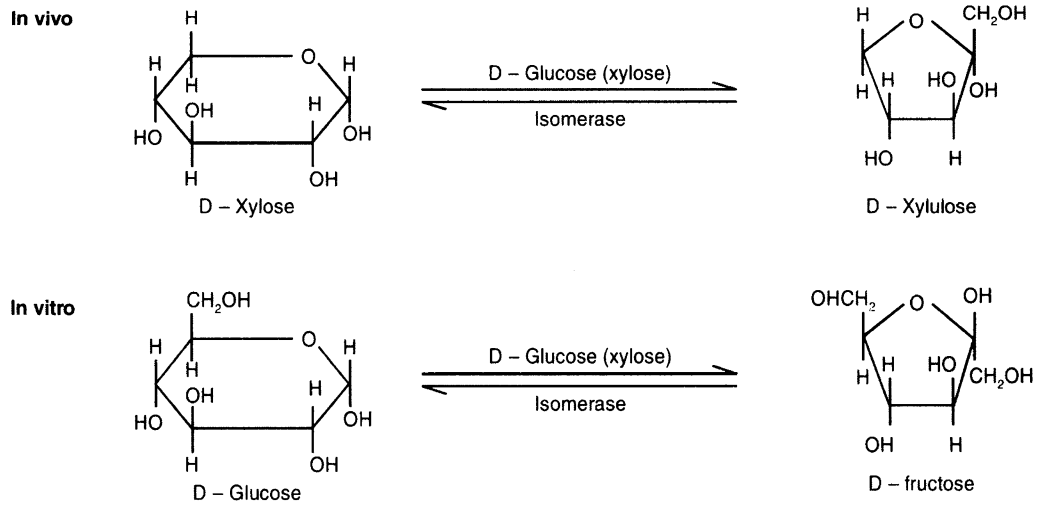

Fig. 1. Reactions catalyzed by GI.

The origin of today's successful development of fructose syrup products lies in the discovery of glucose-isomerizing enzymes. Historically, four different types of enzymes have been termed glucose isomerases. The discovery by Marshall and Kooi (1957) of the glucose isomerizing capacity of the enzyme from *Pseudomonas hydrophila* was the starting point of the exploitation of this enzyme for the manufacture of HFCS as a substitute for cane sugar. Although the affinity of this enzyme was 160 times lower for glucose than for xylose, it was sufficient for the enzyme to be commercially significant. Production of the enzyme required xylose in the growth medium and was enhanced in the presence of arsenate. Later, a xylose isomerase activity which was independent of xylose was found in *Escherichia intermedia* (Natake and Yoshimura, 1964). The enzyme was a phosphoglucose isomerase (EC 5.3.1.9), which could isomerize the unphosphorylated sugar only in the presence of arsenate. Takasaki & Tanabe (1962, 1963) isolated from *Bacillus megaterium* A1 a glucose isomerase (EC 5.3.1.18), which was NAD linked and was specific for glucose. A similar glucose isomerase activity, which catalyzed the isomerization of both glucose and mannose to fructose was isolated from *Paracolobacterium aerogenoides* (Takasaki & Tanabe, 1964, 1966a, b). The glucose isomerases produced by heterolactic acid bacteria require xylose as an inducer and are relatively unstable at higher temperatures. Of these glucose isomerizing activities, xylose isomerase (EC 5.3.1.5) is the most suitable for commercial applications. It is heat stable and does not require expensive cofactors such as NAD^+ or ATP for activity. The potential for using sugar substitutes produced from starch was proposed by several workers (Reed 1966, Crueger & Crueger 1984). Enzymatic glucose isomerization was first accomplished on an industrial scale in 1967 by Clinton Corn Processing Co. in the United States. Immobilized GI was commercially available by 1974. The demand for HFCS in the food industry increased and since 1980, practically all major starch-processing companies in the western world are using GI technology. Today, the enzyme commands the biggest market in the food industry.

The chemical conversion of glucose to fructose has been known for the past 100 years and constitutes a group of reactions collectively known as the Lobry de Bruyn-Alberda van Ekenstein transformation. These reactions are usually carried out at high pH and temperature. The possibility of producing fructose chemically from glucose has been studied by Barker et al. (1973). The reaction is non-specific and leads to the formation of non-metabolizable sugars such as psicose and other undesirable colored products. It is difficult to attain a fructose concentration of more than 40% by this method. Moreover, chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied. Therefore, it cannot be used commercially. On the other hand enzymatic conversion of glucose to fructose offers several advantages, such as (i) specificity of the reaction, (ii) requirement of ambient conditions of pH and temperature, and (iii) no formation of side products. Therefore, enzymatic conversion is preferred to chemical isomerization of glucose to fructose, and today the process involving GI has undergone considerable expansion in the industrial market.

1.1 Importance of glucose isomerase

GI has received increased attention by the industries for its use in producing HFCS and for its potential application in the production of ethanol from hemicelluloses. Increasing demands for refined sugar, coupled with its high cost of production and awareness of the adverse effects of sucrose on human health, have necessitated the search for acceptable sucrose substitutes. A large number of non-calorific and non-carbohydrate artificial sweeteners such as saccharine, cyclamate, acesulfame-K, aspartame, and thaumatin have been discovered and dismissed on the basis of health concerns or other drawbacks.

Incorporation of aspartame into soft drinks renders them less sweet after prolonged storage, because aspartame is slowly hydrolyzed at low pH. Thaumatin, an ideal protein sweetener, is 2,000 times sweeter than sucrose but has a distinct, unpleasant flavour. HFCS, an equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. The sweetening capacity of glucose is 70 to 75% that of sucrose, whereas fructose is two times as sweet as sucrose. HFCS is manufactured from a totally non-sweet substance, namely, starch. The price of HFCS is 10 to 20% lower than that of sucrose on the basis of its sweetening power. HFCS is preferred by the food industry since it does not pose the problem of crystallization as is the case with sucrose. Moreover, D-fructose plays an important role as a diabetic sweetener because it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood. The major uses of HFCS are in the beverage, baking, canning and confectionary industries.

2. SOURCES OF GI

GI is widely distributed in prokaryotes. After its discovery in *Pseudomonas hydrophila*, a large number of bacteria and actinomycetes have been found to produce GI that is active in the absence of arsenate. Among the heterolactic acid bacteria, *Lactobacilus brevis* produces the highest yield of enzyme, having activity at low pH but unstable at high temperature and hence not suitable for economic exploitation. Extracellular GI was produced by *Streptomyces glalucescens* (Weber 1976), *S. flavogriseus* (Chen et al. 1979), *Chainia sp.* (Srinivasan et al. 1983) and an alkalothermophilic *Bacillus* sp.(Chauthaiwale and Rao, 1994). Several *Bacillus* species are also good producers of GI. The occurrence of GI in a few yeasts such as *Candida utilis* (Vongsuvanlert and Tani 1988) and *C. boidinii* (Wang et al. 1980) has been documented. A xylose isomerase from *Neurospora crassa* (Rawat et al. 1996) and from a thermophilic

Table 1: Commercial production of GI

Manufacturer	Trade Name	Organism
Gist Brocades and Anheuser-Busch Inc.	Maxazyme	*Actinoplanes missouriensis*
Novo-Nordisk	Sweetzyme	*Bacillus coagulans*
Miles Kali-Chemie	Optisweet	*Streptomyces rubiginosus*
Finnsugar	Spezyme	
Nagase	Swetase	*Streptomyces phaeochromogenes*
Reynolds Tobacco		*Arthrobacter sp.*
Miles Laboratories Inc.		*Streptomyces olivaceus*

mould *Malbranche pulchella* (Banerjee et al. 1994) have been reported. The organisms that are commercially important as GI producers are listed in Table 1.

3. PRODUCTION OF GI

The cost of production of the enzyme is an important factor in evaluation of its suitability for industrial application. Intensive efforts have been made to optimize the fermentation parameters for the production of GI with a view to develop economically feasible technologies. GI research has been focused on three major aspects: improvement of the yields of GI, optimization of the fermentation medium with special reference to replacement of xylose by a cheaper substitute and elimination of requirement for Co^{2+} ions, and immobilization of the enzyme.

The yields of GI from various potent producer organisms range from 1,000 to 35,000 U liter^{-1}. Improvement in the yield and the properties of the enzyme has been achieved by the strain improvement, using either conventional mutagenesis or recombinant DNA technology. A series of constitutive and high GI-yielding mutants were isolated by applying multiple UV irradiations to *Streptomyces acidoduran* (Bok et al. 1984). One of the mutants produced by ethyl methanesulfonate yielded 1,500 U ml^{-1} when grown on only glucose whereas the parent produced 10 U ml^{-1} under similar conditions (Hafner 1985, Hafner & Jackson 1985).

3.1 OPTIMIZATION OF FERMENTATION MEDIUM

GI is generally produced by submerged aerated fermentation. Research efforts have been directed mainly toward replacement of xylose by another, inexpensive inducer, evaluation of the effect of cheaper nitrogen sources on the yield of enzyme, optimization of pH and temperature for maximum enzyme production and substitution of Co^{2+} ions by other divalent metal ions in the fermentation medium. There is no concrete composition of medium for the best production of the enzyme from different microorganisms. Each organism or strain requires its own special conditions for maximum enzyme production (Takasaki & Tanabe 1966b).

Most of the GI-producing organisms have an obligate requirement for D-xylose to induce production of the enzyme. However, xylose is expensive and hence impractical for use on a commercial scale. Starch, glucose, sorbitol, or glycerol could be used in place of 75% of xylose (Drazic et al. 1980). Takasaki & Tanabe (1966b) showed that *Streptomyces* strain YT-5 was able to grow on xylan or xylan-containing material such as corn cobs or wheat bran. This was the first landmark in selecting strains, which could grow in a cheaper medium. Today, several strains are capable of producing GI-utilizing glucose instead of xylose. These include *Actinoplanes* strains, mutant strains of *Bacillus coagulans* and *Streptomyces olivochromogenes*. Another approach to eliminate the requirement of xylose as an inducer is to generate mutants, which can produce GI constitutively. One of the wild type strains of *Actinoplanes missouriensis* was able to produce GI constitutively and has been used for commercial production of the enzyme by Gist Brocades (Anheuser-Busch Inc. 1974). Another approach to achieving constitutive enzyme production involves cloning the *xyl*A gene in front of a strong *Streptomyces* promoter. The P1 –*xyl*A gene has been integrated into the choromosome by using the integrative vector pTS55. The resultant strain (SBSI) gave about seven fold higher activity in the absence of xylose compared with the wild type strain fully induced by xylose (Bejar 1994).

Although complex nitrogen sources are usually used for GI production, the requirement for a specific nitrogen supplement differs from organism to organism. Peptone, yeast extract, or ammonium salts can be used with *B. coagulans*, but urea and nitrate are not suitable. Corn steep liquor was found to be a cheap and suitable source of nitrogen by some workers ((Anheuser-Busch Inc. 1974, Bucke 1983, Hafner & Jackson 1985) but its use is limited by its seasonal and inter-batch variability. Suitable nitrogen sources as substitutes for corn steep liquor are still being evaluated. Soy flour gave a 50% higher yield than corn steep liquor (Shieh 1977). Addition of certain amino acids improves the yield of enzyme in *Streptomyces viocoelicolor* (Vaheri & Kauppinen 1977, Vandamme et al. 1981).

The nature of the nitrogen source affects the pH and consequently the yield of the enzyme. Most GI-producing fermentations are carried out between pH 7.0 and 8.0 without control of pH. *Streptomyces* sp,

Arthrobacter sp and *Actinoplanes missouriensis* are grown at around 30°C. Thermophillic *Bacillus* sp are incubated at 50 to 60°C (Brownwell 1982, Diers 1976). The period of fermentation varies from 6 to 48 h depending on the type of culture used for GI production (Diers 1976).

Divalent cations are required in the fermentation medium for optimum production of GI. However, the requirement for specific metal ions depends on the source of enzyme. Co^{2+} was essential for GI production by *Streptomyces* sp YT-5 (Takasaki & Tanabe 1966b), whereas *B. coagulans* required Mn^{2+} or Mg^{2+} for the production of the enzyme (Outtrup 1974). Generally, cobalt salts are used in the medium of mesophilic *Streptomyces* species but not for thermophilic species. It is important to reduce the addition of Co^{2+} and the environmental pollution problem related to the disposal of spent media. Some organisms such as *Arthrobacter* sp and *S. olivaceus* and some mutants of *S. olivochromogenes* (Anheuser-Busch Inc., 1974) did not require cobalt for optimal production.

4. ASSAY OF GI

Glucose (xylose) isomerase activity can be determined by colorimetric assay, spectrophotometric assay and by the use of automatic analyzers.

(1) Colorimetric assay: Cysteine-HCl-carbazole- H_2SO_4 test efficiently differentiates between keto sugars and aldo sugars (Dische & Borenfreund 1951) and is the most widely used method for estimation of GI. Different hexoses, pentoses, tetroses and trioses give different colours in the reaction; keto sugars give more intense colour than the corresponding aldo sugars. The principle behind this method lies in the reaction of sugars and aldehydes with carbazole in H_2SO_4. The method used for GI assay is a modification of this reaction. Hexoses give a characteristic purple colour in this reaction, which has λmax at 560 nm; pentoses show λmax absorbance at 540 nm and trioses give λmax at 650 nm developing a blue colour. This colour development reaction is a free radical reaction whose mechanism of action is not fully understood.

Reaction mixture (2 ml) contained $MgSO_4$. $7H_2O$ (5 mM), $CoCl_2.6H_2O$ (1 mM), D-glucose (0.1 M), sodium phosphate buffer (0.05 M), pH 7.5 and suitably diluted enzyme solution. After incubation at 70°C for 30 min the reaction was terminated by adding 2 ml of 0.5 M perchloric acid. To an aliquot of 0.05 ml from this mixture, 0.95 ml H_2O, 0.2 ml of 1.5% cysteine hydrochloride, 6 ml of 70% sulfuric acid and 0.2 ml of 0.12% alcoholic carbazole solution were added sequentially. After heating at 60°C for 10 min, optical density of the violet colour formed due to D-fructose was measured at 560 nm. One unit of the enzyme activity was defined as the amount of enzyme which produces 1 µmole of D-fructose min^{-1} under assay conditions.

GI can also be assayed by quantitation of glucose formed in the reaction mixture using 'Glucoset reagent' (International Biochemicals and Equipment Co., Baroda, India) which is based on glucose oxidase-peroxidase system. Another commercial reagent is "Glox reagent" which is also a glucose oxidase –peroxidase system. GI activity is assayed by detecting D-glucose formed in the reaction mixture containing fructose as a substrate by glucose oxidase – peroxidase system. However, the most accurate among colorimetric methods is cysteine-carbazole- H_2SO_4 method.

(2) Spectrophotometric assay: This assay essentially consists of coupling glucose (xylose) isomerase with a dehydrogenase. In xylose isomerase assay, the resultant D-xylose is reduced with excess of D-

arabitol dehydrogenase or xylitol dehydrogenase and finally the activity of the dehydrogenase is followed by the rate of NADH oxidation at 340 nm. This method has many advantages such as (i) since almost all the glucose isomerases are xylose isomerases also, the enzyme activities can be assayed as XI activities and then converted into corresponding GI activities, (ii) the assay is highly specific and is more sensitive, (iii) assay time is short (three min) as compared to colorimetric assays (10 – 30 min), (iv) the resultant xylitol does not show significant inhibitory effect within the time span of estimation, (v) in direct colorimetric assay the glucose isomerase reaction is reversible; coupling of this reaction with the dehydrogenase prevents this reversion of the product, which is readily converted into the corresponding sugar alcohol. D-glucose (xylose) isomerase activity can also be assayed by coupling the reaction with mannitol dehydrogenase automatic analyzers.

(3) Automatic analyzers: Automatic glucose analyzers is another means of assaying glucose isomerase.

5. PURIFICATION OF GI

GI is generally an intracellular enzyme, except in a few cases when the enzyme production is extracellular. The enzyme is extracted from microbial cells by mechanical disruption (such as sonication, grinding, or homogenization) or by lysis of the cells with lysozyme, cationic detergents, toluene, etc. (Chen 1980a). Purification of GI from microbial sources by classical purification methods, such as heat treatment, precipitation with ammonium sulfate, acetone, Mg^{2+} or Mn^{2+} salts, ion-exchange chromatography, and/or gel filtration, has been reported (Chen, 1980b). Literature on the purification of GI by affinity chromatography methods is also available. An affinity adsorbent xylitol-Sepharose was used to purify the GI from *Streptomyces* sp. Other affinity matrices such as Biogel-P 100 coupled with xylose or mannitol immobilized on silochrome-based adsorbents were also used. A single-step, rapid purification of GI from *Streptomyces* sp. strain NCIM 2730 by immunoaffinity chromatography has been reported (Ghatge et al. 1991). Acid stable GI from *Streptomyces sp.* was purified and characterized for development of single step processes for HFCS production (Kaneko et al. 2002). Purification and characterization of thermostable GI from *Bacillus* has been reported (Lama et al. 2001).

6. CHARACTERIZATION OF GI

The enzymatic and physicochemical properties of GI from several organisms have been extensively studied. The knowledge of specific properties of the enzyme, such as its stability, substrate specificity, and metal ion requirement is important to prevent its inactivation and to assess its suitability for application in HFCS production.

The ability of the enzyme to isomerize a wide variety of substrates such as pentoses, hexoses, sugar alcohols, and sugar phosphates was investigated. Although the substrate specificity of the enzyme from different sources changes, the enzyme is able to utilize D-ribose, L-arabinose, L-rhamnose, D-allose and 2-deoxyglucose, as well as its most common substrates, D-glucose and D-xylose. The Km values of the enzyme from D-glucose and D-xylose were in the range of 0.086 to 0.920 M and 0.005 to 0.093 M, respectively.

GI requires a divalent cation such as Mg^{2+}, Co^{2+}, Mn^{2+} or a combination of these cations for maximum activity. Although both Mg^{2+} and Co^{2+} are essential for activity, they play differential roles. While Mg^{2+} is superior to Co^{2+} as an activator, Co^{2+} is responsible for stabilization of the enzyme by holding the ordered conformation, especially the quaternary structure of the enzyme (Callens et al. 1986, 1988, Gaikwad et al. 1992) Direct metal ion-binding studies were carried out by Danno (1971) on GI from *B. coagulans*. Kasumi et al. (1982) have reported the presence of four Co^{2+} ions per tetramer of GI from *S. griseofuscus*.

The catalytic activity of GI was inhibited by metals such as Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} and to some extent by Ca^{2+}. Other known inhibitors of GI are xylitol, arabitol, sorbitol, mannitol, xylose and Tris (Bucke 1983, Smith et al. 1991).

The sedimentation constants and molecular weights of GI vary from 7.55 to 11.45 and from 52,000 to 191,000 Da, respectively. The subunit structure and amino acid composition of GI reveal that it is a tetramer or a dimer of similar or identical subunits associated with non-covalent bonds and is devoid of interchain disulfide bonds. The extracellular GI from *Bacillus* sp is a trimer (Chauthaiwale & Rao 1994).

The optimum temperature of GI ranges from 60 to 80°C and increases in the presence of Co^{2+}. The optimum pH range of GI is generally between pH 7.0 and 9.0. The enzyme from *Lactobacillus brevis* has a lower pH optimum (between 6 and 7), which is desirable for commercial applications of GI. The identities of amino acids involved at or near the active site of GI were deciphered with group-specific chemical modifiers and by X-ray crystallography. Evidence for essential histidine and carboxylate residues in GI has been presented by Gaikwad et al. (1988) and Ghatge & Deshpande (1993). The structural environment of functional amino acid residues has been determined by chemical modification and subsequent differential peptide mapping of GI (Vangrysperre et al. 1990) The presence of a single active site for isomerization of both glucose and xylose was demonstrated (Gaikwad et al. 1989) by using a kinetic method.

The catalytic mechanism of GI has been a subject of great interest to researchers. Earlier GI was assumed to function in a manner similar to sugar phosphate isomerases and to follow the ene-diol mechanism (Rose et al. 1969). Recent studies have attributed the action of GI to a hydride shift mechanism.

GI from different bacterial species such as *Actinomyces, Arthrobacter, Actinoplanes,* and *Bacillus* species has been studied by X-ray crystallography at different levels of resolution, in the presence and absence of inhibitors and metal ions, to understand and explain the mechanism of action. The structures of GI from several *Streptomyces* sp. are accurately known. They are all very similar, especially at the active site. The structure of GI from *S. rubiginosus* as determined at 4-A° resolution (Carrell et al. 1984) has shown that the enzyme consists of eight β-strand-α-helix [$(\alpha/\beta)_8$] units as found in triose-phosphate isomerase. The smaller domain forms a loop away from the larger domain but overlaps the larger domain of another subunit, so that a tightly bound dimer is formed. The tetramer is thus considered to be a dimer of active dimers.

The anomerism and stereospecificity of the enzyme are shown to be fully consistent with the proposed hydride shift mechanism (Collyer & Blow 1990). Quantum dynamics of hydride transfer catalyzed by bimetallic electrophilic catalysis was studied by Garcia-Viloca et al. (2003). Crystallization and preliminary X-ray studies were carried out on Trp 138 Phe/Val 185 Thr xylose isomerase from *Thermotoga neapolitana* and *Thermoanaerobacterium thermosulfurigenes* (Kim et al. 2001). GI from *S. rubiginosus* was crystallized in two forms using Mn positions for SAD phasing of the single-wavelength data sets (Ramagopal et al. 2003).

7. GENETIC MANIPULATION OF GI

One of the ways to increase the production of GI is to identify the GI gene and clone it on a multicopy vector containing a strong promoter such as *lac, tac, or pL.* The GI gene has been cloned from several microorganisms with the primary aims of (i) overproduction of the enzyme by gene dosage effect (ii)

direct conversion of xylose to ethanol by yeasts and (iii) engineering of the protein to alter its properties to suit its biotechnological applications. Molecular cloning and expression of GI have been carried out in both homologous and heterologous hosts and in yeasts.

Homologous hosts offer several advantages for the cloning and expression of exogenous DNA. One of them is the easy recognition of the expression signals by the host RNA polymerase. There are a few reports on the homologous cloning of GI from *Escherichia coli* (Ho et al. 1983, Ho & Stevis 1985, Briggs et al. 1984, Batt et al. 1986, Lastick et al. 1986a, Wovcha et al. 1983, Wilhelm & Hollenberg 1984, Shin & Kho 1985, Wuxiang & Jeyaseelan 1993), and *Streptomyces* species (Kho 1984, Drocourt et al. 1988, Marcel et al. 1987). A few reports on heterologous cloning with hosts other than *E. coli* are also available. The GI gene from *Clostridium thermosulfurogenes* was cloned in *B. subtilis* with *E. coli-Bacillus* shuttle plasmid pMG1. The expression of the GI gene in *B. subtilis* was constitutive and was higher (1.54 mg $^{-1}$) than that produced in *C. thermosulfurogenes* (0.29 U) (Lee et al. 1990).

Saccharomyces cerevisiae and Schizosaccharomyces pombe offer a high fermentation rate, higher end product yield and increased ethanol tolerance. Transfer of GI genes to these yeasts holds a promise for developing an organism which can ferment xylose directly to ethanol (Chan et al. 1989; Amore et al., 1989).

Bhosale et al. (1996) have compiled and performed a multiple alignment of *xylA* sequences from 18 bacterial sources by using the program Clustal V (Higgins & Sharp 1988). In spite of the low homology between the *Streptomyces* sp and *E. coli* or *Bacillus* enzymes, the amino acids involved in the substrate and metal ion binding, as well as acids involved in the substrate and metal ion binding, as well as in catalysis, are completely conserved. The *cis* peptide linkage between the adjacent glutamic acid and the proline which is responsible for the formation of the rigid structure at the active site is also well conserved in all the GIs studied. Thus, the essential structure at the catalytic center of GI appears to be analogous in all the enzymes that were compared. The information on the conserved and homologous regions in the *xylA* sequences will be a valuable tool for isolating novel GIs with desirable catalytic properties. Comparative analysis of genome sequence data from mesophilic and hyperthermophilic microorganisms has revealed that bivalent cations and amino-acid composition contribute to the thermostability of *Bacillus licheniformis* xylose isomerase (Vieille et al. 2001).

Engineering proteins by manipulation of their genes is, at present, a viable approach, which complements structure-function studies performed by pre-existing methods and allows production of tailor-made proteins with desirable properties to give a complete insight into the mechanism of the enzyme. These studies lead to a hypothesis, which can be verified by protein engineering. Site-directed mutagenesis (SDM) of GI has been carried out with several objectives of academic and industrial importance, such as (i) increasing the thermal stability, (ii) lowering of the pH optimum, (iii) changing of the substrate preference, (iv) deducing the functional role of essential amino acid residues, and (v) studying the subunit interactions.

Enhancement of the thermostability of GI from *Thermoanaerobacterium thermosulfurigenes* was obtained as a consequence of the reduction of the water accessible hydrophobic surface by site-directed mutagenesis of aromatic amino acids in the active site (Meng et al. 1993). Thermostable glucose isomerases are desirable for production of 55% fructose syrups at >90°C. Current commercial enzymes operate only at 60°C to produce 45% fructose syrups. Protein engineering to construct more stable enzymes has so far been relatively unsuccessful. The reversible conformational changes in the apoenzyme arising from

electrostatic repulsions in the active site for varying thermostability have been explained by protein engineering for the buried Arg 30 Asp 299 salt bridge (Hartley et al. 2000). Site-specific integration of the double-mutation (G138P-G27D) of GI gene in *Streptomyces lividans* and its stable expression has been reported by Yang et al. (2002).

The specific activity of GI mutant G247D was demonstrated to be increased by 33% and the optimum pH was lowered by 0.6 unit (Zhu et al., 2000). Attempts to over-express GI were made by cloning the mutated (G138P-G247D) GI genes into *E. coli-Streptomyces* shuttle vectors followed by their transformation into *S. lividans* TK 54 strain (Zhu et al. 2002). SDM was used to assess the structural and functional roles of specific amino acid residues in the GI from *Actinoplanes missouriensis*. His-220 and His-54 were important but not essential for catalysis (Lambeir et al. 1992). The characterization of the physicochemical and catalytic properties of three cold-adapted XIs from *thermus thermophilus* through random PCR mutagenesis is described (Lonn et al. 2002). Engineering of direct fructose production in processed potato tubers has been achieved by expressing a bifunctional gene complex consisting of α-amylase (*Bacillus stearothermophilus)* and GI (*Thermus thermophilus*) (Beaujean et al. 2000). Molecular analysis of xylose (glucose) isomerases from *Thermoanaerobacterium thermosulfurigenes* and *Thermotoga neapolitana* for their thermal stability and activity has been carried out by site directed mutagenesis. The engineered glucose isomerase showed improved catalytic activity and thermostability (Sriprapundh et al. 2000).

These studies on protein engineering of GI have contributed substantially to our knowledge about the molecular mechanism of GI and have created new possibilities of producing an enzyme with properties that are better suited for biotechnological applications.

8. CONCLUSIONS AND PERSPECTIVES

The ideal GI should possess a lower pH optimum, a higher temperature optimum, a resistance to inhibition by Ca^{2+} and a higher affinity for glucose than do presently used enzymes. Introduction of all these properties into a single protein is a Herculean task, which has been an obstacle in the development of an economically feasible commercial process for enzymatic isomerization of glucose to fructose. Advances in recombinant DNA technology and protein engineering have opened new and encouraging possibilities of combining the desirable properties in a single organism to produce a tailor-made protein. Reduction of enzyme cost by amplification of the GI gene may cause an increase in fermentation productivity. Isolation of a mutant for the constitutive production of GI and elimination of the requirement of metal ions could contribute significantly to the improvement of the existing processes for HFCS production. Since higher temperatures thermodynamically favour fructose production, the ability to run GI columns at elevated temperatures for extended periods of time would be a major advance in this technology. The other area in which improved enzyme performance targets can be identified is the isomerization step.

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Cellulases

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1. INTRODUCTION

About 1.8 x 10^{12} MT biomass is generated on Earth yearly in terrestrial and marine ecosystems. A large portion of this biomass (about 60%) is lignocellulose, the structural material of plant cell walls. About 30-60% of lignocellulose is cellulose (Philippidis 1994). The lignocellulose and within it the cellulose is a constant source of renewable energy and chemical feedstock.

Cellulose is a crystalline matrix of linear β-1,4-D glucan chains, whereas hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans and mannans, in complex branched structures with a spectrum of substituents, such as acetyl-esters, along its backbone. Hemicelluloses usually hydrogen bound to cellulose, as well as to other hemicelluloses, which helps stabilize the cell-wall matrix and renders the cell wall insoluble in water (Teeri et al. 1992, Atalla 1993, Biely & Tenkanen 1998, Bhat & Hazlewood 2001). Cellulose is composed of long, unbranched glucose polymers packed onto each other to form highly insoluble crystals. Potent cellulolytic organisms such as *Trichoderma* produce complex mixtures of enzymes required for efficient solubilization of the substrate (Wood 1992, Koivula et al. 1998). The extracellular enzyme system produced by *T. reesei* is composed of three major enzyme components: endoglucanases (EC 3.2.1.4, 1,4-β-D-glucan glucanohydrolases), exoglucanases or cellobiohydrolases (EC 3.2.1.91, 1,4-β-D-glucan cellobiohydrolase) and b-glucosidases or cellobiases (EC 3.2.1.21, β-D-glucoside glucohydrolases) (Sternberg 1976, Wood 1992, Beguin & Aubert 1994, Teeri 1997, Koivula et al. 1998, Bhat & Hazlewood 2001). Endoglucanases specifically cleave the internal β-1,4-glycosidic bonds of amorphous, swollen and substituted celluloses and release glucose, cellobiose and cello-oligosaccharides (Wood 1985, Koivula et al. 1998, Bhat & Hazlewood 2001). The endoglucanases make more random cuts in the middle of the long chains thereby producing new chain ends for the cellobiohydrolases. The endoglucanases in contrast to cellobiohydrolases can also hydrolyze substituted celluloses such as carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC). The exoglucanases or cellobiohydrolases (CBH) cleave cellobiose units from the ends of the polysaccharide chains and typically exhibit relatively high activities on crystalline cellulose (Teeri 1997, Koivula et al. 1998). β-glucosidases cleave cellobiose and other soluble oligosaccharides to glucose, which is an important step because cellobiose inhibits the action of many cellulase components (Wood 1992, Beguin & Aubert 1994, Koivula et al. 1998).

In nature, lignocellulose containing organic matter is converted in different physico-chemical and biological degradation processes into simple compounds (Hajny & Reese 1969, Beguin & Aubert 1994, Bhat &

Hazlewood 2001), and in animals metabolized as chief nutrient in symbiotic association with microbes (e. g. in ruminants and termites). The agent for this bioconversion is the lignocellulolytic enzyme complex. The main components of the lignocellulolytic enzyme complex are the cellulases, hemicellulases, pectinases and different lignin degrading enzymes. These enzymes act in symbiotic interaction in decomposition of the plant cell wall. Industrially the most important among these enzymes are the cellulases, a group of several enzymes that hydrolyze cellulose. Since cellulose is a major structural component in textiles, paper and building materials, also the main nutrient for ruminant animals, much attention was devoted to the production and properties of cellulases.

The deterioration of U.S. Army cotton materials in the tropical warfare in World War II gave the first great impetus on cellulase research (Mandels 1975, Claeyssens 1998, Eveleigh & Mandels 1998). In the years between 1950 and 2000 the cellulase system has been described and characterized (Reese et al. 1950, Wood & McCrae 1979, Koivula et al. 1998, Bhat & Hazlewood 2001). The best producing microorganisms have been isolated and genetically improved (Mandels et al. 1971, Montenecourt & Eveleigh 1977b, Mantyla et al.. 1998, Bhat 2000). Cellulase production by submerged fermentation (SmF) has been realized in industrial scale. Currently, cellulase preparations are directed at low-volume, high-value specialty markets such as food processing, textile processing and laundry detergents that can bear the current high price of cellulases (see Section 3.).

In the food industry, cellulases are used for the extraction and/or clarification of fruit and vegetable juices, treatment of wines, extraction of oils and for improving the quality of bakery products. In the textile industry cellulase is used for biostoning and/or fading of denim and for polishing of cellulosic fabric. Cellulases also can be applied to re-use of waste paper in the pulp and paper industry. The most important commercial cellulase preparations are listed in Table 1.

The increasing demand for renewable resources and sustainable resource management directed cellulase research and production to agro-biotechnological applications, mainly toward the bioconversion of lignocellulose into soluble sugars, which can be processed to biofuels or other biochemical products (Sheehan & Himmel 1999, Wyman 1999).

The strategy of industrial processing of lignocellulose aims at the bioconversion of cellulose, hemicellulose and pectin into reducing sugars, leaving lignin as byproduct, or delignifying the feedstock before bioconversion. The key enzymes for bioconversion are thus the cellulases, hemicellulases and pectinases. Cellulase is by far the most important among these enzymes.

Another important agro-biotechnological application for cellulases and related enzymes is in animal feed supplements, to increase the digestibility of lignocellulosic feed ingredients (Bhat & Hazlewood 2001). These agro-biotechnological applications require more specific, target oriented and much cheaper enzymes than currently available. Today cellulases, hemicellulases and pectinases account for about 20% of the world enzyme market (Mantyla, et al. 1998). This should increase dramatically with the increased agro-biotechnological applications.

Cellulase research and production have been reviewed comprehensively in the past (Philippidis 1994, Tengerdy 1996, Bhat 2000, Bhat & Hazlewood 2001). In this chapter recent efforts for economic cellulase production are emphasized, comparing submerged and solid substrate fermentation (SSF) technologies, and the newest trends for developing the best producing microbes for the targeted new applications.

Table 1. Commercial cellulase or cellulase containing enzyme complex preparations

Product name	Enzyme	Microorganism	Recommended application
Products of Novozymes, Inc. (www.novozymes.com)			
Carezyme	mono-component alkaline cellulase	*Humicola insolens* cellulase gene cloned and expressed in *Aspergillus oryzae*	laundry detergent product
Cellubrix	cellulase-cellobiase enzyme		in the fruit juice industry and in the alcohol industry
Celluclast	fungal cellulase	*Trichoderma reesei*	in the cereal food industry and in the brewing industry
Cellusoft	acid-type cellulase	fungal	bio-polishing (finishing) of cellulosic fabrics
Celluzyme	multi-component alkaline cellulase	fungal	laundry detergent product
Denimax	mono-component alkaline cellulase	*Humicola*	for stonewashing of denim
Denimax Acid	acid-type cellulase	*Trichoderma*	for stonewashing of denim
Glucanex	beta-glucanase	*Botrytis cinerea*	for the treatment of wines
Viscozyme	cellulase, hemicellulase, carbohydrase	*Aspergillus aculeatus*	treatment of plant (vegetable) materials
Novozym 342	special cellulase with an alkaline optimum		deinking of mixed office waste paper
Products of Genencor International (www.genencor.com)			
Puradax® HA	high alkaline cellulase	*Bacillus spp.*	laundry detergent product for fabric surface care
Puradax® EG L	alkaline cellulase	*Bacillus spp.*	laundry detergent product
Multifect® GC	xylanase with cellulase and beta-glucanase activities	*Trichoderma reesei*	baking product
Laminex® BG	cellulase complex		standardized for beta-glucanase activity
Multifect® B	xylanase with cellulase and beta-glucanase activities	*Trichoderma reesei*	baking product
Multifect® A40	cellulase complex		for industrial application
IndiAge cellulases	conventional and engineered component cellulases	*Streptomyces spp.* fabrics and denim	for biofinishing of cellulosic finishing
Primafast® 100	conventional cellulase		finishing of lyocell fabric and garments
GC 220	high concentration cellulase complex		for whole-grain feedstock and biomass processing

2. SOURCES

Cellulases are produced in Nature by various terrestrial and marine organisms. The most important sources for industrial cellulase production are filamentous fungi, living in soil, plants and in the marine environment. Some bacteria are also potential sources for large scale cellulase production. Most of the early studies carried out on the biochemistry and enzymology of cellulases were from aerobic mesophilic fungi such as *Trichoderma viride, T. reesei, T. koningii, Penicillium pinophilum, P. funiculosum, Sporotrichum pulverulentum, Fusarium solani* and *Aspergillus niger.* In the past two decades, it has been recognized that other microorganisms, such as thermophilic fungi (*Chaetomium thermophilum, Humicola insolens, Sporotrichum thermophile, Talaromyces emersonii, Thermoascus aurantiacus),* mesophilic anaerobic fungi (*Neocallimastix frontalis, N. patriciarum, Orpinomyces* sp., *Sphaeromonas communis*), mesophilic and thermophilic aerobic bacteria (*Bacillus* spp., *Cellulomonas fimi, Cellvibrio* sp., *Pseudomonas fluorescens* subsp. *cellulosa*), mesophilic and thermophilic anaerobic bacteria (*Bacteroides cellulosolvens, Fibrobacter succinogenes, Ruminococcus albus, R. flavefaciens, Clostridium thermocellum* and *C. stercorarium*), as well as actinomycetes (*Microbispora bispora, Streptomyces flavogriseus, Thermomonospora fusca*) produce highly active cellulase and hemicellulase systems. In addition, hyperthermophilic microorganisms, namely, *Thermotoga maritema, T. neapolitana, Pyrococcus furiosus* and *Anaerocellum thermophilum,* which grow between 85 and 110°C produce stable cellulases and hemicellulases (Beguin & Aubert, 1994, Canevascini 1999, Bhat & Hazlewood 2001, Haki & Rakshit, 2003).

Cellulases, in general, have low specific activities, typically at least 100-fold less than amylases. Research has been conducted to isolate high producing wild strains, then to increase activity via strain mutation to produce enzymes with higher specific activity and to improve the catalytic turnover rate of the active site (Eveleigh 1987, Philippidis 1994). The first mutation program from the wild strain *Trichoderma reesei* QM 6a (formerly known as *T. viride* QM 6a) was carried out at the US Army Natick Laboratories. In the beginning of the 1970's, the mutant QM 9414 was isolated by Mandels et al. (1971). QM 9414 produced approximately two to four times more cellulase (as filter paper activity) than the wild-type QM 6a (Mandels et al. 1971, Mandels 1975).

Montenecourt & Eveleigh (1977a, b) developed a plate clearing assay to screen high-producing cellulase mutants of *Trichoderma.* The method employed the use of either rose bengall or oxgall to limit colony size in combination with Walseth-cellulose (phosphoric acid swollen cellulose) and glucose or glycerol as catabolite repressors of cellulase biosynthesis. For mutagenesis UV-light and nitrosoguanidine were used. This technique has allowed the isolation of a range of mutants of *T. reesei* (e.g. Rut NG-14), capable of synthesizing cellulase under conditions of high catabolite repression (Montenecourt & Eveleigh 1977b). Mutant Rut NG-14 produced approximately three times more cellulolytic enzyme activity than the wild type *T. reesei* QM 6a. After treating *T. reesei* Rut NG-14 with UV-light, *T. reesei* Rut C30 was isolated (Montenecourt & Eveleigh 1979). Mutant Rut C30 produced 4-5 times more cellulase than the wild parent (QM 6a). With similar protocols, additional hypercellulolytic *T. reesei* mutants have been selected in other laboratories such as L27 at Cetus Corporation (Shoemaker et al. 1981), VTT-D-80133 at VTT, Finland (Bailey & Nevalainen 1981) and CL-847 in France (Durand et al. 1988). The plate clearing assay has been used later successfully for isolation of cellulase producing mutants from *Penicillium pinophilum* (Brown et al. 1987), *P. occitanis* (Jain et al. 1990) and *P. purpurogenum* (Anwar et al. 1996).

Though the amount of secreted protein has been improved in mutated *T. reesei* strains, the proportion of the different cellulase components has remained more or less constant compared to the original strains. Thus,

screening of hundreds of thousands of mutant colonies on plates has not offered realistic possibilities for preparation of tailor-made enzyme mixtures for various biotechnical processes (Uusitalo et al. 1991). Novel *T. reesei* strains producing altered mixtures of cellulases have been constructed by genetic engineering starting from the late 80's (Mantyla et al.. 1989, Harkki et al. 1991, Uusitalo et al. 1991, Bower et al. 1998, Mantyla et al. 1998, Miettinen-Oinonen & Suominen 2002, Zandona et al. 2003). Using cloned *T. reesei* genes and their promoters, novel strains with completely different cellulase profiles, e.g. devoid of one or more cellulase components, have been constructed (Mantyla et al. 1998). Other fungal cellulase genes can also be expressed under the control of *T. reesei* cbh1 promoter (Haakana et al. 2004). These techniques allow the production of a selected cellulase component (typically an endoglucanase) on large scale without significant activities of other components. These mono-component enzymes are much more selective in their effect and they can be applied to special fields such as biostoning and fading of denim (blue jeans) and in laundry detergents.

Efforts are on the way for obtaining more efficient cellulase producing mutants and/or recombinants. The US Department of Energy (DOE) has been funding two leading enzyme manufacturers to meet these goals (www.genencor.com 2003; www.novozymes.com 2003). In 2000, Genencor International was awarded a USD 17 million grant from DOE's National Renewable Energy Laboratory, Golden, Colorado to develop low-cost cellulases and other enzymes for the production of ethanol from biomass. The goal is to deliver enzyme systems enabling a 10-fold improvement in the economics of breaking down cellulosic material and other complex carbohydrates into fermentable sugars. DOE also has been funding Novozymes Inc. by USD 14.8 million for three years (2001-2003) to develop more cost-efficient cellulase enzymes for bioethanol production. Currently, the enzyme costs amount to USD 0.50 per gallon of ethanol produced.

3. PRODUCTION

Cellulases are produced commercially by several companies using submerged fermentation (SmF) technology (see Table 1, Nieves et al. 1998). The production technology depends on the intended use of the cellulase preparation. For textile industry, pulp and paper industry and some food industry applications partly or highly purified cellulases, or specific components of the cellulase enzyme complex may be needed. For agro-biotechnological applications a crude enzyme complex, containing cellulases, hemicellulases and pectinases may be suitable. The relatively high cost of cellulases drives continuous efforts to reduce production costs. The currently available hypercellulolytic fungal mutants used for cellulase production by SmF still have a much lower yield than antibiotics or other fine biochemicals. The price of commercial cellulases is too high for economical application in most agro-biotechnological applications. *On-site* production or *in situ* production (where the substrate residue for enzyme production is incorporated in the final product, e.g., feed supplements, saccharification of pretreated lignocellulose for bioalcohol production) may reduce the cost of application.

The cost of production may be reduced by an alternative technology, solid substrate fermentation (SSF), whenever the enzyme enriched fermented substrate can be used directly as enzyme source (see Section 3.4.).

3.1. Substrates

The selection of substrate depends on the intended use of the cellulase preparation. If pure cellulase or cellulase component is the desired end-product, soluble carbon and nitrogen sources, (e.g., lactose and

ammonium salts) or pure cellulose (Avicel, Solka Floc) may be selected, thinking of easier downstream processing in purification. *Trichoderma* can grow on simple and inexpensive media and on materials such as whey and various plant wastes (Penttila 1998). For large volume, bulk commodity agro-biotechnical application, natural lignocellulosic materials may be used. Crop residues, such as corn byproducts, spent brewery grains, extracted sweet sorghum pulp, sugar cane bagasse, wheat straw, wheat bran, forest industry residues, soft and hard woods, grasses are suitable for cellulase production. These natural substrates may be supplemented with inorganic nitrogen for an optimal C:N ratio (usually 10-20 to 1), also with P, S, other trace minerals and inducers (see Section 6.) for optimal growth and enzyme production. SSF has a particular advantage and flexibility in utilizing locally available residues for a desired enzyme production with host specific fungi. Such enzyme enriched residues are targeted as animal feed supplement, or soil conditioner.

The natural lignocellulosic substrates have to be pretreated for optimal cellulase production. Acid or alkali pretreatment, steaming, steam explosion or NH_3 steam explosion, in some cases delignification may be used to loosen the lignocellulose structure and make it accessible to attack by the cellulase complex of the growing fungus (Philippidis 1994, Wu et al. 1999, Tenborg et al. 2001).

In the selection of the substrate its effect on the produced enzyme profile (the proportion of the different cellulase enzyme components and accessory enzymes, hemicellulases and pectinases) should be considered, since the cellulase complex is inducible (see section 6.2.). Xylanases and other hemicellulases are frequently produced by *Trichoderma* parallel with the formation of cellulase on different substrates (Biely & Tenkanen 1998).

In the selection of substrates the host specificity of the fungus also should be considered. Fungi grow best on their native host (substrate). This is particularly important in SSF, which is close to the native way of fungal existence (Tengerdy 1996).

3.2. Inoculum

An important requirement for successful industrial cellulase production is the availability of a reliable starter culture of the producing microorganism. Since the hypercellulolytic fungi or newer genetically modified organisms are physiologically not as robust as their wild ancestors, great care must be exercised in inoculum production. Mycelial inocula have the advantage of giving rapid growth and enzyme production after inoculation, because the hyphal growth is extensive and the cellulase system is partially induced. The disadvantage is the need for precise microbiological expertise and control at the production facility. Spore inocula on the other hand may be prepared in a central facility, and dispensed to users in sterile packages. This would be an advantage to *on-site* and/or *in situ* enzyme production, particularly in local farm level facilities, where microbiological know how is not available. An alternative might be a pregerminated spore inoculum, where the spores of the fungi are coated on corn-cob particles and pregerminated in a liquid fluidized bed reactor (Tengerdy et al. 1991). In the pregerminated spores the cellulase system is already induced.

3.3. Bioreactors

The bioreactors used for cellulase production should provide optimal conditions for fungal growth and enzyme production (temperature, pH, O_2 transfer, reducing inhibition). This may be accomplished in SmF or SSF biorectors.

3.3.1. SmF reactors

Cellulase production by SmF is usually performed in conventional stirred tank reactors (STR). *T. reesei* is grown in fermenters up to 230 m^3 on simple and inexpensive media and on materials such as whey and various plant wastes (Penttila 1998). With *T. reesei* Rut C30 the temperature optimum of fermentation is 32-33 °C and pH 3.5-4.0. In the first phase (0-48 h) of the fermentation the conditions are optimized for maximal growth, in the second phase (48-96 h) for enzyme production.

Cellulase production by SmF technology is summarized in Table 2. The most common operational mode is batch fermentation, as the most suitable mode for a highly viscous slurry of a lignocellulosic substrate. The yield in a batch fermentation is about 10-20 FPU/ml. The overall cellulase activity is given in this review as international filter paper unit (FPU) for uniform comparison (see section 4.). This corresponds to 200-400 FPU/g substrate in a 5 % slurry. The productivity is about 100-400 FPU/hL. The yield may be enhanced by increasing the concentration of the slurry, but the increase is not proportional, and handling the viscous fluid becomes difficult.

Fed batch fermentation increases both yield and productivity in comparison to batch fermentation (see Table 2.), mainly because of optimal growth conditions (fresh substrate, no lag period for growth, dilution of inhibitory product). Continuous fermentation has not been successful for cellulase production, most probably because of the handling problems with the highly viscous slurry.

3.3.2. SSF reactors

An alternative technology for cellulase production is solid substrate fermentation. Although SSF is widely practiced in Oriental countries since long, difficulty of process control, the labor intensity associated with SSF system, etc have not allowed its universal acceptance as an alternate technology (Pandey 1992, Pandey et al. 2000, 2001). Precise mathematical models for growth, mass transfer and product formation are being developed for SSF, but are not widely used yet (Tengerdy & Szakacs 2003). Cellulase production by SSF is summarized in Table 3. Different authors report widely different yields, but it may be stated that with the best *T. reesei* mutants a yield of 100-200 FPU/g and 200-800 FPU/ml productivities may be reached.

The basic reactor for cellulase production by SSF is the *Koji* type tray reactor that is widely used for fungal fermentations in industrial scale reactors in East Asia (Japan, Taiwan, China). A modified tray reactor is the *PlaFractor* that applies solid matrix fermentation for the production of enzymes and pharmaceutical products (Suryanarayan 2003). There is a considerable effort to develop new SSF reactors that would overcome the problem of heat and moisture control and that would allow on-line control of the fermentation. The first of these experimental reactors was the horizontal drum reactor, followed by modifications, such as the rocking drum reactor with on-line evaporative moisture temperature control (Pandey 1991, Ryoo et al. 1991) and lately the pressure oscillation reactor (Zhang et al. 2003). The most promising is the pressure oscillation reactor, in which cellulase production may be doubled compared with a static tray reactor (see Table 3) (Xu et al. 2002). New SSF reactor designs aim at better controlling optimal moisture and temperature levels and facilitating mass transfer of O_2 and nutrients.

3.4. Comparison of SmF and SSF for cellulase production

Cellulases are currently produced commercially mostly in SmF systems, but production by SSF is gaining ground. The advantage of SmF is the relatively high enzyme yield, the vast experience with SmF bioreactors and process control borrowed from the pharmaceutical industry, and the possibility for modeling cellulase

production and regulating biosynthetic pathways for enhancing a desired enzyme component. The disadvantage of SmF is the high production cost and the difficulty of adjusting the system to produce an optimal multienzyme complex for hydrolyzing a particular substrate.

SSF is a low technology, cost effective way for producing crude enzyme complexes, including cellulases, hemicellulases, pectinases and ligninases (Pandey et al. 1999). The use of SSF may be a viable alternative to SmF when the crude enzyme product may be used directly for the bioconversion or enzyme enrichment of a natural substrate (e. g. in the production of biofuels and animal feed supplements).

Weighing the advantages and disadvantages of SmF and SSF for cellulase production, it may be stated that SmF is preferable when pure enzymes or specific enzyme components are needed, and when the desired application can bear the high enzyme cost. SSF is preferable when the crude fermented product can be used directly e.g. as feed supplement, silage additive, or crude enzyme source for biofuel production.

In comparing cellulase production by SmF and SSF technologies (Tables 2 and 3), it may be stated that the overall yield in SmF is higher (200-400 FPU/g DM substrate in a 5% slurry) than in SSF (100-200 FPU/g DM), but productivity is higher in SSF (200-800 FPU/hL) than in SmF (100-400 FPU/hL). The volumetric productivity in SSF is favourable than in SmF. Assuming an average packing density of 0.33 (1 g substrate occupies 3 ml fermentation volume), a 100 FPU/g yield would correspond to 33 FPU/ml, compared to 10-20 FPU/ml in SmF.

The following cost estimation intends to illustrate the main elements of production costs in SmF and SSF processes. This comparison is based on a conventional stirred tank reactor (STR) for SmF and a Koji type SSF reactor as described above. A more detailed cost estimation was given elsewhere (Tengerdy & Szakacs 2000, Szakacs et al. 2001).

SmF: Assuming $\$160/m^3$ for an average fermentation cost in a STR that is used in the antibiotics industry and an average cellulase yield of 20 FPU/ml from SmF, the estimated production cost would be $\$8/10^6$ FPU.

SSF: Using \$150/MT from the Koji industries for an average fermentation cost in SSF and an average cellulase production yield of 50 FPU/g substrate, the estimated production cost would be $\$3/10^6$ FPU.

The actual numbers may vary, but the fermentation cost would always be significantly lower in SSF than in SmF. Major cellulase producers may have achieved higher yields and productivities than reported here, using new genetically modified fungi, but this is proprietary information that can not be considered in this review.

3.5. Process improvement

Considerable research and development is invested in improving cellulase production. The first avenue is improving the producing microorganisms as discussed in Section 2. The second avenue is improving the fermentation. Modeling both SmF and SSF fermentations promises advances in reactor design and process control. Modeling of cellulase production by SmF is well advanced but SSF models are still being developed. A better regulation of temperature and/or moisture coupled with optimal mass transfer would increase the attractiveness of SSF for cellulase production.

An interesting possibility for process improvement is mixed culturing of different fungi. This is particularly relevant for SSF, since SSF closely resembles the natural way of living for fungi, usually in commensal or

Table 2. Cellulase production using submerged fermentation (SmF)

Mode of fermentation	Microorganism	Substrate	Substrate concentration (g/L)	Cellulase yield		Cellulase productivity (FPU/hL)	Reference
				(FPU/mL)	(FPU/g DM)		
Batch (14 L)	*Trichoderma reesei* RUT C30	Cellulose	2% slurry	4.2	210	29	Hendy et al. 1984
1-stage continuous	*Trichoderma reesei* RUT C30	Cellulose	2% slurry	2.1	105	97	Hendy et al. 1984
2-stage continuous	*Trichoderma reesei* RUT C30	Cellulose	2% slurry	3.3	165	58	Hendy et al. 1984
Fed-batch (14 L)	*Trichoderma reesei* RUT C30	Cellulose	10% slurry	22.4	224	247	Hendy et al. 1984
Fed-batch (20 L)	*Trichoderma reesei* RUT C30	Pulp cellulose	2.3% slurry	57	246	427	Watson et al. 1984
Shake culture	*Trichoderma* cultures					158	Tsao et al. 2000
Shake culture	*Trichoderma reesei* RUT C30	Steam-pretreated willow	(15 g/L)	0.61			Reczey et al 1996
4-L fermenter	*Trichoderma reesei* RUT C30	Steam-pretreated willow	(18.2 g/L)	0.8		12.2	Reczey et al. 1996
Shake culture	*Trichoderma* species A-001	Filter paper		18 IU/ml			Gashe 1992
Bubble column	*Aspergillus niger* KKS	Rice-straw			84	9.7	Kim et al. 1997
Shake culture	*Trichoderma reesei* RUT C30	1% Avicel and 5% wheat bran		12.85 IU/ml			Yu et al. 1998
Stirred tank reactor	*Chaetomium globosum* 414	Oil palm empty fruit bunch fibre		2.5		20.8	Umikalsom et al. 1998
3.7-L fermentor	*Trichoderma reesei* RUT C30	Exploded wood	2% slurry	4.3	215/g S or 363/g cellulose		Shin et al. 2000
Fermentor	Po16 mutant of *Penicillium occitanis*	Paper pulp		23			Belghith et al.2001

Table 3. Cellulase production using solid substrate fermentation (SSF)

Reactor	Microorganism	Substrate	Cellulase yield (FPU/g DM)	Cellulase productivity (IU/hL)	Reference
Rotating drum	*Thermoascus aurantiacus*	Wheat straw	5.5		Kalogeris et al. 2003
Tray fermenter	*Trichoderma reesei* ZU-02	Corncob residue	158		Xia & Cen 1999
Deep trough fermenter with forced aeration	*Trichoderma reesei* ZU-02	Corncob residue	128		Xia & Cen 1999
Tray fermenter with static culture	*Penicillium decumbens* JUA 10	steam-exploded straw/bran (8/2)	10.8		Xu et al. 2002
Tray fermenter with periodically dynamic changes of air	*Penicillium decumbens* JUA 10 (8/2)	steam-exlploded straw/bran	20.4		Xu et al. 2002
Static tray culture	*Penicillium* system	*decumbens* JUA 10	10		Zhang et al. 2003
Solid-state bioreactor coupled with forced aeration and pressure oscillation	*Penicillium decumbens* JUA 10		15		Zhang et al. 2003
Solid-state bioprocessing	*Bacillus subtilis* CBTK 106	Banana fruit stalk waste	2.8		Krishna 1999
SSF in 1000 mL flasks	*Trichoderma* cultures			234	Tsao et al. 2000
SSF with pressure pulsation and repeated extraction	*Trichoderma* cultures			806	Tsao et al. 2000

symbiotic associations in mixed cultures. This natural coexistence may be approximated in SSF by mixed culturing of different fungi, e.g. a hypercellulolytic mutant and a host-specific "helper" fungus. Since the current hypercellulolytic mutants have been developed for SmF processes, in SSF they may benefit from the help of host-specific fungi, promoting better colonization, host penetration, and possibly metabolic enhancement from co-metabolites. For example the co-culturing of a *T. reesei* hypercellulolytic mutant and an *Aspergillus* spp. improved the cellulase/β-glucosidase ratio, and increased overall growth and cellulase production (Gutierrez-Correa & Tengerdy 1997). Mixed culturing offers the possibility to "design" a desired enzyme complex with an enzyme profile most appropriate for the hydrolysis of a given natural lignocellulolytic substrate.

4. ASSAYS

Cellulase is multienzyme complex including three groups of individual enzymes such as endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases. Assays are designed to detect overall hydrolytic activity on selected substrates, or to detect the activity of an individual enzyme component on selected substrates. The assays in use do not measure directly the amount of enzymes. The assays are thus relative and differ markedly in sensitivity and specificity, depending on the substrates used for assay, the conditions of the assay and the microbial cellulase system studied. Principal methods used for detecting enzyme activity include (1) reduction in viscosity of a solution of a cellulose derivative; (2) production of reducing sugar from a soluble cellulose derivative; (3) production of reducing sugar or loss of residue weight from solid cellulose; (4) dye release from a dyed cellulose (Mandels & Weber 1969, Bhat & Hazlewood 2001).

The most widely accepted and used assay for overall cellulase activity is the filter paper activity (FPA) assay (Ghose 1987). It measures the capacity of an enzyme preparation to hydrolyze Whatman # 1 filter paper stripe (1 x 6 cm, approx. 50 mg) into reducing sugars. In best commercial cellulase preparations 1 mg total protein represents 0.54-0.57 FPU activities (Nieves et al. 1998). It was assumed that 1 mg pure cellulase protein was equivalent with approx. 1 FPU activity.

For determination of endo-β-1,4-glucanase activity, soluble cellulose-derivatives such as carboxymethyl-cellulose (Ghose 1987) or hydroxyethyl-cellulose (Bailey & Nevalainen 1981) are used.

It is well known that the level of β-glucosidase in a cellulase enzyme preparation may effect the result of cellulase assays, in particular the assay of FPA (Sternberg 1976, Bailey 1981). Therefore, determination of β-glucosidase ("cellobiase") activity is also important. Substrates for β-glucosidase determinations are cellobiose (Ghose 1987) or p-nitrophenyl-β-D-glucoside (Herr et al. 1978).

Due to the relative nature of the FPA, it must be performed under strictly controlled standardized conditions. Fungal cellulases are secreted into the culture fluid, but some remain adsorbed on the substrate, therefore, standard dilution is needed for assay. For example SmF fermentations usually are conducted at 5-15% substrate concentration whereas SSF is performed at 20-40% dry matter (DM) content. With the increasing importance of comparing cellulase production by SmF and SSF, particular attention must be paid to equivalent dilution before assay (Urbanszki et al. 2000). In expressing FPA, convertibility between SmF and SSF assays must be observed. For SmF, FPA is expressed as FPU/ml, as a direct measure of cellulase activity in the culture fluid, but for SmF/SSF comparison FPU/g DM is more appropriate, as it does not depend on the slurry concentration in SmF. To express the efficiency of cellulase production, specific cellulase activities should be given FPU/g biomass and FPU/g protein. The first figure expresses the relationship between fungal growth and enzyme production, the second the efficiency of enzyme expression.

An automated FPA method has been developed recently using a Cyberlabs C400 robotics deck equipped with customized incubation, reagent storage, and plate-reading capabilities (Decker et al. 2003). Enzyme-linked immunosorbent assay (ELISA)-based methodology also has been developed for quantitative determination of cellulase concentration in fermentation liquids (Kolbe & Kubicek 1990, Harkki et al. 1991, Lynd & Zhang 2002, Zhang & Lynd 2003).

5. PURIFICATION

The degree of purification of the cellulase enzyme complex depends on the intended use of the enzymes. For fundamental research and specific applications, highly purified enzyme components may be needed,

but for most agro-biotechnical applications a crude enzyme or partially purified culture fluid may be not only adequate, but desirable, because for the hydrolysis of natural lignocellulolytic substrates the interaction of the entire cellulase complex and accessory enzymes are necessary.

The first step in industrial downstream processing of SmF is the separation of the culture fluid, where most of the enzymes are secreted, from the insoluble part of the fermentation beer such as fungal mycelium, non-utilized medium components, etc. This can be carried out by rotary vacuum filters and/ or by decanter centrifuges (Stanbury et al. 1995). The next step usually is ultrafiltration of the liquid (supernatant). During this procedure simultaneous concentration and partial purification can be achieved. Vacuum evaporation also may be used for concentration. If the industrial (commercial) enzymes are marketed as liquid concentrates (e.g., Celluclast 1.5 L), stabilization of liquid for a long-term storage should be arranged. This task might be fulfilled by addition of sugar alcohols such as sorbitol or sugars such as lactose in higher concentration. Preservatives to suppress bacterial and fungal contamination also must be applied. For laboratory purification of cellulases and cellulase components different methods can be used such as ultrafiltration, gel filtration, ion exchange chromatography, fast protein liquid chromatography (FPLC), capillary electrophoresis, etc. (Medve et al. 1998, Jorgensen et al. 2003a, b)

6. CHARACTERIZATION

6.1. The cellulase complex

Cellulases are modular proteins composed of a large catalytic domain connected to a small cellulose-binding domain (CBD) via an O-glycosylated linker peptide. Removal of the CBD reduces activity on crystalline cellulose whereas the catalytic domains remain fully active on soluble substrates (Koivula et al. 1998). All *T. reesei* CBDs belong to the family 1 carbohydrate-binding modules whereas the catalytic domains are found in many different glycosyl hydrolase families. Besides the active cellulase components some other proteins apparently showing no cellulase activity also take part in the breakdown of the cellulose molecule. Non-hydrolytic decomposition of cellulose by specific low molecular weight proteins resulting in short fiber (fibril) formation without any detectable reducing sugars have been reported by many authors (Halliwell 1966, Krull et al. 1988, Liu et al. 1996, Banka et al. 1998, Saloheimo et al. 2002, Wang et al. 2003).

6.2. Inducers

Cellulase is an inducible enzyme complex with cellulose, cellobiose and other oligosaccharides, lactose, sophorose, and other readily metabolizable carbohydrates acting as apparent inducers for virtually all cellulase synthesizing organisms (reviewed by Bisaria & Mishra 1989, Kubicek & Penttila 1998). It has been reported that cellulose or cellulose-rich materials are the best carbon sources for the production of high levels of cellulases by many microorganisms. Since the polysaccharides cannot enter the microbial cell, it is generally accepted that the low levels of cellulases constitutively produced by the microorganisms hydrolyze the corresponding polysaccharides to soluble sugars, which enter the microbial cell and either acts as, or get converted into, true inducers and stimulate the transcription of cellulase genes (Kubicek et al. 1993, Kubicek & Penttila 1998). It is believed that in *Trichoderma* conidial-bound cellobiohydrolases initiate the degradation of cellulose to cellobiose, which is taken up by the fungus and then induces enzyme synthesis and secretion (Kubicek et al. 1993). However, the actual inducing molecule remains unknown, possibly a β-glycoside.

Sophorose (β-1,2-glucobiose), a positional isomer of cellobiose, is reported to be an effective inducer of cellulases in *T. reesei* and other species of *Trichoderma*, even at low concentrations (Mandels et al. 1962,

Bhat & Hazlewood 2001). Interestingly, some inducers such as sophorose are required at extremely low concentrations, whereas others are needed at high levels (Philippidis 1994).

6.3. Inhibitors

Cellulases are often inhibited by the presence of high concentrations of their hydrolysis products. For example, most cellobiohydrolases are inhibited by cellobiose, even though cellobiose (>10 mM) stimulates the activity of cellobiohydrolase II from *P. pinophilum* (Wood & McCrae 1986). Similarly, endoglucanases are inhibited by cellobiose at or above 100 mM (Bhat et al. 1989). However, glucose (up to 100 mM) showed little effect on many CBHs and endoglucanases (Wood et al. 1988, Bhat et al. 1989). Presence of higher amounts of glucose causes catabolite repression in cellulase biosynthesis (Kubicek & Penttila 1998, Suto & Tomita 2001). Also, β-glucosidases are inhibited by glucose and other mono- and disaccharides, such as xylose, fructose, galactose, maltose, lactose and meliobiose. Nojirimycin and gluconolactone are also known to be potent inhibitors of β-glucosidases (Bhat & Hazlewood 2001). Product inhibition may be decreased by proper fermentation technology, e.g. fed batch or continuous fermentation.

7. CONCLUSIONS AND PERSPECTIVES

Cellulase is one of the most important industrial enzymes because it makes possible the rational use of the largest renewable resource on Earth, the lignocellulose. The shift of emphasis in cellulase application from the earlier textile industry and food industry applications to agro-biotechnological applications will govern efforts for improving cellulase production. The biofuel industry will require much cheaper, much higher activity enzymes with specific enzyme profiles suitable for a given substrate. The animal feed industry will need enzymes for a maximal feed digestibility. Integral crop management will need different microorganisms, including cellulase producers for biofertilizer, biostimulant, biopesticide functions.

The viability of these agro-biotechnological applications depends on the availability of cheap, target oriented cellulases. The high enzyme price is still one of the limiting factors in the cost efficient lignocellulose bioconversion processes, such as biofuel production and in using lignocellulose as the primary feedstock for organic chemical products. The widespread use of animal feed supplements, containing cell wall digesting enzymes requires a cheap multienzyme complex, containing cellulases, hemicellulases and pectinases.

To meet these requirements three approaches promise success.

1. The first one is the selection and improvement of the best cellulase producing microorganisms. The further genetic improvement of the currently most successful producers, the *Trichoderma* genus, will follow new directions. The emphasis will be on increased specific activity and improved enzyme profile and/or enhancing critical components of the cellulase enzyme complex. (Sheehan & Himmel 2001)

Good advances have been made in the selection and genetic improvement of fungal strains for cellulase production by SmF. The best strains now produce about 20 FPU/ml cellulase activity, but it appears there is a limit for overall yield improvement.

An important but in the past largely neglected aspect of selecting cellulase producers is the host specificity of the fungus, and the substrate specificity of the enzyme profile produced by a given fungus. In nature, host specific fungi inhabit and degrade their host, e.g. in wood decay. In commercial cellulase production

hypercellulolytic mutants of a single fungal species, *T. reesei,* are used predominantly, with no regard on what substrate they would be used, yet it is known that the enzyme profile of the complex produced by *T. reesei* Rut C30 depends on the substrate on which it is grown (see Section 3.).

The host specificity of fungi and the substrate specificity of enzyme production are even more important in SSF, which resembles closely the natural way of living of fungi. In SSF a much greater variety of substrates may be used for cellulase production, e.g. as feed supplement or bioconversion of agricultural and forestry residues, therefore, a much broader screening program is needed to select the most efficient host specific fungus for a given agricultural residue.

An additional aspect of fungal selection is their ability to cooperate with other fungi in mixed culturing for producing a desired optimal multienzyme complex, e.g. as animal feed supplement. Genetic improvement of selected wild fungi should consider these aspects of cellulase production, as well as the different physiological conditions in the selected mode of production, SmF or SSF. When designing a bioconversion process, such as biofuel production targeted for large volume agricultural and forestry residues, e.g. corn byproducts, grasses, wood residues, host specific fungi should be selected first, than genetically improved for maximal cellulase production. If SSF is the preferred mode of fermentation, the strain improvement should include criteria that are specific for SSF:

a. good growth on the particular substrate, including better colonization and penetration of the substrate, tolerance of high temperature, low moisture and high osmotic pressure

b. enhanced symbiotic association with other fungi in mixed culturing

2. The judicial choice of the technology is another approach for improving cellulase production. For highly specific applications in the pulp and paper industry, textile and food industry SmF will be the preferred choice of technology. For agro-biotechnological applications the choice between SmF and SSF should be carefully weighed. For biofuel production with new hypercellulolytic mutants SmF may be preferable, because of the refined process control, especially if on-site production can make it economical. Alternatively, SSF enzymes should be considered for better economy. For animal feed supplement production SSF enzymes would have a clear advantage in cost, direct applicability and diversity of the enzyme profile. The potential of using host specific fungi possibly in mixed cultures producing an optimal multienzyme complex for the hydrolysis (digestion) of a given substrate is a good argument for choosing SSF for animal feed supplement.

3. The use of transgenic plants is a future possibility for producing cellulases in food or feed ingredients making lignocellulose directly digestible. The possible lateral gene transfer cautions about widespread application of this technology.

In conclusion, the demand for the rational use of lignocellulose will drive microbial cellulase production in the near future. Genetic improvement of fungi and improved technology with greater emphasis on SSF will drive the price of cellulase down, acceptable for agro-biotechnological applications. Cellulase will have a major share of the word industrial enzyme production. Cellulase by transgenic plants looms in the future.

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Pectinases

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1. INTRODUCTION

Pectinases are a group of at least seven different enzymatic activities that contribute to the breakdown of pectin from a variety of plants. Pectin is a structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables. Pectin has a complex structure; the predominant structure consists of homopolymeric partially methylated poly-α-(1,4)-galacturonic acid. Sections of α-(1,2)-L-rhamnosyl- α-(1,4)-D-galacturonosyl containing branch-points with L-arabinose and D-galactose can be incorporated in the main polymeric chain. Pectin may also contain residues of D-glucuronic acid, D-apiose, D-xylose and L-fucose attached to poly- α-(1,4)-D-galacturonic acid sections (Pérez et al. 2003). As other enzymes, pectinases contained in natural ingredients have been used long time ago, e.g. the production of coffee and chocolate, where pectinases produced by wild microorganisms improved the fermentation step to remove grain mucilage. Pectinases are very useful to determine the structural characteristics of pectic substances and to prepare plant cell protoplasts for studies of genetic engineering (Shubakov & El'kina 2002). Due to its ability to degrade cell wall, pectinases have been used in juice and wine processing for the last 70 years. They are extensively used in food industry to increase juice yields, to accelerate juice clarification and to produce juice concentrates from grapes, berries, pears, apples, carrots, beets, green peppers and citrus fruits. Pectinases are also used to increase the colour of juices, promoting antioxidants formation and favour the extraction of colour, flavour components and fermentable sugars when added to grapes of musts during wine production. Removal of the inner wall of lotus seed, garlic, almond and peanut is also carried out by pectinases. Pectinase world-wide consumption is above 7 x 10^6 tons per year.

2. SOURCE AND TYPE OF PECTINASES

Although plants and microorganisms produce them, the most common source of commercial pectinases is the filamentous fungus *Aspergillus* sp. that produces a complex of pectinolytic enzymes, including the de-esterifying and chain-splitting enzymes. They are also obtained from tomatoes and oranges.

According to the reaction mechanisms, pectinases splitting homopolymeric partially methylated poly-α-(1,4) galacturonic acid (homogalacturonan) can be classified as: i) esterases, ii) hydrolytic depolymerases, and iii) eliminative depolymerases (Table 1). Pectinases degrading hairy regions (rhamnogalacturonan I) are not described in this chapter, but they have been described by Voragen et al. (2003) and include rhamnogalacturonan-hydrolases and lyases.

Table 1. Classification of pectinases from enzyme nomenclature

Name	EC number	Reaction type
Pectinesterase	3.1.1.11	Hydrolysis of carboxylic ester
Polygalacturonase	3.2.1.15	Hydrolysis of *O*-glycosyl bond
Galacturan 1,4-α-galacturonidase	3.2.1.67	Hydrolysis of *O*-glycosyl bond
Exopoly-α-galacturonosidase	3.2.1.82	Hydrolysis of *O*-glycosyl bond
Endo-pectatelyase	4.2.2.2	Elimination (C-O bond cleavage)
Exo-pectatelyase	4.2.2.9	Elimination (C-O bond cleavage)
Endo-Pectinlyase	4.2.2.10	Elimination (C-O bond cleavage)

2.1. Pectinesterase

Pectinesterase (E.C. 3.1.1.11) is an enzyme that catalyses the hydrolysis of methylated carboxylic ester groups in pectin into pectic acid and methanol. It is used in the treatment of certain foodstuffs and can be produced by a wide variety of plants and micro-organisms. Table 2 presents some of the micro-organisms producing pectinesterase.

Although pectin is the natural substrate of pectin esterase, methyl pectate (Versteeg 1979) and methylated oligogalacturonides (Shevchik et al. 1996) have also been used as substrates. Pectinesterase activity is stimulated by $(NH_4)_2SO_4$ (Versteeg 1979), Mg^{2+}, NaCl (Lim et al. 1983) and inhibited by Cu^{2+}, Hg^{2+} (Lim et al. 1983), D-galacturonate, polygalacturonate (Pitkänen. et al. 1992) and pectate (Markovic et al. 1985). Studies carried out on pectinesterase immobilisation revealed a 5-fold increase in K_m value after immobilisation (Markovic et al. 1985). The immobilised enzyme did not act on pectin with a high esterification degree and presents lower activity than the free enzyme. Table 3 presents the optimal values of pH and temperature of pectinesterases produced by different microorganisms. Plant pectinesterases release a methoxyl group adjacent to a free galacturonic acid and slide along the homogalacturonan to produce pectins with blocks of free carboxyl groups, whilst fungal pectinesterases saponify methyl esters in more or less random fashion (Ralet et al. 2002).

2.2. Polygalacturonase

Endo-polygalacturonases (E.C. 3.2.1.15) are important enzymes in fruit ripening and in fungal and bacterial attack on plants and are used in treatment of certain vegetables such as tubers, apples, etc. The enzymatic reaction involves random hydrolysis of *O*-glycosyl bonds in 1,4-α-D-galactosyluronic linkages in homogalacturonans. Most of the studies dealing with endo-polygalacturonase characteristics have been carried out with strains of *Aspergillus*, (particularly, *A. niger*), *Erwinia carotovora* and *Saccharomyces fragilis*. However, several plant pathogenic and saprophytic fungi and bacteria have also been studied. Table 4 lists some of the microorganisms reported as endo-polygalacturonase producers.

Homogalacturonan is the natural substrate of endo-polygalacturonases. However, a wide variety of oligogalacturonides are used as substrates, producing several products, depending on the nature of substrate. For example, molecules containing two or more monomers of galacturonate act as substrate for this enzyme. Soluble (homogalacturonan and methylated homogalacturonan) and insoluble (protopectin) substrates can be used as inducers for microbial endo-polygalacturonases. Hydrolysis of α-D-

Table 2. Microorganisms producing pectinesterases

Microorganism	References	Microorganism	References
Aspergillus carbonarius	Versteeg 1979	*Corynebacterium sp.*	Versteeg 1979
Aspergillus foetidus	Markovic & Machova 1985	*Erwinia chrysanthemi*	Heikinheimo et al. 1991, Pitkänen et al. 1992, Shevchik et al. 1996
Aspergillus japonicus	Versteeg 1979	*Fusarium oxysporum*	Versteeg 1979
Aspergillus kawachii	Contreras-Esquivel 2003	*Fusarium roseum*	Versteeg 1979
Aspergillus niger	Versteeg 1979		
	Lobarzewski et al. 1985	*Gibberella* sp	Versteeg 1979
Aspergillus oryzae	Ueda et al. 1982		
	Lim et al. 1983		
	Kitamoto et al. 1999	*Kluyveromyces fragilis*	Versteeg 1979
Aureobasidium pullulans	Manachini et al. 1988	*Penicillium chrysogenum*	Versteeg 1979
Botryosphaeria ribis	Versteeg 1979	*Pseudomonas solanacearum*	Versteeg 1979
Botrytis cinerea	Versteeg 1979	*Saccharomyces cerevisiae*	Gainvors et al. 1994
Chaetomium globosum	Versteeg 1979	*Torulopsis candida*	Versteeg 1979
Clostridium multifermentans	Versteeg 1979	*Trichoderma lignorum*	Versteeg 1979
Colletotrichum trifolii	Versteeg 1979	*Trichoderma reesei*	Markovic et al. 1985
Corticium rolfsii	Yoshihara et al. 1977	*Xanthomonas malvacearum*	Versteeg 1979

Table 3. Optimal values of pH and temperature for activity of microbial pectinesterases

Micro-organism	pH	Temperature (°C)	References
Aspergillus foetidus	4.8	52	Markovic et al. 1985
Aspergillus oryzae	8.2	50	Lim et al. 1983
Aureobasidium pullulans	4.5	50	Manachini et al. 1988
Erwinia chrysanthemi	7.5	40	Shevchik et al. 1996

galacturonosyl-(1,4)-*O*- α-D-galacturonate has been reported for the endo-polygalacturonases produced by *Botrytis cinerea* and *Aspergillus niger* (Deuel & Stutz 1958). Endo-polygalacturonase activity produced by *Rhizopus stolonifer* was stimulated by Co^{2+}, Fe^{3+}, Mg^{2+} (Manachini et al. 1987) and was inhibited by Ba^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+} (Manachini et al. 1987, Trescott & Tampion 1974). Other endo-polygalacturonases were also inhibited by Ag^{+}, $Ba^{2+}$and Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+} and Pb^{2+} (Sakai et al. 1982, 1984, Lim et al. 1980). Values of K_m of endo-polygalacturonases decrease as the number of monomers into the galacturonide oligomer increases. Endo-polygalacturonases produced by some bacteria, yeast and fungi has been in the range of $0.01mM<K_m<12.5mM$. The physiological significance of this variability is not yet understood.

Table 4. Microorganisms producing endo-polygalacturonases

Microorganism	References	Microorganism	References
Aspergillus foetidus	Deuel & Stutz 1958	*Galactomyces reesei*	Sakai & Yoshitake 1984
Aspergillus japonicus	Schejter & Marcus 1988 Ishii & Yokotsuka 1972	*Kluyveromyces marxianus*	Sakai et al. 1984
Agrobacterium vitis	Herlache et al. 1997	*Mucor pusillus*	Foda et al. 1984
Aspergillus aculeatus	Foda et al. 1984	*Neurospora crassa*	Deuel & Stutz 1958
Aspergillus alleaceus	Sreenath et al. 1986	*Penicillium capsulatum*	Gillespie & Coughlan 1989 Gillespie et al. 1990
Aspergillus aureus	Deuel & Stutz 1958	*Penicillium expansum*	Deuel & Stutz 1958
Aspergillus kawachii	Contreras-Esquivel 2003	*Penicillium frequentans*	De Fatima-Borin et al. 1996
Aspergillus niger	Cooke et al. 1976 Heinrichova & Rexová-Benková 1977 Heinrichova & Dzurova 1981 Raab 1992 van Santen et al. 1999 Parenicova et al. 2000	*Pseudomonas solanacearum*	Ofuya 1984
Aureobasidium pullulans	Sakai & Takoka 1985	*Rhizopus arrhizus*	Schejter & Marcus 1988
Bacillus mesentericus	Deuel & Stutz 1958	*Rhizopus stolonifer*	Manachini et al. 1987 Trescott & Tampion 1974
Bacillus sp.	Horikoshi 1972	*Rhizopus tritici*	Deuel & Stutz 1958
Botrytis cinerea	Deuel & Stutz 1958	*Saccharomyces cerevisiae*	Blanco et al. 1994 Blanco et al. 1998 Hirose et al. 1999 Gainvors et al. 2000
Clostridium felsineum	Deuel & Stutz 1958	*Saccharomyces fragilis*	Lim et al 1980
Corticium rolfsii	Tagawa and Kaji 1988	*Sclerotinia sclerotiorum*	Oliva et al. 1999 Fraissinet-Tachet et al. 1995
Erwinia carotovora	Ried & Collmer 1986 Saarilahti et al. 1990 Lei et al. 1992 Herlache et al. 1997 Palomaeki & Saarilahti 1997 Pickersgill et al. 1998 Pickersgill et al. 1999	*Trichoderma koningii*	Schejter & Marcus 1988 Fanelli et al. 1978 Tagawa & Kaji 1988
Erwinia chrysanthemi	Collmer et al. 1988 Ried & Collmer 1986	*Trichoderma reesei*	Markovic et al. 1985
Fusarium monoliforme	Deuel & Stutz 1958 Caprari et al. 1996	*Verticillium albo-atrum*	Schejter & Marcus 1988
Fusarium oxysporum	Strand et al. 1976 di Pietro & Roncero 1996	*Verticillium dahliae*	Deuel & Stutz 1958

Table 5. K_m values of some endopolygalacturonases.

Microorganism	Substrate	K_m (mM)	References
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	3.67	Heinrichova & Dzurova 1981
Galactomyces reessii	(1,4-α-D-galacturonaide)$_2$	3.98	Sakai & Yoshitake 1984
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	4.26	Sakai et al 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	12.5	Heinrichova & Rexova-Benkova 1977
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	1.82	Heinrichova & Dzurova 1981
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	2.2	Sakai et al 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	3.31	Heinrichova & Rexova-Benkova 1977
Galactomyces reessii	(1,4-α-D-galacturonaide)$_2$	0.71	Sakai & Yoshitake 1984
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	0.87	Sakai et at 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	0.89	Heinrichova & Dzurova 1981
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	1.85	Heinrichova & Rexova-Benkova 1977
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	0.7	Heinrichova & Dzurova 1981
Aspergillus niger	Pectate	0.01	Heinrichova & Rexona-Benkova 1977
Trichosporun penicillatum	Polygalacturonic acid	0.04	Sakai et al 1982
Aspergillus niger	Polygalacturonic acid	0.34	Heinrichova & Dzurova 1981

Depending on the type of microorganisms, endo-polygalacturonases have a wide range of optimal pH and temperature values (Table 6). Such variability may be useful for special conditions to be applied at industrial operations and may be the justification for looking at biodiversity as a source of special strains for such industrial applications.

Table 6. Optimal values of pH and temperature and molecular weight for endopolygalacturonases produced by some microorganisms

Microorganism	pH	Temperature (°C)	Molecular weight (kDa)	References
Aspergillus aculeatus	3.0	60		Foda et al. 1984
Aspergillus niger	5.0	40	46	Heinrichova & Rexová-Benková 1977
Clostridium thermosulfurogenes	5.5	75	320	Schink & Zeikus 1983
Erwinia carotovora	5.5	37		Lei et al. 1992 Saarilahti et al. 1990
Kluyveromyces marxianus	5.0	60	44	Hirose et al. 1999
Mucor pusillus	4.5	50		Foda et al. 1984
Penicillium capsulatum	4.7	52	51.6	Gillespie & Coughlan 1989 Gillespie et al. 1990
Saccharomyces cerevisiae	6.0	45	45	Hirose et al. 1999

Table 7. Optimal values of pH and temperature for activity of exo-polygalacturonases produced by some microorganisms

Microorganism	pH	Temperature (°C)	References
Fusarium oxysporum	5.6	60	Vásquez et al. 1993
Aspergillus niger	5.2	45	Heinrichova & Rexová-Benková 1976
Geotrichum lactis	5.0	40	Pardo et al. 1991 Pardo & Gacto 1992
Fusarium oxysporum	5.0	40	Martínez et al. 1991

Table 8. Bacteria and fungi producing endo-pectatelyase

Microorganism	References
Bacteria	
Bacillus subtilis	Nasser et al. 1990 Nasser et al. 1993 Sakamoto et al. 1994
Bacteroides thetaiotaomicron	McCarthy et al. 1985
Erwinia carotovora	Sugiura et al. 1984 Ried & Collmer 1986 Lei et al. 1987 Yoshida et al. 1991 McMillan et al. 1992 Heikinheimo et al. 1995
Pseudomonas solanacearum	Ofuya 1984
Streptomyces nitrosporeus	Sato & Kaji 1977,1980
Thermoanaerobacter italicus	Kozianowsky et al. 1997
Thermomonospora fusca	Stutzenberger 1987
Xanthomonas campestris	Nasuno & Starr 1967
Fungi	
Fusarium oxysporum	Huertas-González et al. 1999
Fusarium solani	Crawford & Kolattukudy 1987 Guo et al. 1995a,b Guo et al. 1996
Penicillium oxalicum	Ikotun 1984

2.3. Exo-polygalacturonases

Galacturan 1,4-α-galacturonidases (E.C. 3.2.1.67) are enzymes that degrade polygalacturonan by hydrolysis of the glycosidic bonds from the non-reducing ends yielding the corresponding 1,4- α-D-galacturonide and galacturonic acid. They are produced by a wide variety of plants and microorganisms. Strains of fungi such as *Aspergillus*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium* and *Trichoderma* have been used for biochemical characterisation of these enzymes. They are also produced by some

bacterial strains of the genus *Bacillus* and *Butyrivibrio*. Although the natural substrates of exo-polygalacturonases are pectic acid and pectin, these enzymes have a preference for oligogalacturonates and present activity with some arabinogalactans. The exo-polygalacturonase was inhibited by Ca^{2+} (Vásquez et al. 1993), glucose (Pardo et al. 1991, Pardo & Gacto 1992) and D-galactopyranuronic acid (Kester et al. 1996, 1999). When (1,4)- α-D-(galacturonide)$_n$ was used as substrate with n = 2 to 7, the exo-polygalacturonase activity of *Aspergillus tubingensis* showed preference for the high molecular weight molecules (K_m = 1.44 and 0.32 mM for n = 2 and n = 7, respectively). The optimum pH for activity of these enzymes is below 7 and has been 3.0 as minimum for polygalacturonase produced by the plant pathogen fungal *Colletotrichum capsici*. The specific activity of the exo-polygalacturonase produced by this fungus was near to 25 times higher than that produced by *Aspergillus niger* (Behere et al. 1993). Table 7 presents the optimal values of pH and temperature of exo-polygalacturonases produced by different microorganisms.

2.4. Exo-poly-α-galacturonosidases

Exo-polygalacturonosidases (E.C. 3.2.1.82) hydrolyse the pectic acid from the non-reducing end, releasing digalacturonates. These enzymes are produced by bacterial strains such as *Clostridium thermosaccharolyticum* (van Rijssel et al. 1993), *Erwinia carotovora* (Kegoya et al. 1984), *E. chrysanthemi* (Ried & Collmer, 1985), *Pseudomonas sp.* (Hatanaka & Imamura, 1974), *Ralstonia solanacearum* (Huang & Allen, 1997) and *Selenomonas ruminantum* (Heinrichova et al. 1989). Their natural substrate is pectin and has K_m values as high as 1159 mM measured with trigalacturonate for the enzyme produced by *Selenomonas ruminantum* (Heinrichova et al. 1989).

2.5. Endo-pectatelyases

Endo-pectatelyases (EC 4.2.2.2) do eliminative cleavages of pectate at the C-O bonds to give oligosaccharides with 4-deoxy- α-D-gluc-4-enuronosyl groups at their non-reducing ends resulting in formation of products with a double bond between C-4 and C-5. A wide variety of bacteria and some groups of plants and phytopathogenic fungi produce these enzymes. The enzyme production was constitutive in *Bacillus* sp. and *Fusarium solani*, but was induced by pectin or pectic acid in *E. anoideae* (Kamimiya et al. 1977). Its production by *B. subtilis* was repressed by glucose (Sakamoto et al. 1994). Table 8 presents some of the bacteria and fungi that produce this enzymatic activity.

Table 9. Molecular weight, pH and temperature optimal values for endo-pectatelyases produced by some microorganisms

Microorganism	K_m(mM)	pH	Temperature (°C)	References
Bacillus macerans	45000	9.0	60	Kamimiya et al. 1977
Bacillus subtilis	32000	8.0	60	Sakamoto et al. 1994
Colletotrichum gloesporioides	39000	8.9	35	Wattad et al. 1994
Erwinia anoideae	36000	9.0	35	Kamimiya et al. 1977
Streptomyces nitrosporeus	39000	9.3	5	Sato & Kaji 1977
Thermoanaerobacter itallicus	148000	9.0	80	Kozianowsky et al. 1997

Table 10. Microorganisms producingendo-pectinlyases

Micro-organism	References	Micro-organism	References
Bacteria		*Aspergillus niger*	Mayans et al. 1997 Kester & Visser 1994 Hanisch et al. 1978
Bacillus sp.	Jong-Chon et al. 1998	*Aspergillus oryzae*	Lim et al. 1983
Erwinia aroideae	Kamimiya et al. 1974	*Aspergillus sojae*	Ishii & Yokotsuka 1972
Erwinia carotovora	Itoh et al. 1982	*Colletotrichum lindemuthianum*	Wijesundera et al. 1984
Lachnospira multipara	Silley 1986	*Fusarium oxysporum*	Guevara et al. 1996
Pseudomonas fluorescens	Schlemmer et al. 1987	*Penicillium expansum*	Silva et al. 1993
Pseudomonas marginalis	Sone et al. 1988 Nikaidou et al. 1995	*Penicillium italicum*	Alana et al. 1991
Fungi		*Penicillium paxilli*	Szajer & Szajer 1982
Aspergillus japonicus	Ishii & Yokotsuka 1972 Dinnella et al. 1995	*Rhizoctonia solani*	Bugbee 1990

Oligogalacturonates, pectate, pectic acid, polypectate, polygalacturonic acid and pectin are used as substrates of endo-pectatelyase. The endo-pectatelyase activity required Ca^{2+} and was stimulated by Mg^{2+} (Sato & Kaji 1977, Brühlmann 1995), Mn^{2+} (Kobayashi et al. 1999, Miyazaki 1991, Guo et al. 1995a,b) and Sr^{2+} (Kobayashi et al. 1999, Brühlmann 1995); it was inhibited by several metal ions such as Mn^{2+}, Mg^{2+}, Cu^{2+} (Sugiura et al. 1984), Ba^{2+}, Cd^{2+} (Kamimiya et al. 1977, Sakamoto et al. 1994) and Ca^{2+} (Tardy et al. 1997). The K_m values of the enzyme produced by *Streptomyces nitrosporeus* for penta, tetra and trigalacturonic acids were 0.23, 0.19 and 1.5 mM, respectively (Sato & Kaji 1977). Endo-pectatelyase has maximal activities at alkaline pH values. Table 9 shows the molecular weight and the optimal pH and temperature values for endo-pectatelyase activities produced by different microorganisms.

2.6. Exo-pectatelyases

Exo-pectatelyases (E.C.4.2.2.9) do eliminative cleavages at the C-O bonds of 4-(4-deoxy- α-D-galact-4-enuronosyl)-D-galacturonate at the reducing end of unesterified pectin. These enzymes are produced by *Clostridium multifermentans* (Macmillan & Phaff 1966, Miller & Macmillan 1970, Macmillan & Vaughn 1964), *Erwinia chrysanthemi* (Shevchik et al. 1998, 1999a,b), *E. carotovora* (Ikeda et al. 1984, Kegoya et al. 1984), *Streptomyces nitrosporeus* (Sato & Kaji 1979) and *S. massasporeus* (Sato & Kaji 1980). Low molecular weight galacturonates are better substrate than polygalacturonate (Shevchik et al. 1999a). The exo-pectatelyase produced by *C. multifermentans* was inactive with pectin of high methoxyl content (Macmillan & Vaughn 1964). Production of exo-pectatelyase by *S. massasporeus* required Ca^{2+} and was stimulated by Mn^{2+} and Sr^{2+} (Sato & Kaji 1980). Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Sr^{2+} stimulated exo-pectatelyase from *C. multifermentans* (Macmillan & Phaff 1966). Mn^{+2} also stimulated exo-pectatelyase production by *E. chrysanthemi* (Shevchik et al. 1999a). Ba^{2+}, Cu^{2+}, Hg^{2+} and Mg^{2+} inhibited the enzyme activity (Hatanaka & Ozawa 1972, 1973, Ikeda et al. 1984). The exo-pectatelyase produced by *E.*

Table 11. Effect of metal ions on pectinlyase activity

Microorganism	Stimulated by	Inhibited by	References
Aspergillus japonicus	Ca^{2+}		Ishii & Yokotsuka 1975
Aspergillus niger	Ca^{2+},Na^{+}		Kester & Visser 1994
Aspergillus oryzae	Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}		Lim et al. 1983
Aspergillus sojae	Ca^{2+}, Co^{2+}, K^{+},	Hg^{2+} Mg^{2+}, Mn^{2+}, Na^{+}	Ishii & Yokotsuka1972
Bacillus sp.	Ca^{2+}	Co^{2+}, Fe^{2+},Hg^{2+}, Mn^{2+}, Zn^{2+}	Jong-Chon et al. 1998
Colletotrichum lindemuthianum	Ca^{2+}		Wijesundera et al. 1984
Erwinia areideae	Ba^{2+},Ca^{2+},Co^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}		Kamimiya et al. 1974
Fusarium oxysporum	Ca^{2+}		Guevara et al. 1996
Penicillium expansum		Ca^{2+}	Silva et al. 1993

Table 12. Characteristics of pectinlyases produced by different micro-organisms

Microorganism	pH	Temperature (°C)	Molecular weight (kDa)	References
Aspergillus japonicus	6.0	55	32	Ishii & Yokotsuka 1975
Aspergillus oryzae	8.5	5	34	Lim et al. 1983
Aspergillus sojae	7.0	-	32	Ishii & Yokotsuka 1972
Bacillus sp.	6.0	40	52	Jong-Chong et al. 1998
Colletotrichum lindemuthianum	9.3	-	27	Wijesundera et al. 1984
Erwinia aroideae	8.0	40	28	Kamimiya et al. 1974
Penicillium expansum	7.0	4	36.5	Silva et al. 1993
Pseudomonas marginalis	8.5	-	32	Sone et al. 1988

carotovora had K_m value as low as 1.3 μM with polygalacturonate (Ikeda et al. 1984). The pH and temperature optimal values for these enzymes are from 8.5 to 9.5 and 40 to 45°C, respectively. Molecular weights of 39 kDa and 54 kDa were found by gel filtration for the exo-pectatelyases produced by *S. nitrosporeus* and *S. massasporeus*, respectively (Sato & Kaji 1979, 1980).

2.7. Endo-pectinlyases

Endo-pectinlyases (E.C. 4.2.2.10) or pectinlyases give oligosaccharides with terminal 4-deoxy-6-methyl-α-D-galact-4-enuronosyl groups by eliminative cleavage of pectin. They are produced by some bacterial

and fungal strains (Table 10). Endo-pectinlyases produced by *A. japonicus* (Ishii & Yokotsuka 1975, Dinnella et al. 1995) and *A. niger* (Kester & Visser 1994, Mayans et al. 1997) have been studied and characterised.

Pectin, polygalacturonic acid, methoxylated pectic acid and polymethylpolygalacturonate methyl glycoside are used as substrate by the pectinlyases. The enzyme produced by *A. niger* was stimulated by Ca^{2+} and Na^{+} (Kester & Visser 1994) and was inhibited by acetate, formate, propionate, iso-butyrate and butyrate. The soluble enzyme had a pH optima of 5.2 (Hanisch et al. 1978) and a molecular weight around 35.5 kDa. Table 11 presents the stimulatory and inhibitory effect of some metal ions on pectinlyases produced by several microorganisms.

K_m values for the different pectinlyases have been generally estimated with citrus pectin as substrate, obtaining values of 3.2 mg/mL for *Pseudomonas fluorescens* (Schlemmer et al. 1987) and 15.0 mg/mL for *Penicillium italicum*. When pectin was used as substrate, K_m values of 1.36, 2.5 and 9.0 mg/mL were obtained with pectin lyases from *A. oryzae* (Lim et al. 1983), *Penicillium paxilli* (Szajer & Szajer 1982) and *P. expansum* (Silva et al. 1993), respectively. Optimal pH values from 5 to 9.8 have been reported. Table 12 presents some of the characteristics of pectinlyases produced by different microorganisms.

3. PRODUCTION OF PECTINASES

Pectinases are produced by a few microorganisms and strains of *A. niger*, *A. oryzae* and *A. aculeatus* are mainly used. Other species such as *Penicillium expansum* are also used for pectinase production at industrial scale. Pectinase production at large scale is carried out mainly by submerged culture (deep-tank process), although solid-state fermentation (SSF) is also used.

3.1 Submerged fermentation

Industrial fermentors for submerged fermentation (SmF) have volumes from 20 to several hundred cubic meters. Since most of the microorganisms used for pectinases production are aerobic, air must be supplied at rates from 0.1 to 2.0 vvm. Oxygen transfer from the gas phase to the liquid phase is enhanced by mechanical or airlift agitation of the culture medium and agitation helps to maintain homogeneous conditions of pH, temperature and dissolved oxygen in the broth. Pectinases production by a mutant strain of *A. niger* growing in a culture medium with sugar beet slices supplemented with malt extract and mineral salts was strongly influenced by the oxygen uptake rate (Zetelaki-Horvath & Vas 1981). While growth was stimulated at an oxygen uptake rate (OUR) of 100 mmol/Lh, pectinesterase, endo-polygalacturonase and pectinlyases production was stimulated at OUR values of 13, 49 and 60 mmol/Lh, respectively. However, the macerating activity, due to pectinesterase and endo-polygalacturonase activities, gave two maximal values at 12 and 14 mmol/Lh. On the other hand, some yeast strains such as *Kluyveromyces marxianus* produced nine isoenzymes forms of endo-polygalacturonases growing under anaerobic conditions with glucose as sole carbon source (Harsa et al. 1993). Most of the pectinases are inducible enzymes that require the presence of the inducer to be synthesised. Although pectin is the natural inducer for pectinases production, its elevated cost makes difficult its use at industrial level. A number of agricultural products containing pectin and other polysaccharides have been used for pectinase production. Depending on the raw material used as source of inducer and carbon, the culture

medium needs to be supplemented with minerals to improve the microbial growth and enzymes production. Ammonium sulphate is widely used as nitrogen source. Macro elements such as P, K, Mg are generally supplemented as KH_2PO_4 and $MgSO_4$ and micro elements such as Mo, Zn, Fe, Mn and Co are added as mineral salts at low concentrations (below 0.05%, w/w). A comparative study of polygalacturonases production by *A. niger* and *P. dierckxii* from different pectin sources showed that sugar beet pectin (at 10 g/L) was the most active inducer and ammonium sulphate the best source of nitrogen for polygalacturonase production by both the strains (Shubakov & El´kina 2002). The use of a mixture of sugar beet pulp and alkaline extracted sugar beet pulp instead of sugar beet pulp alone slightly increased the polygalacturonase production by *Trichoderma reesei* (Olsson et al. 2003).

As stated above although pectinases production by fungi has been mostly inducible, a constitutive exo-pectinase was produced by *Aspergillus* sp. CH-Y-1043 grown on glucose, sucrose, fructose, glycerol and galacturonic acid (Aguilar & Huitrón 1990). Pectinases production by *Aspergillus* sp. (Solís et al. 1990, Aguilar & Huitrón 1987) and *Neurospora crassa* (Polizeli et al. 1991) was induced by pectin and repressed by glucose and by the degradation products of pectin. In contrast, *Erwinia carotovora* (Tsumuyu, 1977) produced a pectatelyase that was induced by the breakdown products of pectic acid. The complexities of the regulatory mechanisms involved on pectinase production require permanent programs for the selection of catabolic resistant strains. However, technological aspects such as fed-batch cultures or solid- state fermentation can be used to minimise the catabolic repression by glucose or by the breakdown products of pectin (Solís-Pereira et al. 1993, Díaz-Godínez et al. 2001).

3.2. Solid-state fermentation

Solid-state fermentation (SSF) used for the production of polygalacturonases by *A. niger* with sugar cane bagasse as solid support showed that endo-polygalacturonase and exo-polygalacturonase productivities were 18.8 and 4.5 higher in SSF than in SmF (Solís-Pereira et al. 1993). Apparently, the regulatory phenomena such as induction-repression related to pectinases synthesis by *A. niger* are different in the two types of fermentation (Solís-Pereira et al. 1993). The use of sugar cane bagasse as a sole carbon source allowed higher production (6-fold) of pectinesterase and polygalacturonase by *A. niger* in SSF as compared with that obtained in submerged fermentation. Moreover, glucose addition improved pectinase production in SSF but it was decreased in submerged fermentation (Maldonado & Strasser 1998). Similar results were obtained with polyurethane (as inert support) and a culture medium containing pectin as carbon source, showing that protease production is lower in SSF than in SmF (Díaz-Godínez et al. 2001). However, Morita and Fujio (1999) compared specific polygalacturonase activities from *Rhizopus* sp. MKU 18 of a metal-ion-regulated liquid medium and a wheat bran solid medium. Their work suggested that some advantages can be found in producing polygalacturonase by metal-ion-regulated liquid medium. The use of washed sugar cane bagasse as support, impregnated with a defined culture medium showed that pectinases produced by SSF were more stable at pH and temperature than those produced by SmF (Acuña-Argüelles et al. 1995). Although pectinases production at low water activity (a_w) values was lower than that obtained at high a_w values, the specific activity increased up to 4.5 times in SSF (Acuña-Argüelles et al. 1994). Polygalacturonase production by thermophilic *Thermoascus aurantiacus* by SSF using a mixture of sugar cane bagasse and orange bagasse (1:1) at different

culture conditions (pH, substrate moisture and temperature) was highest at pH 5, substrate moisture 70% and 50°C (Martins et al. 2003).

4. PECTINASE ASSAYS

4.1. Pectinesterases

Pectinesterase activity can be estimated by automatic or manual titration of the H^+ produced by the reaction in a solution containing the enzyme and its substrate. To 10 mL of 0.5% (w/v) pectin in 0.1 M NaCl, 2 mL of the enzyme solution is added. The pH is adjusted to 4.5 with 0.1 M NaOH and the mixture is incubated for 65 min at 35 °C (Maldonado & Strasser de Saad 1998). Pectinesterase activity is measured by determining the carboxyl groups released by titration with 0.02 N NaOH. Pectinesterese activity from plant sources is usually determined at pH 7.5 (Rexová-Benková & Slezárik 1966), but fungal pectinesterases are assayed at pH 4.5. The activity is expressed as the number of milliequivalents of methyl ester groups cleaved by enzyme per min. Pectins with degrees of esterification above 65% have been used to determine pectinesterase activity. Pectinesterase activity can be determined in a pH-stat at pH 4.0 to 7.7 (Whitaker 1984). Free methanol can be also measured by gas chromatography and used to assay the enzyme activity (Versteeg 1979). Pectinesterase activity can also be estimated spectrophotometrically by monitoring the colour changes of the indicator dye added to the reaction mixture (Vilariño et al. 1993).

4.2. Hydrolytic depolymerases

Enzymatic activities such as endo- and exo-polygalacturonase and endo and exo-polymethylgalacturonases are usually assayed by the release of reducing groups or by the reduction of viscosity of a solution containing pectin (Solís et al. 1990), sodium pectate (Aguilar and Huitrón 1986) or polygalacturonic acid (Manachini et al. 1988) as substrate. The endo-PG activity of the filtrate is determined by adding 2 ml of filtrate to 8 ml of a 1% (w/v) apple pectin solution and measuring the viscosity in a viscometer. One endo-polygalacturonase activity unit (U) is defined as the quantity of enzyme, which caused a reduction in viscosity of 50% in 30 min of reaction under standard conditions (Dartora et al. 2002). To measure the release of reducing sugars, 0.3 mL of the enzymatic solution is added to a solution containing 1 mL of 0.9% of substrate and 0.7 mL of buffer; samples are incubated at 45°C for 30 min and the reducing groups determined by a colorimetric method (see below). One exo polygalacturonase activity unit is defined as the amount of enzyme that liberates 1 mmol of reducing group per minute (Solís-Pereira et al. 1993). The reaction mixture for the hydrolytic depolymerising enzymes contains from 0.2% (Miyairi et al. 1985) to 1.0% (Kumar and Lonsane 1988) of substrate and are carried out at temperatures from 30°C (Miyairi et al. 1985) to 50°C (Zetelaki 1976) and pH values from 3.8 (Zetelaki 1976) to 5.5 (Manachini et al. 1988). Different sources of pectin can be used as substrate for determination of these enzymatic activities. The Miller (1959) and Somogyi-Nelson (Somogyi 1952) reagents are used to measure released reducing groups. The exo-poly-α-D-galacturonosidase activity is assayed in a reaction mixture containing 0.5% D-galacturonan at pH 6.0 (Collmer et al. 1982). In order to inhibit eliminative activities, 2mM EDTA is added to the reaction mixture. The amount of released reducing groups is estimated by the arsenomolybdate method (Nelson 1944). The thiobarbituric acid assay (Ayers et al. 1966) is used to check if the pectolytic activity was due to transelimination action and/or to hydrolase activity. The occurrence of peaks at 510 and 550 nm is indicative of the presence of hydrolase and lyase activities respectively (Manachini et al. 1988).

4.3. Eliminative depolymerases

Pectinlyase and pectatelyase are assayed spectrophotometrically by monitoring the increase in absorbance at 235 nm of a solution containing from 0.25% to 1% of substrate. The reaction mixture containing 1mL of substrate and 0.5 mL of enzyme solutions is incubated at 30°C for 60 min. The reaction is stopped by adding 3.5 mL of 0.5 M HCl. One unit of eliminative depolymerase activity is defined as the amount of enzyme that releases 1 μmol of 4,5 unsaturated digalacturonic acid per minute. A molar extintion coefficient of 5550 M^{-1} cm^{-1} is used for this calculation (Manachini et al. 1988). The enzymatic reaction is carried out at pH values from 5.5 (Manachini et al. 1988) to 9.0 (Durrands & Cooper 1988) and temperatures from 30°C to 35°C. Since calcium is an activator of these enzymatic activities, 10^{-4} M $CaCl_2$ is added to the reaction mixture (Tsuyumu 1979). Before the spectrophotometrical determination, excess of the substrate and the enzyme can be precipitated, adding successively 0.6mL of a 9% solution of $ZnSO_4$, 9mL of H_2O and 0.6mL of a 0.5N solution of NaOH. The mixture is shaken vigorously and centrifuged for 15 min at 8000 rpm, then subjected to photometry at 235 nm. (Bravova et al. 1982). For pectatelyase determination, pectin with a low methoxyl content or polygalacturonate can be used as substrate, whilst high methylated pectin is used for pectinlyase determination. Molar extinction coefficients of 4600 M^{-1} cm^{-1} and 5500 M^{-1} cm^{-1} are used to calculate the pectatelyase and the pectinlyase activities, respectively. These enzymatic activities can be also colorimetrically assayed after reaction with thiobarobituric acid, being one unit of pectinlyase defined as the amount of enzyme which creates 1 nM of unsaturated product (4-dehydro-5-ketouronic acid) per min (Szajer & Szajer 1982).

5. PURIFICATION OF PECTINASES

Pectinases produced by different microorganisms have been purified (partially or to homogeneity). The first purification/concentration step involves precipitation with ammonium sulphate or with ethanol. Ultrafiltration is also employed to concentrate the enzymatic extract followed by different steps of gel filtration, affinity or ion exchange chromatography, which may produce a homogeneous preparation of the enzymes present in the extract.

An enzymatic preparation from *A. niger* containing five endo-polygalacturonases and one exo-polygalacturonase was purified by affinity chromatography, followed by chromatofocusing and gel permeation chromatography. The exo-polygalacturonases were purified to homogeneity and endo-polygalacturonase was partially purified (Kester & Visser, 1990). Three endo-polygalacturonases produced by *A. carbonarius* were isolated to apparent homogeneity by molecular sieve chromatography on Sephacryl S-200 followed by ion exchange chromatography on CM Sephadex and gel filtration on Sephadex G-50 (Devi & Rao, 1996). Endo-polygalacturonase I from *Stereum purpureum* was purified to homogeneity by ion exchange chromatography on CM-52 followed by gel filtration on Sephadex G-100 (Miyairi et al. 1985). An enzymatic extract produced by *Neurospora crassa* was precipitated by ethanol, followed by chromatography on Biogel P-60. The pooled fractions were applied to a DEAE-cellulose column and finally applied to a CM-cellulose column. Polygalacturonase was eluted as a single peak with a purification factor of 56.14. Pectatelyase and pectinlyase eluted in three fractions with purification factors from 5 to 21 (Polizeli et al. 1991). Cross flow filtration was used to concentrate the endo-polygalacturonase present in the filtrated culture broth of *Mucor flavus*. The dialysed concentrate was purified to homogeneity by two ion-exchange chromatography steps on CM-Sepharose CL-6B (Gadre et al. 2003). An exo-polygalacturonatelyase produced by *Bacillus sp.* was purified to homogeneity by salting-out with ammonium sulphate followed by gel filtration on Superose 12 HR10/39, hydrophobic

interaction chromatography on a Phenyl-Superose HR5/5 column and ion exchange chromatography on a Mono-Q- HR5/5 column. The final yield and purification factor were 20% and 45.4%, respectively (Singh et al. 1999). An aqueous two-phase system (polyethyleneglycol/potassium phosphate) was used to purify 4 different pectinolytic activities from a commercial enzyme preparation (Lima et al. 2002). The best purification factors were observed in the upper phase for the systems containing high molecular weight polyethyleneglycol without NaCl, obtaining a purification factor of 5.49, 16.28, 16.64 and 14.27 for exo-polygalacturonase, endo-polygalacturonase, pectinesterase and pectinlyase, respectively.

6. CONCLUSIONS

Microbial pectinases are complex group of polymer-splitting enzymes that breakdown homogalacturonan and rhamnogalacturonan pectin-regions. They are widely used as aid processing in food industries. However, the fields of new industrial and analytical applications are being extended in recent years making necessary to study more deeply into these enzymes. Although extensive studies have been carried out on the production of microbial pectinases under various conditions from different carbon sources employing different microbial species and fermentation systems, yet there is not yet a comprehensive set of models of pectinase regulation. Apparently much is yet to be understood on kinetic studies in fermentors for pectinase production in SSF as SSF could be potential tool for its commercial production with better economic feasibility. The regulatory phenomenon such as induction-repression or activation-inhibition could be different in submerged culture and SSF. Similary, it is necessary to carry out efforts to understand the importance of enzymes related to the degradation or modification of hairy regions and those enzymes considered as accessories.

7. PERSPECTIVES

From the description as above, it would be worth to peruse the production of pectinases related to the degradation of rhamnogalacturonan region and those considered as accessories. More work is needed on bioreactor kinetics to analyse the whole process: Since, high concentrations of carbon source inhibit enzyme synthesis in submerged fermentation, it could be worth to explore the production using fed batch fermentation with the corresponding optimisation studies. Continuous production process is yet another aspect to explore. Furthermore, the fact that pectinase production by SSF is not strongly repressed by carbon sources makes this field an attractive way to produce pectinases. It should also be stressed that literature on high cell density cultivation for pectinases production is not available. Further studies on design of level reactors for the production of pectinases can be attempted. An interesting opportunity is the production of pectinases by SSF of these enzymes using genetically modified strains suitable to SSF conditions.

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Lipases

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1. INTRODUCTION

The annual market for industrial enzymes has reached around US$1.6 billion in 2000 (Demain 2000). Twelve major suppliers and four hundred minor producers assure the world demand of enzymes. Europe alone produces around 60% of the total world supply of industrial enzymes which are mainly (at least 75%) hydrolytic including proteases, carbohydrases and lipases (Sharma et al. 2001).

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. Their physiological role is to hydrolyze triglycerides to diglycerides, monoglycerides, fatty acids and glycerol. Lipase producing bacteria were reported nearly one century ago by the microbiologist C. Eijkmann (Jaeger & Eggert 2002) but it is not until the 1950's that the first works on fungal lipases were reported (Ghosh et al. 1996). Lipases catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides. As hydrolases, they do not require cofactors. They usually exhibit good chemioselectivity, regioselectivity and enantioselectivity. Finally, lipases possess broad substrate specificity and can be found with optimum activities over a wide range of temperatures. These interesting properties make lipases the most versatile biocatalyst. Their applications are found in the detergent, food, leather, textile, oil and fat, cosmetic, paper and pharmaceutical industries (Kademi et al. 2004). Commercial use of lipases in various applications is a billion-dollar business (Jaeger & Eggert 2002, Pandey et al. 1999a). In the detergent industry, about 1000 tons of lipases are sold every year (Godfrey & West 1996). This chapter describes the production, purification and characterization of lipases from different microbial sources.

2. SOURCES

Lipases are widely distributed in animals, plants and microorganisms. Several lipases are currently commercialized but the majority are produced by fungi, yeasts and bacteria due to the facility to cultivate these microorganisms on a large scale. Pancreatic lipase, one of the exocrine enzymes of pancreatic juices, is obtained from human and pig pancreas. Among all lipolytic enzymes, pancreatic lipase is the best known and the most often investigated (Ferrer et al. 2001). This enzyme can be purchased from different suppliers. Among bacterial lipases, attention has usually been focused on lipases from the genus *Pseudomonas* which are especially interesting for biotechnology because they exhibit the most versatility, reactivity and stability in catalyzing reactions in a nonaqueous environment (Gao et al. 2000). Lipases from *Geotrichum candidum* and *Rhizopus* are attractive catalysts for lipid modification.

G. candidum lipase shows preference for fatty acids containing a *cis*-9 double bound (Burkert et al. 2003) while *Rhizopus* lipases belong to a group of lipases that are active only against esters of primary alcohols (Haas et al. 1999).

Thermostable lipases such as lipases from *Thermus aquaticus*, *T. flavus* and *T. thermophilus* are gaining much attention because working industrial processes at elevated temperatures offers numerous advantages such as a decrease in the risk of contamination, high mass transfer and an increase in solubility of lipid substrates. Table 1 presents some commercial lipases from different suppliers. Due to their high performance, some commercial lipases are associated to special applications such as the Palatase from *Rhizomucor miehei* (Novozymes) launched for the dairy industry. On the other hand, other lipases such as from *Candida rugosa* can be very efficient in different fields of applications (Benjamin & Pandey 1998).

3. PRODUCTION

The cost of microbial lipase production depends on the enzymatic yield which is defined by the cost of the amount of enzyme produced, the cost of downstream processing and the stability of the enzyme post-harvest (Kanwar et al. 2002). Lowering cost, increasing enzyme productivity and increasing enzyme stability all contribute to a more viable process. The increase of enzyme production can be achieved by selecting the best fermentation technique and optimization of culture conditions.

3.1 Fermentation techniques

3.1.1 Submerged fermentation

Submerged fermentation (SmF) is the most commonly used for lipase production. Lipase production in batch process is mostly growth-associated and occurs during the early exponential rate. High lipase yield is obtained when cell growth reaches stationary phase (Lin et al. 2001) where some essential substrates become limiting. Nakashima et al. (1989) developed a computer–assisted fed-batch culture for controlling the specific feed rate of meat extract into the culture medium to study the intracellular lipase production by *Rhizopus chinensis*. The productivity of the fed-batch culture of *Pseudomonas fluorescens* was eight times that of the batch method while the apparent yield was also increased by 1.6-fold using the controlled feeding of olive oil (Suzuki et al. 1988). The intracellular lipase activity of immobilized *R. chinensis* computer-assisted fed-batch culture was also increased 1.3-1.5 times compared to the one obtained in batch cultivation when the specific feed rate of meat extract was controlled (Nakashima et al. 1989).

Recently, the production of microbial lipases in organic solvent is gaining much attention. This technique offers many advantages such as higher solubility of the substrates, easy product recovery into the organic phase, possibility to isolate unwanted inhibitory substrates and/or products by using solvents-water two-phase systems and finally reaction equilibrium may be shifted towards synthesis by continuously removing the products (Hun et al. 2003).

3.1.2 Immobilization

In view of the advantages of whole cells immobilization technique, studies have been made on lipase production as well. Immobilized cells of *R. arrhizus* on polyurethane foam were viable and produced lipase (70-80 U/l) for 120 h period (Elibol & Ozer 2000a). Entrapment in agar, alginate and polyacrylamide were tested to enhance lipase production by *Ralstonia pickettii*. Polyacrylamide at 15% (p/v) was found to be the best matrix and lipase production reached 24.75 U/ml after 96 h with 2.0 g of cells/l of saline. Immobilized cells in polyacrylamide could be reused three times with only 10% loss at each consecutive use (Hemachander et

Table 1. Some commercially available lipases

Source	Trade name	Supplier
Bacteria		
Achromobacter sp.	Lipase ALC, Lipase ALG	Meito Sangyo Co.
Alcaligenes sp.	Lipase PLC, Lipase PLG, Lipase QLC, Lipase QLG	Meito Sangyo Co.
Burkholderia cepacia	Lipase SL	Meito Sangyo Co.
Chromobacterium viscosum	Lipase CV	Genzyme Calbiochem
Pseudomonas cepacia	Lipase PS "Amano"	Amano
Pseudomonas fluorescens	Lipase AK "Amano"	Amano
Pseudomonas stutzeri	Lipase TL	Meito Sangyo Co.
Thermus aquaticus		Fluka
Thermus flavus		Fluka
Thermus thermophilus		Fluka
Fungi		
Aspergillus niger	Lipase A "Amano" 6	Amano
	Lypolyve AN	Lyven
Penicillium camembertii	Lipase G "Amano" 50	Amano
Penicillium roquefortii	Lipomod™ 338P - L338P	Biocatalysts Ltd
Mucor javanicus	Lipase M "Amano" 10	Amano
Mucor miehei	Piccnate	Gist-Brocades
Rhizomucor miehei	**Palatase®***	Novozymes
Rhizopus arrhizus		Fluka
Rhizopus delemar		Fluka
Rhizopus oryzae	Lipase F-AP15	Amano
	Lipase L036P – L036P	Biocatalysts Ltd
	Lipase F-DS	Amano
Rhizopus oryzae	Lipomod™ 627P – L627P	Biocatalysts Ltd
	Lipopan® F	Novozymes
	NovoLime® (with protease)	Novozymes
	Greasex®, NovoCor® AD	Novozymes
Rhizopus niveus	Newlase F	Amano
	Lipozyme® TL IM	Novozymes
Rhizopus sp.	Lipase UL	Meito Sangyo Co.
Thermomyces lanuginosus	**Lipolase®, Lipolase® Ultra**	Novozymes
	Lipo Prime™, Lipex®	
Penicillium sp./*Aspergillus* sp.	Lipomod™ 621P - L621	Biocatalysts Ltd
fungal sources	Lipomod™ 187P - L187P	Biocatalysts Ltd

Source	Trade name	Supplier
Yeasts		
Candida antarctica	**Novozym® 435**	Novozymes
	Noopazyme®	Novozymes
Candida cylindracea	Lipomod™ 34P – L034P	Biocatalysts Ltd
	Lypolyve CC	Lyven
	Lipase MY	Meito Sangyo Co.
Candida lipolytica		Fluka
Candida rugosa	Lipase AY "Amano" 30	Amano
	Resinase®	Novozymes
	Lipase AYS "Amano"	Amano
	Lipase II	Genzyme
		Seikagaku, Calzyme
Candida utilis		Fluka
Mammalians		
Porcine pancreas	Lipomod™ 224P - L224P	Biocatalysts Ltd
	Lipase 30, Lipase–CE	SPL
		Seikagaku, Calzyme, Worthington
Vegetal		
Wheat germ		Sigma
Mixtures		
Candida cylindracea + porcine pancreas	Lipomod™ 29P – L029P	Biocatalysts Ltd

* Lipases in bold are recombinant

al. 2001). Polypropylene powders (Accurel MP 1000) were used as adsorbents for organic solution containing *n*-hexadecane and olive oil to immobilize *Acinetobacter radioresistens* cells (Liu & Tsai 2003). After successive optimization providing a maximal interfacial area, controlling the release of oleic acid and avoiding formation of protease, the lipase production reached 44.3 U/ml after 16 h.

3.1.3 Solid-state fermentation

Solid-state fermentation (SSF) offers many advantages over submerged fermentation (SmF) for the production of various enzymes, including lipases (Pandey 1992, Pandey et al. 1999b, 2000). High lipase productions were obtained by cultivation of *Rhizopus* sp. (Christen et al. 1995, Ul-Haq et al. 2002), *Aspergillus* sp. (Olama & El-Sabaeny 1993, Kamini et al. 1998, Mahadik et al. 2002) and *Penicillium* sp. (Riviera-Muñoz et al. 1991, Chen et al. 1999, Miranda et al. 1999). SSF was carried out using inert support (amberlite, a polymeric resin) which was supplemented with culture medium to study the lipase production by *R. delemar* (Christen et al. 1995). Recently, cheap agricultural by-products have been gaining a great interest as suitable substrates in SSF: rice bran (Rao et al. 1993a, 1993b, Bhushan et al. 1994), wheat bran (Mahadik et al.

2002), almond meal (Ul-Haq et al. 2002), babassu oil cake (Chen et al. 1999), gingelly oil cake (Kamini et al. 1998), coconut oil cake (Benjamin & Pandey 1997) and olive oil cake (Cordova et al. 1998, Kademi et al. 2003a). Comparing the potential of nylon sponge as inert support to food and agroindustrial wastes (Barley bran and triturated nut) for lipase production by *Yarrowia lipolytica*, Dominguez et al. (2003) found that lipase activities obtained on wastes were 5-fold higher than those on nylon sponge. SSF represents, therefore, a good technology for recycling agroindustrial wastes and reducing lipase production cost. However, despite all the advantages of SSF over SmF, the use of this technique for the production of lipases is still far to be applied on industrial scale (Ferrer et al. 2001).

3.1.4 Recombinant DNA technology

Recombinant DNA technology represents a very attractive technology that can be used to increase lipase production mainly in the case of isoenzymes whose purification leads to very low yields. This technology can allow up to 40% decrease in the cost of raw material, water, steam and electricity compared to the cost of native enzyme production (Anonymous, Novozymes web site: www.novozymes.com). Early successes in the production of heterologous proteins were achieved using *Escherichia coli* as host (Itakura et al. 1977) and various kinds of proteins were expressed in *E. coli*. However, expression of eukaryotic proteins in *E. coli* became very difficult due to formation of inclusion bodies, protein misfolding and safety issues. Other expression systems have been developed among yeasts, fungi, plants and animals. Main microbial expression systems are *A. oryzae*, *Saccharomyces cerevisiae* and *Pichia pastoris*. Production of heterologous microbial lipases by yeasts was discussed recently by Kademi et al. (2003b). The first lipase produced by recombinant DNA technology was Lipolase® introduced in the market by Novozymes in 1988. Originating from *Thermomyces lanuginosus*, formerly *Humicola lanuginosa*, this lipase was expressed in *A oryzae*. The growing number of recombinant lipases is attributed to the recent progress in molecular technologies (cloning and sequencing). Around 12 recombinant lipases are now commercialised by Novozymes.

3.2 Culture conditions

Lipases are enzymes of secondary metabolism with the function to initiate the metabolism of lipids when they become available in the extracellular environment. Lipids, insoluble in water, must then be hydrolyzed to more polar compounds, which are furthermore absorbed as nutrients by microbial cells. The production of lipases, which are mostly extracellular, is therefore often regulated in response to the presence of lipids in growth medium (Haas et al. 1999). Microbial lipase fermentations are affected by different factors such as medium composition (carbon and nitrogen sources), temperature, pH, aeration, agitation, size and age of inoculum. Each factor can have various effects on lipase production depending on culture conditions and microorganism under examination (Haas & Bailey 1993).

Lipases are mostly induced in presence of fats or oil in the culture medium. On the contrary, *Pseudomonas* sp. lipase was produced constitutively and presence of organic, natural oil/fats or fatty acid esters had no significant effect on the production (Gao et al. 2000). *Mucor* sp. lipase was also produced constitutively in absence of lipids in batch culture, but the regulatory system of lipase synthesis seemed to be stimulated in presence of oil palm on solid medium (Abbas et al. 2002). Triglycerides such as tributyrin (5 g/l) were used to induce the lipase production by *Kluyveromyces marxianus* (Deive et al. 2003). Lipase C (acting on *sn*-2 position of the triglyceride) was produced when *Geotrichum* sp. FO401B was grown in a medium containing tributyrin while lipase A acting on *sn*-1,3 position was not. Tributyrin increased the

production of extracellular and cell-bound enzyme (Ota et al. 2000). *Cryptococcus* sp. S-2 lipase activities were influenced by the presence of triolein (Kamini et al. 2000). Olive oil is the most used lipid substrate to induce lipase production by bacteria (Rathi et al. 2002), yeasts (Corzo & Revah 1999, Muralidhar et al. 2001) and fungi (Omar et al. 1987, Gulati et al. 2000, Lima et al. 2003). The production of alkaline lipase by *Acinetobacter radioresistens* was stimulated when a combination of olive oil and *n*-hexadecane was added to the culture medium (Liu & Tsai 2003). The best volumetric ratio *n*-hexadecane:olive oil was 7 for a maximum lipase activity of 21.86 U/ml at 24 h. Using GYP media, Sarkar et al. (1998) showed that olive oil at a concentration of 7% (v/v) resulted in an increase in lipase production by *Pseudomonas* sp., but at higher concentration the activity ceased drastically. The inhibition of lipase synthesis at higher olive oil concentrations could be due to poorer oxygen transfer into the medium which can alter fungal metabolism and consequently the production of lipases (Elibol & Ozer 2000b).

In some conditions, fatty acids also stimulated the lipase production as reported for *A. calcoaceticus* BD413 (Mahler et al. 2000), *A. radioresistens* (Liu & Tsai 2003), *C. rugosa* (Dalmau et al. 2000), *R. oligosporus* (Nahas 1988) and *G. candidum* (Shimada et al. 1992). However, the presence of short chain fatty acids inhibited the lipase production by *Pseudomonas* sp. (Gao et al. 2000).

Other oils from vegetable or animal sources such as corn oil (Corzo & Revah 1999, Ozer & Elibol 2000, Lima et al. 2003), sesame oil (Maia et al. 2001), rape oil (Hiol et al. 1999), castor oil (Omar et al. 1987), whale oil (Omar et al. 1987) and tung oils (Omar et al. 1987) are less expensive than olive oil and also have positive effect on enzyme production and in some cases higher lipase production than on olive oil can be obtained (Maia et al. 2001). These oils can be used to replace olive oil and consequently can help to reduce production cost.

Tween 80 was showed in several reports to be an effective substrate for lipase production (Bhatnagar & Johri 1988, Muralidhar et al. 2001, Gopinath et al. 2003). *Yarrowia lipolytica* lipase was enhanced with the presence of Tween 80 between 0.5 and 2 g/l in the culture medium (Corzo & Revah 1999). Addition of Tween 80 to the culture medium gave similar extracellular enzyme activities and lower cell-bound lipase of *C. rugosa* compared to media containing other lipidic compounds. This result indicated that enzyme biosynthesis was followed by an almost complete secretion (Dalmau et al. 2000). It seemed that Tween 80, in addition to its induction effect on lipase biosynthesis, increased cell permeability, and consequently cell-bound lipase decreased significantly.

Use of fats or oils to induce lipase production also has some disadvantages such as limitation of nutrient and energy availability due to low solubility of carbon source, difficulties in maintaining lipid in an emulsified state in culture, and the fact that lipid causes turbidity in the growth medium, which hinders optical or spectral assessment of the organism's growth (Haas et al. 1999). Haas & Bailey (1993) suggested that glycerol, a highly soluble final product of triglyceride hydrolysis, might be the real inducer molecule for lipase production. In glycerol medium, the maximum lipase level was achieved much earlier during growth and the enzyme activity remains stable at its peak value for a considerable period of time (compared to glucose and olive oil). The use of glycerol as main carbon source rather than glucose or lipid supported mycelial growth and lipase production of *R. oryzae* (formerly *Rhizopus delemar*) (Haas et al. 1999).

Gum Arabic used to emulsify lipid substrates can enhance enzyme production by improving the availability of the substrates (Schmidt-Dannert et al. 1994, Gulati et al. 1999, Mahler et al. 2000). It is believed that Gum Arabic helps in releasing the enzyme bound to the membrane into the medium (Schmidt-Dannert et al. 1994).

Various sugars, including fructose (Kamini et al. 2000), lactose (Kamini et al. 2000), maltose (Fadiloglu & Erkmen 2002), galactose (Gopinath et al. 2003) and glucose (Elibol & Ozer 2000a), can have a stimulating effect on enzymatic production. However, in some cases, high levels of glucose cause catabolite repression on enzyme production (Elibol & Ozer 2000a).

Various nitrogen sources were tested in the study of lipase production. A minimal culture medium can be used with an organic nitrogen source such as urea (Deive et al. 2003), corn steep liquor (Gao et al. 2000, Burkert et al. 2003), or an inorganic nitrogen source such as sodium nitrate (Kanwar et al. 2002, Gopinath et al. 2003), ammonium sulphate and ammonium nitrate. Also, a rich medium containing peptone (Fadiloglu & Erkmen 2002, Benjamin & Pandey 1997), tryptone or yeast extract can be used. Organic nitrogen sources can be costly and a burden in downstream processing.

Aeration has a highly variable effect on the production of lipases. Some microorganisms produce great amounts of lipases when the culture medium is gently aerated, while significant aeration is required for other microorganisms. Oxygen concentration is often used as a criterion for determining whether fermentation is oxygen-dependent or oxygen-independent. However, oxygen transfer rate (OTR) appears to be the critical factor in the production of lipases (Chen et al. 1999, Elibol & Ozer 2000b). Improving OTR either by aeration, agitation or perfluorocarbon (PFC) inclusion resulted in an increase in lipase production of *Rhizopus arrhizus* (Elibol & Ozer 2000b).

Ogino et al. (1999) reported the growth of *P. aeruginosa* in presence of organic solvents. The strain produced a solvent-stable enzyme which is also active in presence of various solvents. The strain grew in media containing solvent of log *P* of 7.6 and 6.6, *n*-tetradecane and *n*-dodecane respectively as well as in absence of solvent. *Bacillus sphaericus* 205y was also able to grow in presence of solvents up to 75% and also could produce a solvent-stable lipase (Hun et al. 2003). The enzyme activity was increased by the presence of *n*-hexane and *p*-xylene for the lipase of *Pseudomonas* sp. G6 (Kanwar et al. 2002). When *n*-hexadecane was used as sole carbon source in a mineral medium, growth and lipase production were stimulated.

Advantages to work at higher temperatures have already been mentioned (higher diffusion rates, increasing mass transfer effects, increased solubility of lipid substrates in water and reduced risk of contamination) (Abdel-Fattah 2002). Contamination would end up with less biomass, unwanted end-product and enzyme degradation by proteolysis (Johri et al. 1985, Gabelman 1994).

3.3 Modelling

Mathematical models have been developed and applied to study metabolite production by microorganisms. The aim of these models is to understand the mechanisms of lipase production and improve its productivity. Ferrer et al. (2001) reviewed different models used for lipase production by *C. rugosa*. From these models, it was observed that production of high levels of lipase by *C. rugosa* was associated with consumption of fatty acids released by hydrolysis of triglycerides. Montesinos et al. (1995) developed a simple structured model for lipase production by *C. rugosa* in batch culture. In this model, lipase production was induced by oleic acid present in extracellular medium. Oleic acid was transported into the cell, where it was consumed,

transformed and stored. Lipase was then excreted into the medium and distributed between available oil-water interface and aqueous phase. Cell growth was modulated by intracellular substrate concentration.

4. ASSAY

Numerous methods for detection or quantification of lipolytic activity have been developed and improved. Agar media methods are mainly applied in the detection of microbial lipases and are classified into two categories:

i) Methods where lipolysis results in a change of substrate appearance: clear zones around colony are observed after hydrolysis of tributyrin while opaque halos of calcium oleate appear in the case of Tween 80 hydrolysis (Sierra 1957).

ii) Methods where lipase activity is detected with indicator dyes: release of FFAs during lipolysis decreases the pH of the medium around the lipolytic colony. This pH decrease can be detected by a color change of an indicator dye present in the medium such as Victoria blue B, Spirit blue, Nile blue sulphate and night blue (Shelley et al. 1987). Fluorogenic reagents can also be used to detect lipolytic activity on solid media. For instance, substrate hydrolysis can be visualised with rhodamine B dye which formed an orange fluorescent halo around lipolytic colony visible under UV light (Kouker & Jaeger 1987).

Usually these methods are applied for qualitative detection of lipolytic activity. However, some authors have demonstrated a good correlation between lipase concentration and the diameter of lipolysis zone allowing a quantification of lipase activities (Kouker & Jaeger 1987, Samad et al. 1989, Lima et al. 1991). Raw materials such as natural fats (animal fats and vegetable oils), synthetic triglycerides (tributyrin and triolein) or other synthetic esters (Tween 20, Tween 80) are employed as substrates for the detection of microbial lipases.

In practice, lipase producing bacteria can be detected using the protocol reported by Kouker & Jaeger (1987) with little modifications. Rhodamine B (1 mg/ml) is dissolved in distillated water and sterilized by filtration. Olive oil is sterilized in an oven (180°C, 1 h). The growth medium contains per liter nutrient broth 8 g, NaCl 4 g and agar 10 g. The pH is adjusted to 7.0. After autoclaving, the medium is cooled to 60°C. Olive oil (3% (v/v)) is added and emulsified by a vigourous mixing in a homogenizer for 1-2 minute, incubated at 60°C for 10 minutes to reduce foaming. Rhodamine B solution (1% (v/v)) is added with vigorous stirring and the solution is poured into Petri dishes. Bacterial cultures can be streak-plated and incubated at the respective optimal growth temperature of bacteria during 24-48 h and then exposed to UV light (350 nm) to monitor lipase production. To quantify lipase activity, 3 mm diameter holes are punched into the agar and 10 µl of lipase solutions are used to assess the activity. The assay can be adapted to detect lipase producing fungi or yeasts by using their respective culture medium.

4.1 Methods for assaying lipase activity

Various quantitative methods have been developed to measure lipase activity and most of them are based on the principle of product appearance such as titrimetry, photometry, conductimetry, chromatography and IR spectroscopy methods. Turbidimetric method is based on the clearing of substrate emulsion during hydrolysis. The interfacial tensiometric method measures changes in oil/water interface properties. These methods are presented in Table 2.

Table 2. Assays for the quantification of lipase activity

Assay	Principle	References
Titrimetry		
pH-stat	Titration by NaOH of FFAs released with time during hydrolysis	Desnuelle et al. (1955)
Spectroscopy		
Photometry	Conversion of FFAs to copper soaps and reading of absorbance	Lowry & Tinsley (1976)
	Measure of appearance of coloured hydrolytic products such as p-*nitrophenol (synthetic substrates)*	Winkler & Stuckman (1979) Pencreac'h & Baratti (1996)
Fluorimetry	Measure of the increase with time of the fluorescence intensity of the product at a given wavelength	Berto et al. (1997)
Infra Red	Monitoring the lipolysis by recording the Fournier-transform IR spectrum of the reaction mixture	Walde & Luisi (1989)
Chromatography (HPLC/GC/TLC)	Analysis and quantification of the hydrolytic products (FFAs, mono- and diacylglycerols)	Kates (1986)
Radioactivity	Use of Triacylglycerols containing radio-labelled acyl chains	Briquet-Laugier et al. (1999)
Interfacial tensiometry	Monitoring the decrease in surface pressure due to reduction of surface density during hydrolysis of monomolecular substrate film	Verger (1980)
Turbidimetry	Monitoring the rate of clearing the substrate emulsion	Arzoglou et al. (1989)
Conductimetry	Measure of conductance change in the reaction mixture during lipolysis	Ballot et al. (1984)
Immuno-chemistry	Use of antibodies recognizing an epitope on a lipase	Grenner et al. (1982)
Microscopy	*In situ* Detection of FFAs by electron microscopy	Nagata (1974)

4.2.1 pH-stat method

The pH-stat method is the most popular quantitative assay. This method offers adequate sensitivity in many applications and is generally used as reference lipase assay with olive oil or triolein as substrates. FFAs released with time by lipolysis of natural or synthetic triglycerides are neutralized by addition of titrating NaOH to maintain the pH at a constant end point value. The sensitivity of the pH-stat method is within one μmole of released fatty acid per min. (Beisson et al. 2000). Substrates are prepared as emulsion in buffer solution by sonication or by using a blender. This emulsion is usually stabilized by the presence of gum Arabic. Usually tributyrin is used as lipase substrate instead of olive oil or triolein. However, the former is not specific for lipases and can be hydrolysed also by esterases. It is generally believed that the positive effect of calcium ions in lipase assays is due to the water insolubility of long-chain fatty acids calcium soaps. The micro-precipitation phenomenon drives the chemical equilibrium by virtue of the mass action law (Beisson et al. 2000).

The pH-stat method has the disadvantage of restricted working range of pH values because the detection of protons released during the hydrolytic reaction requires a partial ionisation of released fatty acids. The pH end point values in the reaction medium must therefore be roughly equal to, or preferably higher than apparent pk_a value of released fatty acid. It was reported that apparent pk_a values of oleic acid could be as high as 7.5 under assay conditions (Benzonana & Desnuelle 1968). The pH-stat method is also time-consuming. During purification experiments, photometric assays (mainly colorimetric assay) which are time-saving, reproducible, easy-to-handle and more sensitive are routinely used (Vorderwulbecke et al. 1992).

The pH-Stat equipment is composed of a pH-meter, an autotitrator, an autoburette, a recorder and a magnetic stirrer. Triolein solution (1 g) prepared in 30 ml of Triton X-100 and 200 ml aqueous solution of NaCl (0.9% (w/v)) is separately heated to 55°C. NaCl solution is added to the triolein solution under stirring by portions of 50 ml. The final assay solution (230 ml) is cooled to room temperature before pH adjustment to 7.5 with 1.0M NaOH.

Twenty five ml of the assay solution is heated to 30°C and put in the thermostated vessel. The pH is adjusted and the reaction is started by injection of lipase sample. Liberated fatty acids are titrated by continuous addition of 10 mM NaOH. One unit of enzyme liberates 1 μmol of fatty acid min^{-1} which is equivalent to 0.1 ml of the consumed NaOH (Vorderwülbecke et al. 1992).

5. PURIFICATION

The importance of lipases has been widely recognized in numerous fields of applications (Houde et al. 2004). Although for most of these applications, a high degree of purity is not required, in some instances, when purified biocatalyst is indeed needed, industry looks for large quantities of highly stable and active enzyme preparations purified with a minimum number of steps that will keep the price of the process down. The source and nature of enzyme, the eventual application and degree of purity needed contribute to determine the purification schemes. This subject has been recently and extensively reviewed (Palekar et al. 2000, Saxena et al. 2003). Enzyme purity is evaluated after each step with the measurement of overall activity (U) and specific activity (U/mg). The purification efficiency is estimated by the determination of the enzyme activity yield and the purification factor. As a general rule, four steps of chromatography are needed to reach high purity for lipase (Palekar et al. 2000). In general, purification of lipases from animals or higher plants needs more steps than lipase from microbial sources to obtain a similar degree of purity. However, they seem to be more stable during the entire process.

Pre-purification steps include separation of microorganism from the medium supernatant by either centrifugation (Schmidt-Dannert et al. 1994) or filtration or both (Kamini et al. 2000, Abdou 2003). Enzymes are concentrated by precipitation from a large supernatant volume with ammonium sulphate (Patkar et al. 1993, Talon et al. 1995, Abbas et al. 2002), acetone, ethanol (Rua et al. 1993), acids or by ultrafiltration (Snellman et al. 2002). These steps involve the treatment of large supernatant volumes and precipitation techniques usually end up with higher enzyme yields (Aires-Barros et al. 1994).

Purification process goes through one or combination steps involving mainly chromatography procedures. The choice of chromatography is governed by enzyme characteristics (pI, molecular size, etc). The step sequence is oriented by practical considerations such as sample volume, protein concentration, viscosity of the sample, degree of purity needed, presence of nucleic acid or proteolytic enzymes and the ease to wash the adsorbents from contaminants (Palekar et al. 2000). Ion exchange chromatography using mostly weak anion exchanger DEAE-sepharose was applied to purifiy lipase from *Staphylococcus warneri* (Talon et al. 1995) and *C. antartica* (Patkar et al. 1993), DEAE-Sephacel for *C. rugosa* (formerly *C. cylindracea*) (Rua et al. 1993), DEAE-Sephadex A-50 for lipase from *G. candidum* (Gopinath et al. 2003), CM-Cellulose (weak cation) followed by DEAE cellulose for *Serratia marcescens* (Abdou 2003), and strong anion exchanger such as Mono Q for *Acinetobacter* sp. RAG-1 lipase (Snellman et al. 2002), Q-Sepharose for lipase from *B. thermocatenulatus* DSM 730 (Schmidt-Dannert 1994) and for *M. hiemalis f. himalis* lipase (Hiol et al. 1999). Cation exchangers have also been reported for the purification of lipase from *Cryptococcus* sp. S-2 on SP-5PW (Kamini et al. 2000), on CM-Cellulose for lipase from *Serratia marcescens* (Abdou 2003), on CM-Sepharose for *C. antartica* lipase (Patkar et al. 1993), on SP-Sepharose for *R. oryzae* lipase (Hiol et al. 2000) and on CM-Sephadex for lipase from *R. delamar* (Haas et al. 1999) and *Mucor* sp. (Abbas et al. 2002).

Hydrophobic interaction chromatography (HIC) is often considered since very high levels of purification can be achieved with this step. Lipases are known to possess hydrophobic patches on their surface that result in strong hydrophobic bonds between enzyme and hydrophobic substrates or matrices (Gabelman 1994). In equilibrating buffer, water molecule availability is decreased by addition of salting-out salts that results in an increased surface tension and promotes ligand and protein interaction. Elution is obtained by decreasing salt concentration in buffer (Palekar et al. 2000). HIC separates proteins on the basis of varying strength of the hydrophobic bonds between protein and the gel matrix. HIC has been used for the purification of lipases from *Acinetobacter* sp. RAG-1 (Snellman et al. 2002), *Saphylococcus warneri* (Talon et al. 1995), *C. rugosa* (Ferrer et al. 2001), *Candida antartica* (Patkar et al. 1993) and *Rhizomucor miehei* (Wu et al. 1996).

Gel filtration is widely used for lipase purification even if it is known to have a low capacity (Saxena et al. 2003). Gel filtration has been used for the lipases from *Bacillus* sp. THL 027 (Dharmsthiti & Luchai 1999), *Serratia marcescens* (Abdou 2003), *G. candidum* (Gopinath et al. 2003), *M. hiemalis f. hiemalis* (Hiol et al. 1999), *Mucor* sp. (Abbas et al. 2002) and *R. oryzae* (Hiol et al. 2000). Using aqueous two-phase system, Gulati et al. (2000) purified 11-fold the *A. terreus* lipase without any loss in activity.

6. CHARACTERIZATION

Among the wide variety of sources, lipases exhibit different characteristics in terms of a) optimal temperature and pH and their range for stability, and b) sensibility to activators and inhibitors. In Table 3, characteristics of some microbial lipases are summarized. Molecular weight of lipases generally ranges between 22-69

Table 3. Characteristics of purified microbial lipases

Organism Bacteria	pI	Temperature optimal activity	Temperature stability	pH optimal activity	pH stability	Activators	Inhibitors	Molecular Mass (kDa)	Reference
Acinetobacter sp. RAG-1		55°C	Up to 70°C	9.0	5.8-9.0	Stabilizer: Ca^{2+}	EDTA, Hg^{2+} Cu^{2+} Pyridine	33	Snellman et al. 2002
Pseudomonas sp.		45°C	Up to 60°C	9.0	7.0-11.0	K^{+}, Ca^{+}, Mg^{2+} Activate	Hg^{2+}, Cu^{2+}, Sn^{2+} Inactivate Surfactants, bile salt		Gao et al. 2000
Bacillus thermocatenulatus DSM 730		60-70°C		7.5-8.0		CHAPS, taurocholi acid, octyl-glucoside	Tween 20, Tween 80	16	Benjamin & Pandey 1998
Bacillus sp. THL 027		70°C	Up to 75°C	7.0	At neutral pH	Na^{+}	Fe^{3+}, EDTA (metalloenzyme)	69	Darmsthiti & Luchai 1999
Staphylococcus warneri	6.8, 7.1, 7.4	25°C (45 kDa)	20-40°C	9.0 (45 kDa)	5.0-9.0	Ca^{2+} Mn^{2+}	Co^{2+}, Zn^{2+}, serine protease inhibitors, EDTA	90 (pro-lipase), 45 (mature form)	Talon et al. 1995
Serratia marcescens		37°C, high activity at 5°C	65°C	8.0-9.0	8.0			52	Abdou 2003
Bacillus thermocatenulatus		60°C-70°C	Up to 40°C	7.5-8.0	5.0-11.0	CHAPS, taurocholic acid, octyl-glucoside	Tween 20, Tween 80	16	Schmidt-Dannert et al. 1994

Organism Yeasts	pI	Tempe-rature optimal activity	Tempe-rature stability	pH optimal activity	pH stability	Activators	Inhibitors	Molecular Mass (kDa)	Reference
Geotrichum candidum		40°C		7.0	6.5-8.5	Tween 1.0% Ca^{2+}, Ba^{2+}	$AgNo_3$, $NiCl_2$, $HgCl_2$, EDTA	32	Gopinath et al. 2003
Kluyveromyces Marxianus CECT 1018 GRAS			Up to 100°C in acidic pH		2.0-8.0				Deive et al. 2003
Geotrichum sp. FO401B						Plant oils, tributyrin	Soybean oil, long-& shor-chain fatty acids	62 (lipase A) 58 (lipase C)	Ota et al. 2003
Candida antartica				7.0 for both				45 (lipase A) 35 (lipase B)	Paktar et al. 1993
Candida cylindracea	Lipase A: 5.5 Lipase B: 4.80, 4.84			7.0 for lipase A & B				64 (lipase A) 62 (lipase B)	Rua et al. 1993
Yarrowia lipolytica 681		37°C		6.0	5.0-7.0	Olive of corn oil low levels aeration Tween 80	Oleic acid		Corzo & Revah 1999
Cryptococcus sp. S-2		37°C	Up to 50°C	7.0	5.0-9.0	Sardine oil, soy bean oil, triolein DMSO, diethyl ether	Benzene Hg^{2+} Cu^{2+}	22	Kamini et al. 2000

Organism Fungi	pI	Temperature optimal activity	Temperature stability	pH optimal activity	pH stability	Activators	Inhibitors	Molecular Mass (kDa)	Reference
Mucor sp	6.2	35°C	30°C-45°C	7.0	5.0-7.0	Ca^{2+}, Co^{2+}, (1mM), Sodium cholate (10mM)	Triton X-100; SDS, Fe^{2+}, Fe^{3+}, Hg^{2+}	42	Abbas et al. 2002
Humicol lanuginosa S-38		60°C	60°C	8.0	4.0-11				Liu et al. 1972
Rhizopus delamar		25°C-35°C		7.5-8.0		Ca^{2+}, Ba^{2+}, Ma^{2+}		30.3	Haas et al. 1999
Rhizomucor Miehei	3.8	50°C olive oil (hydrolysis) 37°C 1-butyl oleate (synthesis)	30°C	8.0	8.0		K^{+}, Li^{+}, Ni^{+}, Co^{2+}, Zn^{2+}, Mg^{2+}, Sn^{2+}, Cu^{2+}, Ba^{2+}, Ca^{2+}, Fe^{2+}, SDS	31.6	Wu et al. 1996
Mucor hyemalis f. hiemalis	4.6	40°C	45°C	7.0	4.0-9.0	Ca^{2+}, Mg^{2+}, Co^{2+} Mn^{2+}, Na^{+}	Fe^{2+}, Cu^{2+}, Ba^{2+} Triton X-100, Tween 20, Taurocholic acid	49	Hiol et al. 1999
Rhizopus oryzae	7.6	35°C	45°C	7.5	4.5-7.5	Sodium cholate, taurocholate	Triton X-100, SDS Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}	32	Hiol et al. 2000

kDa. However, the molecular weight of lipase from *B. thermocatenulatus* was 16 kDa (Schmidt-Dannert 1994). Most lipases show their optimal activity between 25°C-40°C. Abdou (2003) reported that purified lipase from *Serratia marcescens* had optimum activity at 37°C but retained 90% of its total activity at 5°C. This feature could be of great interest for some applications at low temperature. On the other hand, thermostable lipases offer several advantages. Table 3 also lists some thermostable lipases. Lipase can be active in extreme pH conditions, as low as 2 for *Kluyveromyces marxianus* CECT 1018 lipase (Deive et al. 2003) and as high as 11 for *Pseudomonas* sp. (Gao et al. 2000), *B. thermocatenulatus* lipase (Schmidt-Dannert 1994) and *H. lanuginosa* S-38 lipase (Liu et al. 1972).

Bacterial lipases are of interest because they are generally more stable than those from other organisms, especially against high temperatures and severe operational conditions and/or storage conditions (Dharmsthiti & Luchai 1999). *Bacillus* THL027, a thermophilic strain isolated from oil-contaminated area close to a seashore restaurant, was found to grow with up to 3 % NaCl and its lipase was active with up to 5 % NaCl (Dharmsthiti & Luchai 1999).

The ability of lipases to catalyse a wide variety of substrates makes them a useful tool in biocatalysis with vivid applications. Some factors affecting substrate specificity are the state of the substrate interface, lipid distribution, effect of metal ions, etc (Abbas et al. 2002). Broad substrate specificity has been reported for *B. stearothermophilus* lipase for soluble and insoluble substrates (Kambourova et al. 2003). The highest activity was against tributyrin, and triacylglycerols were preferred over monoacylglycerols. This lipase expressed also activity towards soluble esters. Tributyrin, tricaprylin, tripalmitin and triolein were hydrolyzed by lipases from *Cryptococcus* sp. (Kamini et al. 2000) and *Mucor hiemalis sp. hiemalis* (Hiol et al. 1999). Lipase from *Acinetobacter* sp. was active against medium and long chain acyl triglycerides (Snellman 2002). Lipase from *Mucor* sp. hydrolysed vinyl esters and triglycerides (Abbas et al. 2002). Oils such as palm oil, sunflower oil, rape seed oil and olive oil were hydrolysed by lipases produced by *R. oryzae* (Hiol et al. 2000), *Cryptococcus* (Kamini et al. 2000) and *Mucor* sp. (Abbas et al. 2002).

Substrates for lipases are often insoluble in aqueous solution. The presence of solvent increases the substrate solubility. Since numerous biocatalysts are denatured or inactivated by organic solvents, lipases tolerant to solvent are considered useful in the industry. Stability in organic media has been reported for *Mucor* sp. immobilized lipase in hexane, cyclohexane and MTBE but ethanol inactivated the enzyme (Abbas et al. 2002). *M. hiemalis f. hiemalis* was reported to be active in hexane, heptane and isooctane and slightly activated in acetonitrile (Hiol et al. 1999). Good solvent stability in methanol, ethanol, isopropanol, acetone, glycerol, sorbitol and *n*-hexane has also been reported for lipase from *B. stearothermophilus* (Kambourova et al. 2003). Lipase isolated from *B. sphaericus* 205y was stable and showed an increased activity in presence of *p*-xylene and *n*-hexane (Hun 2003). Enzyme activity of lipase from *Cryptococcus* sp. S-2 was also enhanced with dimethylsulfoxide and diethyl ether. The enzyme was stable in all organic solvent tested (Kamini et al. 2000). The same stability in dimethylformamide, acetone, dimethylsulfoxide, tetrahydrofuran and acetonitrile was observed for lipase from *Acinetobacter* sp. RAG-1 (Snellman et al. 2002). *P. aeruginosa* LST-03 produced a lipase stable in the presence of cyclohexane (Ogino et al. 1999). High stability of lipase from *R. oryzae* in hexane, heptane, cyclohexane and isooctane was also observed (Hiol et al. 2000).

7. CONCLUSIONS

Lipases have several interesting potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries. However, their high production costs limit their industrial use. Nevertheless,

different production techniques, such as recombinant DNA technology, solid-state fermentation, immobilization, fed-batch culture and continuous culture, have been developed with the aim to optimize lipase production and/or to reduce the costs of downstream processing. Development of strategies to apply these techniques at industrial scale should allow a significant decrease in lipases cost and consequently stimulate the development of more industrial applications.

8. PERSPECTIVES

In the near future, development of new and more-efficient expression systems in combination with improvement of protein engineering technologies (rational protein design and directed evolution) should constitute powerful tools to produce in bulk lipases with improved properties and new selected catalytic functions for industrial technology purposes.

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Proteases

16

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1. INTRODUCTION

The catalytic activity of enzymes have been exploited by man for thousands of years in various processes such as cheese making, wine and beer production, release of free fermentable sugar from barley grains during malting, etc. Out of the total enzymes being used industrially, over half are extracted from fungi and yeast, one-third from bacterial sources and the remaining from animal (8%) and plant (4%) sources.

Proteases are a complex group of enzymes capable of hydrolyzing the peptide bond in a protein molecule. They account for about 60% of the total worldwide sale of enzymes (Singh et al. 2001). The versatility of proteases ranging from being the major armour of protein degrading saprophytes to the signal sequence cleaving peptidase enzymes of higher organisms, clearly illustrates their influence in the biosphere. They belong to Class 3, the hydrolases and subclass 3.4, the peptide hydrolases or peptiodases. Depending on the site of action, proteases are broadly classified into two major classes such as exopeptidases and endopeptidases. Exopeptidases hydrolyse the peptide bond from the amino or carboxy termini of the polypeptide chain, while endopeptidases cleave internal peptide bond of the polypeptide. Proteases are also classified based on the functional group present at the active site into 4 groups: serine protease, cysteine (thiol) protease, aspartic protease and metallo protease. And, depending on the pH at which they are active, they are also classified as acid, alkaline and neutral proteases. Table 1 gives a detailed description on classification of proteases (adapted from Rao et al. 1998).

1.1. Historical developments in commercial production of proteases

The first successful attempt to apply proteases in detergent industry dates back to 1913 when Rohm and Hass marketed a pre-soak detergent 'Burmin' using pancreatic enzyme. The extensive use of proteases as detergent additives in 1960's stimulated their commercial development. By 1969, 80% of all laundry detergents contained protease. The action of proteinases on more selective substrates and highly specific and selective amplification of proteins by limited hydrolysis paved the way for novel applications.

Recently, there has been an increasing demand for proteases because of their suitability for versatile applications, mainly as a detergent additive. About 500 tons of protease is now produced per year. Among the major protease producers of the world, Novo Industries, Denmark occupies about 40% of the market share whereas Gist- Brocades, Netherlands and Genencor International, United States occupies 20 and 10%, respectively (Rao et al. 1998).

A good number of bacterial alkaline proteases are commercially available such as subtilisin Carlsberg,

Table 1 Classification of proteases

Protease	Enzyme Classification number (EC No)
Exopeptidases	
Aminopeptidases	3.4.11
Carboxypeptidase	3.4.16-3.4.18
Serine type protease	3.4.16
Metalloprotease	3.4.17
Cysteine type protease	3.4.18
Endopeptidases	3.4.21-3.4.24
Serine protease	3.4.21
Cysteine protease	3.4.22
Aspartic protease	3.4.23
Metalloprotease	3.4.24
Endopeptidases of unknown catalytic mechanism	3.4.99

subtilisin BPN' and Savinase, with their major application as detergent enzymes. However, mutations have led to newer protease preparations with improved catalytic efficiency and better stability towards temperature, oxidizing agents and changing wash conditions. Many newer preparations, such as Durazym, Maxapem and Purafect, have been produced, using techniques of site-directed mutagenesis or random mutagenesis. Protease preparations derived from genetically modified organism like *A.oryzae* containing *Rhizomucor* gene are available for food use. However search for novel proteases from uncultured microorganisms from natural habitat will allow biotechnological exploitation of this important enzyme.

Proteases regulate complex processes in the normal physiology of the cell as well as in abnormal pathological conditions. They are potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS because of their involvement in the life cycle of the causative organisms. They also collaborate with the transport of secretory proteins across membranes.

The wide specificity of hydrolytic action of proteases finds extensive applications in different industries viz. food, pharmaceutical, silk and for recovery of silver from used X-ray films (Phadatare et al. 1993, Banerjee et al. 1999, George et al. 1995, Reichelt 1983, Chiplonkar et al. 1985, Ogino et al. 1995).

In this chapter, we discuss the sources of proteases, production strategies employed, purification and characterization of these enzymes as well as the different methods available for determining protease activity.

2. SOURCES OF PROTEASES

Proteolytic enzymes are available from plant, animal and microbial sources. However, microbes are considered preferred source of protease due to their broad biochemical diversity and susceptibility to genetic manipulation. Micro organisms degrade proteins and utilize the degradation products as nutrients for their growth. They account for approximately 40% of the total worldwide enzyme sales. Table 2 gives a list of microbial protease producers, type of protease and their application in industry. Among

Table 2. Some sources of protease and their industrial applications

Microorganism	Type of protease	Industry
Bacteria		
Bacillus licheniformis	Alkaline	Detergent
Bacillus amyloliquefaciens	Alkaline	Detergent
Bacillus firmus	Alkaline	Detergent
Bacillus megaterium	Alkaline	Detergent
Bacillus pumilis	Alkaline	Detergent
Streptomyces fradiae	Alkaline	Detergent
Streptomyces griseus	Alkaline, neutral	Detergent, leather, food
Streptomyces rectus	Neutral	Detergent
Bacillus subtilis	Neutral	Leather, food
Bacillus cereus	Neutral	Leather, food
Bacillus megaterium	Neutral	Leather, food
Pseudomonas aeruginosa	Neutral	Leather, food
Fungi		
Aspergillus niger	Alkaline	Detergent
Aspergillus sojae	Alkaline, neutral	Detergent, leather, food
Aspergillus oryzae	Alkaline, neutral	Detergent, leather, food
Aspergillus flavus	Alkaline	Detergent
Pericularia oryzae	Neutral	Leather, food
Endothia parasitica	Acid	Pharmaceutical, food
Mucor miehei	Acid	Pharmaceutical, food
Mucor pusillus	Acid	Pharmaceutical, food

these, fungi as enzyme producers have many advantages, considering that the produced enzymes are normally extracellular, making easier its recuperation from the fermentation broth. Besides, use of fungi as enzyme producers is safer than bacteria, since they are normally GRAS (generally regarded as safe).

The widely known proteases of plant origin are papain, bromelain, keratinases, and ficin. The major factors that limits the use of plants as protease source are availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants in a time-consuming process. The production of animal proteases depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies. Some examples of animal proteases are pancreatic trypsin, chymotrypsin, pepsin, and rennin.

3. PRODUCTION OF PROTEASES

Protease production has been studied by submerged (SmF) as well as solid-state fermentation (SSF) processes. The first step to be considered while studying an industrial fermentation process is isolation of microorganisms capable of producing economically viable yields of desired product. Usually a plate assay on milk agar medium containing 1% casein is carried out to isolate protease producing organisms

by measuring the clear zone of casein hydrolysis formed on milk agar (Ellaiah et al. 2002, Rajamani and Hilda 1987, Adesh et al 2002). On the contrary, it has been reported the *B. licheniformis* produces narrow zones of hydrolysis on casein agar despite being very good producer in submerged culture. Another screening technique for microbial proteases was developed by Wikstroem (1983), where protein adsorbed to hydrophobic surfaces was used for determination of proteolytic acitivity. A method developed by Vermelho et al. (1996) allows detection of extracellular proteases from microorganisms using different substrates (gelatin, BSA, hemoglobin) on agar plates.

While developing a production medium, an important factor to be considered is the cost effectiveness of the medium. This can be achieved by employing cheaply available agroindustrial residues as substrates for microbial growth and enzyme production.

Any variation in the environmental conditions of the microorganism such as change of temperature, pH, substrates, moisture, supply of air, inoculum concentration, carbon and nitrogen sources etc. influence the metabolic activity of the microorganism. The effect also varies widely from species to species for each of the organism. Hence, it is necessary to optimize the environmental conditions of the microorganism to attain the maximum production.

3.1. Submerged fermentation (SmF)

Most industrial fermentations are carried out in liquid media, which range from simple stirred tank or non-stirred containers to complex, aseptic, integrated systems. The production medium can be a chemically defined (synthetic) media or crude digests of substances such as casein, beef, soybeans, yeast cells, etc. Synthetic media have several advantages such as enhanced process consistency, better control and monitoring, improved process scale up, product recovery, simplified purification and improved product quality.

In a batch bioreactor containing a defined medium, an uncontrolled pH operation starting at pH 7.1 was found to be favourable for serine alkaline protease production by recombinant *Bacillus licheniformis* carrying a gene pHV1431: SubC (Calik et al. 2003). A fed-batch strategy was employed by Beg et al. (2002) to enhance protease synthesis by separating biomass production phase and enzyme production phase, i.e. by depression and subsequent induction during the growth of *Bacillus mojavenis*. It was found that the time of addition of inducer (casamino acids) had a greater influence on protease production as cells in depressed state reacted best to induction as compared to actively growing cells. A four fold increase in production was obtained during this process.

3.1.1. Factors affecting SmF

The significance of temperature and pH in the development of a biological process is such that they could determine effects such as protein denaturation, enzymatic inhibition, promotion or inhibition on the production of a particular metabolite, cell deaths, etc. (Nehra et al. 2002). Carbon and nitrogen sources are other significant component of the medium, which significantly affect protease production in SmF.

Carbon source could be a simple monosaccharide compound such as glucose or a complex such as polymeric molecules (e.g. cellulose, starch). Generally pure carbon sources inhibit protease production, indicating the presence of catabolite repression of protease biosynthesis. However, Mabrouk et al. (1999) showed that the highest yield of alkaline protease by *Bacillus licheniformis* was achieved using

a mixture of lactose (4%) and glucose (1.5%) as carbon source. Sutar et al. (1992) found that protease production by *Conidiobolus coronatus* NCIM 1238 was increased by glucose and sucrose but arabinose, lactose, maltose and raffinose resulted in poor enzyme activity. Among the sugar alcohols studied only glycerol showed enhanced production while erythritol, mannitol and myo-inositol gave lower production.

Different types of inorganic or organic nitrogen sources, such as pure salts (e.g. ammonium salts) and complex sources such as soybean extract can be supplemented in the medium. Mabrouk et al. (1999) found soybean (6%) and ammonium phosphate (1.2%) as the best nitrogen sources for the production of alkaline protease by *Bacillus licheniformis.* Protease production by *Bacillus* sp.P-2 was found to be highest with a combination of yeast extract and peptone in the basal medium while supplementation of inorganic nitrogen sources reduced the enzyme production to 90% (Kaur et al. 2001). The media are usually supplemented with 10-15% dry substance and high protein content. Free amino acids are avoided as they often inhibit protease formation (Menon and Rao 1999).

Factors such as inoculum concentration, aeration, agitation, presence of surfactants and metal ions in the fermentation medium also strongly influence microbial protease production by SmF.

3.2. Solid-state fermentation (SSF)

In SSF, agro-industrial residues such as wheat bran, rice bran, rice husk, soybean meal, soybean flour, coffee husk, sweet potato residues, etc. have been used as solid support and carbon source for protease production (Pandey 1992, Pandey et al. 1999, 2000a, 2001, Pandey & Soccol 2000b, Germano et al. 2003, Sandhya et al. 2004). Inert supports such as polyurethane foam also can be employed (Ozawa et al. 1996). Protease production by *A.flavus* under SSF showed that of all the brans tested, wheat bran and a mixture of wheat bran and rice bran gave maximum enzyme yield (Mulimani & Patil 1999). A comparative study on protease production in SSF and SmF showed that the total enzyme activity present in 1g bran was equivalent to100 ml liquid broth (George et al. 1997). Supplementation of different carbon sources on production of alkaline protease by *Aspergillus flavans* under SSF showed that all sugars except lactose, repressed enzyme synthesis. Enrichment of wheat bran medium with 10% corn steep liquor or casitone increased production by 58 and 42%, respectively over the control medium without additional nitrogen source (Malathi & Chakraborty 1991). Supplementation of surfactants, vitamins, metal ions and plant growth factors to the medium affects protease production by SSF (Tunga et al. 2001).

The important parameters that influence large-scale production of protease by SSF were accumulation density, bed height, agitation etc. Effect of height of culture medium on protease production showed that a height up to 4.0 cm was suitable, an increase to 5.5 cm led to a 20% reduction in the enzyme yield (Chakraborty & Srinivasan 1993). The optimum conditions for protease production by *R. oryzae* consisted of an accumulation density of 0.3125 g/cc and a bed height of 1 cm without aeration (Tunga et al. 1999).

Maximal protease activity by *B. amyloliquefaciens* in SSF was achieved at 37°C with aeration of 4l/min in a horizontal tray reactor (George et al. 1995). However, in trays, the mould grew poorly and produced less protease. This could have been due to evaporation, which resulted in drying of the substrate, causing growth inhibition. The shear effects on the surface of the lumps in rolling drum fermentor may prevent growth and protease production. Aikat & Bhattacharya (2001) compared protease production by *R. oryzae* on wheat bran in a stacked plate fermenter (SPF) and packed bed fermenter (PBF), incorporating the liquid culture medium recycle strategy. PBF showed four times higher activity than SPF.

3.2.1. Factors affecting SSF

There are a number of factors which affect protease production in SSF. Among these, the most critical are substrate moisture and water activity, temperature of incubation, heat and mass transfer effect, etc. The importance of water in the fermentation system is due to the fact that the great majority of viable cells are characterised by moisture content of 70-80%. A study on protease production by *Pseudomonas* sp showed highest enzyme titre with 74% initial moisture content (Chakraborty & Srinivasan 1993). In fungal and bacterial SSF, lower moisture levels lead to reduced solubility of the nutrients in the solid substrates, a lower degree of substrate swelling and higher water tension (Zandrazil & Brunert 1981). Similarly, higher moisture contents were reported to cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume, decreased gas exchange and enhanced formation of aerial mycelium (Pandey et al. 2001). Microbial growth is determined by the degree of availability of water, commonly expressed as water activity.

Temperature affects the rate of biochemical reactions and is an important parameter in SSF, but its control is more difficult in SSF process than in SmF. Optimum temperature for protease production by most of the cultures has been reported in mesophilic range.

3.3 Membrane surface liquid fermentation

A new culture method (membrane surface liquid fermentation, MSLF) was carried out by Yasuhara et al. (1994), where fungal mycelia were grown on the upper side of a microporous membrane facing the air, while the opposite side was in contact with the liquid culture medium. Neutral protease produced by *A. oryzae* IAM 2704 was two times as high as in SSF and 10 times that in the liquid culture. The MSLF has advantages of control of pH or substrate concentration during culture and repeated batch or continuous culture may also be performed. Purification is simplified, since mycelia may be separated readily from the medium and the membrane may be used repeatedly.

3.4. Production using immobilised cells

Cell immobilization techniques have been used for protease production (Elibol & Moreira 2003, Chellapandian & Sastry 1992). Alkaline protease when immobilised on vermiculite showed lower specific activity towards high molecular weight substrates but a broader pH tolerance and higher thermal stability than the free enzyme (Chellapandian 1998). The increase in thermal stability was due to the multipoint covalent attachment and stabilization of weak bonds between protease and the support, which protected the enzyme from inactivation. pH tolerance may be due to partition effects that cause different concentrations of hydrogen ions in the environment of immobilized enzyme when coupled to a carrier possessing electrostatic interactions.

3.5. Correlation between growth and protease production

Enzyme production exhibits a characteristic relationship with regard to the growth phase of that organism. Proteases are generally constitutive or partially inducible in nature and under most growth conditions, *Bacillus* sp. produce extracellular proteases during post exponential and stationary phases (Ward 1985, Kole et al. 1988). Protease production by *Rhizopus* strains was found to be dependent on mycelial growth (Elegado & Fujio 1993). A study on the effect of fungal morphology on protease production showed that growth of mycelium in the form of large pellets was associated with lower specific protease activity (Papagianni & Moo-Young 2002). Effect of light on growth and alkaline protease production in *Bacillus* fermentation showed that fluorescent light (9000 lux) induced greater cell mass with lower protease yields compared with dark growth conditions (Dhandapani 1993).

3.6. Mathematical modelling

The complexities and uncertainities associated with large-scale fermentation usually come from the lack of knowledge of the sophisticated interactions among various factors. Mathematical modelling is an important tool which enables the elucidation of underlying metabolic regulatory mechanisms for microbial growth and enzyme production, which could facilitate the optimization of production for up-scaling. The trial and error approach commonly used for fermentation process now becomes economically nonviable, since the errors may lead to big losses both in terms of money and time. Models are considered empirical (statistical) or structured (obtained from functions or mathematical expression deduced from theoretical deductions of the system). Recent efforts to develop mathematical models of SSF in tray, packed bed and rotating drum bioreactors have addressed inter-particle heat and mass transfer and provide insights into the design and operation of bioreactors (Sangsurasak et al. 1996).

The possibility was examined by developing a predictive model that combined microbial growth with extracellular proteolytic activity of a cocktail of four strains of *Pseudomonas* sp and one strain each of *Acinetobacter* sp and *Shewanella putrefaciens*. The environmental conditions examined were temperature 2-20^{0}C, pH value 4.0-7.5, water activity (a_w) 0.95-0.98 and a model was constructed which predicted growth, based on increase in cell number. Data on protease activity were generated but no function could be identified which was a good fit to these data, since most enzymatic production and activity occurred as expected during transition from exponential to stationary phase (Braun & Sutherland 2003).

4. ASSAY METHODS

Different methods developed to assay proteolytic activity are listed in Table 3. Among these, widely employed procedure for determining protease activity in microbial cultures is using sulphanilamide azocasein substrate according to the method of Leighton et al. (1973). Azocasein is a chemically modified protein

Table 3. Assay methods for determining proteases

- Colorimetric method (Keay & Wildi 1970)
- Measuring the release of azocasein-derived amino acids and small peptides (Girard & Michaud 2002)
- Using an insoluble chromogenic substrate where the new substrate is simple to prepare , assay is fast and reproducible and requires no precipitation step (Safarik 1988)
- By immobilising substrate protein to an insoluble particle and linking it to a reporter enzyme which is resistant to proteolysis than the substrate proteins. This method is more sensitive than passive dye method (Andrews 1982)
- Using enhanced green fluorescent protein fused with staphylococcal protein A as substrate. This assay was sensitive enough to measure picogram levels of tyrosine and chymotrypsin and applicable to various other proteases (Hiroyoshi et al. 2002)
- A fluorescent zymogram in-gel assay using SDS polyacrylamide gel copolymerised with a peptide-MCA (4-methyl-coumaryl-7-amide) substrate helps in simultaneous determination of protease cleavage specificity and molecular weight (Yasothornsrikul & Hook 2000)
- Developing a fluorescence polarisation technique, where changes in molecular volume due to cleavage of intact fluorescein thiocarbamoyl (FTC)-casein molecules to smaller FTC peptides were measured. This assay is more sensitive than other non radioactive protease assays and requires no separations, precipitations or transfers of the reaction mixture (Bolger & Checovich 1994)

containing sulphanilamide groups (orange in colour), covalently linked to peptide bonds of milk protein casein. During incubation for 1h, proteases hydrolyse peptide bonds, liberating shorter peptides and amino acids from the chain. Trichloroacetic acid (TCA) is then added to precipitate macromolecules including the enzyme and undigested azocasein, which can be removed by centrifugation. Short peptide chains and free amino acid liberated are not precipitated by TCA and thus, remain in solution, which is orange in colour. The intensity of colour is measured spectrophotometrically to determine protease activity.

4.1. Procedure

The colorimetric assay is carried out at 45^{0}C with 250 µl 1% w/v azocasein in 0.2M citrate phosphate buffer, pH 6.5. The reaction is started by the addition of 150µl of the crude extract of enzyme. After incubation for 1 h, the enzyme is inactivated by addition of 1.2 ml of TCA solution (10%v/v) and the solution is neutralised using 1M NaOH solution. The absorbance is read against a blank of the inactivated crude extract (100^{0}C for 10 minutes) at 440 nm. One unit of proteolytic enzyme activity is defined as the amount of enzyme that produces absorbance difference during 1 h incubation at 45^{0}C for one millilitre of solution of crude extract.

5. PURIFICATION AND CHARACTERISATION OF PROTEASES

A better understanding of the function of enzyme could be determined by purification of enzyme. The primary objective in the purification programme is the removal of excessive amount of water in the cell free extract. A direct method is concentration of extract using salts or solvents with a high affinity which results in the precipitation of protein. In addition to this, purification can be achieved by chromatographic and electrophoretic procedures (Manonmani & Joseph 1993, Su &Lee 2001). Column techniques have the advantage that the resulting fractions are amenable to quantitation using general analytical methods. Gel permeation chromatography separates proteins based on their size while ion exchange chromatography use substituted cellulose ion exchangers,diethylaminoethylcellulose (DEAE) and carboxymethyl cellulose (CM). Electrophoretic seperation of protease can be achieved by difference in size and charge.

Table 4 gives an overview of procedures commonly employed for purification of microbial protease as well as characteristics of the purified enzyme. Characterisation of proteases can be carried out by determining the properties and kinetic parameters of the enzyme (El Assar 1995, Datta 1992). Protease inhibitors are chemicals that block proteolytic action, hence while characterizing the enzyme, influence of inhibitors also has to be determined. Reactions with specific inhibitors determine the type of protease. The common inhibitors affecting protease are ethylene diamine tetraacetic acid (EDTA), N',N'-tetraacetic acid (EGTA), pepstatin, phenyl methyl sulphonyl fluoride (PMSF), etc. Gelatin zymography involves electrophoresis of secreted proteases through discontinous polyacrylamide gels containing enzyme substrate. This technique helps to determine different proteases present in the crude culture extract.

Partial characterisation of protease from *Pencillium* sp showed that the enzyme was quite stable at pH 6.0-8.0 with optimum value at pH 6.5 and 45^{0}C. Effect of metallic ions on enzyme activity showed that Co^{2+}, Mg^{2+} and Zn^{2+} reduced enzyme activity while Ca^{2+} and Na^{+} resulted in increased activity. Reaction with specific inhibitors showed that it was a serine protease. These characteristics enabled the enzyme to be compatible with commercial detergents and oxidizing agents, suggesting possibility of its applicability in detergent industry (Germano et al. 2003).

Table 4 Purification and characterisation of microbial proteases

Microorganism	Purification steps	Characteristics of protease	Reference
Bacillus polymyxa (mutant)	Ammonium sulphate fractionation (40-80%), DEAE cellulose fractionation, Sephadex G 100 fractionation	Molecular weight –31kD (SDS PAGE), Optimum pH-9.25, Optimum temperature-70^{0}C, Inhibited by Cu^{2+}, Hg^{2+}, EDTA and PMSF. Suitable for detergent industry.	Madan et al. 2002
Nocardiopsis sp	Ammonium sulphate fractionation (0-20%), Sephadex G-75 fractionation	Stable at alkaline pH, Optimum pH-8.0, Optimum temperature-50^{0}C, Use of specific inhibitors showed that it is a serine protease	Moreira et al. 2003
Aspergillus sp	Ultrafiltration, lyophilisation, DEAE Sepharose CL-6B fractionation, Isoelectric focusing, Sephacryl S-200HR fractionation	Molecular weight –28kD, pI-3.75, Optimum pH-7.0-9.0, Optimum temperature-65^{0}C, Stable pH range 5.0-9.0, temperature stability- 55^{0}C for 3 hr. Inhibited by $HgCl_2$.	Su & Lee 2001
Phanerochaete chrysosporium ATCC 24725	Concavalin A-Sepharose fractionation, Sephadex G-25 fractionation, FPLC on Mono Q, Ultrafiltration, Gel filtration	Molecular weight –40kD (SDS PAGE), pI-5.6, Optimum pH-4.0, Inhibited by mercury ions, p-hydroxy mercuribenzoic acid and N-bromosuccinimide. Response to inhibitors suggested that it has properties of both aspartate and thiol type proteases	Datta 1992
Trichoderma koningii	Ion exchange chromatography, Biogel P-100, Affinity chromatography, Poly acrylamide gel electrophoresis (PAGE)	Molecular weight –85kD, Optimum pH-10.5, Optimum temperature –50^{0}C, P^i-9.0, Rich in glycine, serine, alanine and aspartic acid residues	Manonmani & Joseph 1993
Fusarium pallidoroseum	Sephadex G-100 fractionation, DEAE cellulose fractionation	Optimum temperature-40^{0}C, Optimum pH-7.0, Inhibited by EDTA, sensitive to heat treatment at 55^{0}C, Active on human fibrin	El Assar 1995

6. CONCLUSIONS

From the above discussion, it is clear that microbes are the preferred source of protease and environmental factors such as temperature, pH moisture content, aeration, carbon and nitrogen sources have a strong influence on protease production. Generally pure carbon sources repressed enzyme production, although lactose has been described to be an inducer in many reports. Supplementation of organic nitrogen sources showed better production than inorganic nitrogen sources. SSF appears a promising alternative technology, which could use cheap and abundantly available agro-industrial residues as substrates for large-scale production of proteases, thereby minimising the high cost.

7. PERSPECTIVES

Microbial proteases possess versatile applications. Recently there is increased interest in proteases as targets for developing therapeutic agents. As a commodity product, the pressure on the protease market will continue to be on price reduction and increasing performance. In spite of their wide popularity, industrial application of microbial proteases faces several problems and challenges such as enhancement of thermostability, prevention of auto inactivation, altering of pH optimum, etc. Thus, future studies should take place in the direction involving new or improved microorganisms capable of producing protease with these desired characters. Optimization of existing fermentation processes and equipments would include activities in SSF as SSF processes are economical at low scale of operation due to higher production and do not require large product demand, infrastructure such as bioreactor, etc. Another area of future development is use of molecular biology techniques to develop versatile proteases, which will offer an exciting area of research.

Acknowledgements

Authors thank grants under CSIR Task Force project for protease research. AS acknowledges award of a JRF from CSIR, New Delhi.

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Xylanases

P Prema

1. INTRODUCTION

The plant cell wall is a composite material of cellulose (35-50%), hemicellulose (20-30%), and lignin (20-30%), and they are closely associated with each other. Xylan, the major hemicelluloses is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone of β-1, 4, -linked xylopyranose units. It is bound to lignin, cellulose and other polymers by covalent and non-covalent interactions (Subramaniyian & Prema 2002). Lignin is linked to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues. The depolymerisation of xylan by xylanases results in the conversion of the polymeric substance into xylooligosaccharides and xyloses. Xylanases are one of the important industrial group of enzymes finding significant applications in paper and pulp industry, as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent. Vikari and his group [Viikari (1991) Viikari et al. (1994)] were the first to demonstrate the application of xylanases for delignification in bleaching process. Xylanases are applied to produce the dissolving pulp (Bajpai & Bajpai 2001), i.e. the pure form of cotton fibre free from other carbohydrates.- for the preparations of rayon, cellophane and several chemicals like cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose, methyl and ethyl cellulose). Xylanases also find application on areas like - bioconversion of lignocellulosic material and agro-wastes to fermentative products; clarification of juices; improvement in consistency of beer; and the digestibility of animal feed stock etc. Exploitation of xylanase in the saccharification of xylan in agrowastes intensifies the potential need of them in biotechnology.

Xylan, the second most abundant polysaccharide next to cellulose is present in all terrestrial plants and accounts for 30% of the cell wall material of annual plants, 15-30% of hard woods and 7-10% of softwoods. Similar to most of the other polysaccharides of plant origin, xylans also display a large polydiversity. They occur in almost all variety of plant species distributed in several types of tissues and cells. However, all terrestrial plant xylans are characterised by a β-1, 4-linked D-xylopyranosyl main chain carrying a variable number of neutral or uronic monosaccharide subunits or short oligosaccharide chains. In the case of soft wood plants, xylan fraction is mainly arabino-4-O-methyl glucuronoxylan which in addition to 4-O-methyl glucuronic acid is also substituted by α-arabinofuranoside units linked by α-1, 3-linkage to the xylan backbone and the ratio of arabinose side groups to xylose residue is 1:8. Rarely, acetyl groups are attached to the softwood xylan. The reducing ends of the xylan chains are reported to be linked to rhamnose and galacturonic acid in order to make alkali resistant end groups of xylan chain. (Kulkarni et al. 1999) Arabinoxylan is usually found in *Poaceae* (Fig. 1). Similar to other

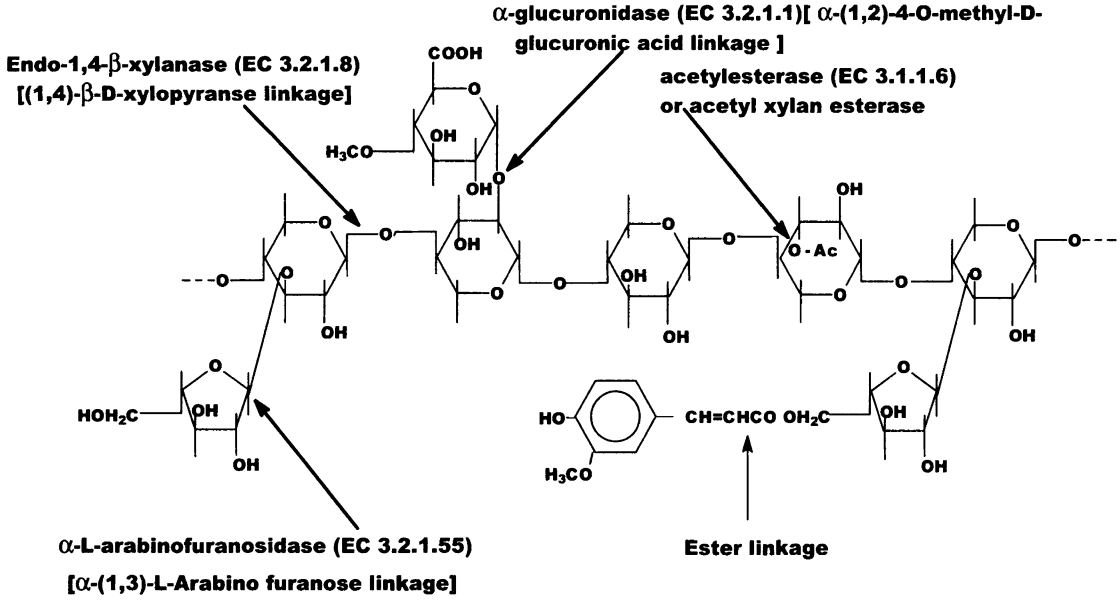

Fig. 1. Structure of arabinoxylan from grasses. The substituents are: Arabinose, 4-O-methyl-D-glucuronic acid, O-Ac (Acetyl group) and there is also ester linkage to phenolic acid group (Subramaniyian 2000)

biopolymers, xylan is also capable of forming intrachain hydrogen bonding, which supports a two fold extended ribbon like structure. The β(1-4) D-xylan chain is reported to be more flexible than the two fold helix of β(1-4) cellulose as there is only one hydrogen bond between adjacent xylosyl residues in contrast with two hydrogen bonds between adjacent glycosyl residues of cellulose. The absence of primary alcohol functional group external to the pyranoside ring as in cellulose and mannan has a dramatic effect on the intra and inter chain hydrogen bonding interactions. Intra-chain hydrogen bonding is occurring in unsubstituted xylan through the O-3 position which results in the helical twist to the structure. Acetylation of xylose monomers and substitution to the backbone of the polymer disrupt and complicate this structure. An arabinose to xylose ratio of 0.6 is usually found in wheat water-soluble xylans.

The hard wood xylan is *O*-acetyl-(4- *O*-methylglucurono) xylan and the backbone consists of β-(1-4)-D-xylopyranose residues, with, on average, one α-(1-2)-linked 4- *O*-methyl glucuronic acid substituent per 10-20 such residues. Approximately 60-70% of the xylose units are esterified with acetic acid at the hydroxyl group of carbon 2 and/or 3 and on an average every tenth xylose unit carries an α-1,2-linked uronic acid side groups. Reports regarding the covalent lignin carbohydrate bonds by ester or ether linkages to hemicelluloses are identified and the covalent attachment of hemicelluloses to cellulose is less certain (Puls 1997). In primary plant cell walls, xyloglucans form the interface between the cellulose microfibrils and the wall matrix, but in some monocots (eg. Maize) this position is occupied by glucurono arabinoxylans (Teleman et al. 2002). Hemicelluloses are also associated with pectins and proteins in primary plant cell walls and with lignin in secondary walls, exact composition of which varies between organism and with cell differentiation. The complex structure of xylan needs different enzymes for its

complete hydrolysis and an array of xylanases occur in different microbial species as well as in few plant species (Biely 1985, Kulkarni et. al 1999, Subramaniyian & Prema 2002). Endo-1, 4-β-xylanases (1,4-β-D-xylanxylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan backbone and 1,4-β-D-xylosidases (1,4,β-D-xylan xylohydrolase E.C.3.2.1.37) split off small oligosaccharides. The side groups present in xylan are liberated by α-L-arabinofuranosidase, α-D-

Table1. 1. Xylanase producing microorganisms (Subramaniyian 2000)

Microorganism
Fungi
Aspergillus awamori VTT-D-75028
Aspergillus niger KKS
Aspergillus niger sp.
Fusarium oxysporum VTT-D-80134
Thermomyces lanuginosus strain
Phanerochate chrysosporium
Piromyces sp.strain E 2
Schizophyllum commune
Talaromyces emersonii CBS 814.70
Thermomyces lanuginosus
Trichoderma reesei RUT C-30 ATCC 56765
Trichoderma reesei
Trichoderma viride
Bacteria
Bacillus pumilis
Bacillus circulans
Bacillus stearothermophilus StrainT6
Bacillus sp.
Bacillus sp.
Bacillus sp. strain NCL 87-6-10
Bacillus circulans AB 16
Bacillus stearothermophilus SP
Clostridium absonum CFR – 702
Rhodothermus marinus
Streptomyces cuspidosporus
Streptomyces roseiscleroticus NRRL-B-11019
Streptomyces sp.
Streptomyces sp. QG-11-3
Thermoactinomyces thalophilus sub group C

glucuronidase, galactosidase and acetyl xylan esterase

Endo xylanases are produced by many of the bacteria, actinomycetes and fungi. However, there are reports regarding xylanase origin from plants i.e. endo-xylanase production in Japanese pear fruit during the over-ripening period. Some members of higher animals, including fresh water molluscs are able to produce xylanases.

α-Arabinofuranosidases (EC 3.2.1.55) hydrolyse the terminal, non-reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans. A number of microorganisms including fungi, actinomycetes and other bacteria have been reported to produce α-arabinosidases.

Potential application of xylanases in biotechnology include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels etc.

2. CLASSIFICATION OF XYLANASES

Microbial xylanases are classified into two groups on the basis of their physicochemical properties such as molecular mass and isoelectric point. One group consists of high molecular mass enzymes with low pI values the other of low molecular mass enzymes with high pI values, with few exceptions (Fujimoto et al. 2000, Subramaniyian & Prema 2002). The high molecular weight endoxylanases with low pI values belong to glycanase family 10, formerly known as family 'F', while the low molecular mass endoxylanases with high pI values are classified as glycanase family 11 (formerly family G). Recently there has been the addition of 123 proteins in Family 11, Biely (1985) after extensive study on the differences in catalytic properties among the xylanase families concluded that endoxylanases of family10 are capable of attacking the glycosidic linkages next to the branch points and towards the non-reducing end and also require two unsubstituted xylopyranosyl units. Endoxylanases of family 10 liberate terminal xylopyranosyl attached to a substituted xylopyranosyl residue and also exhibit aryl-D-xylosidase activity. Family 11 xylanases are divided into three groups- I, II, and III, which are mainly fungal enzymes. The enzymes in groups I and II are generally 20 kDa enzymes from *Ascomyceta* and *Basidiomyceta*. Enzymes of group III are produced by anaerobic fungi. Bacterial xylanases are divided in to three groups (A, B and C); group A belongs to enzymes produced by *Actinomycetaceae* and the *Bacillaceae* families, strictly aerobic gram-positive ones. Groups B and C are more closely related and contain mainly enzymes from anaerobic gram-positive bacteria, which usually live in the rumen. Xylanases from aerobic gram-negative bacteria are found in subgroup Ic as they closely resemble the fungal enzymes of group I. Unlike previous classifications, they also reported a fourth group of fungal xylanses consisting of only two enzymes.

3. SOURCES OF XYLANASES

Several microorganisms including fungi and bacteria produce1,4- β-D endoxylanases (E.C. 3.2.18) and β-xylosidases (EC.3.2.1.37) (Kulkarni et al. 1999, Subramaniyian & Prema 2002). According to early reports on xylanases from plant pathogens β-xylanases together with cellulose degrading enzymes play a role during primary invasion of the host tissues. Most of the fungal plant pathogens produce plant cell wall polysaccharide degrading enzymes and soften the region of penetration by partial degradation of cell wall structures, but xylanases from *Bacillus*, *Streptomyces* and other bacterial genera do not exhibit any role related to plant pathogenicity.

3.1. Fungal xylanases

Variety of wood degrading and cellulolytic fungi produce xylanases along with other hydrolases. Although high xylanase activities were reported for several fungi, the presence of considerable amount of cellulase activities and lower pH optima make the enzyme less suitable for certain applications such as in pulp and paper industries. *Phanerochaete chrysosporium, Thermomyces lanuginosus* and *Aspergillus niger* are considered as potent producers of xylanases (Subramaniyian & Prema 2002)

3.2. Bacterial xylanases

Bacteria produce higher levels of xylanases than most of the fungal enzymes with few exceptions. Noteworthy members producing high levels of xylanase activity at alkaline pH and high temperature are *Bacillus* spp. (Subramaniyian & Prema 2000, Subramaniyian. et al. 2001). Few strains of filamentous bacteria, including *Streptomyces* sp, *S roseiscleroticus, S. cuspidosporus* are also potent producres of endoxylanases, xylanase and polygalacturonase (Maheswari & Chandra 2000). The strict thermophilic anaerobe *Caldocellum saccharolyticum* and *Dictyoglomus* sp. produced xylanases and endoxylanase.

4. PRODUCTION OF XYLANASES

Production of xylanase was carried out by Submerged (SmF) and Solid-State Fermentation (SSF) (Kimuro et al. 2000, Subramaniyian & Prema 1998, 2001, Rani & Nand 2000, Pandey et al. 1999, 2000, 2001) and the enzyme yields varied according to the nature of the organism, mode of fermentation and the substrates used.(Prema, P. 2003)

However, most of the studies on xylanase production were by SmF and the enzyme activity in many cases were reported as International Units per ml of the culture filtrate obtained. Rarely, the enzyme was expressed as specific activity. The earlier expression was not uniformal and even could be misleading too sometimes. A comparative study on the production of endoxylanases by SmF and SSF using *B. pumilis* culture used for SmF (g/dl) KH_2PO_4 - 0.1; $(NH_4)_2SO_4$ 0.165; $MgSO_4.7H_2O$- 0.1; xylan – 0.5 ; pH

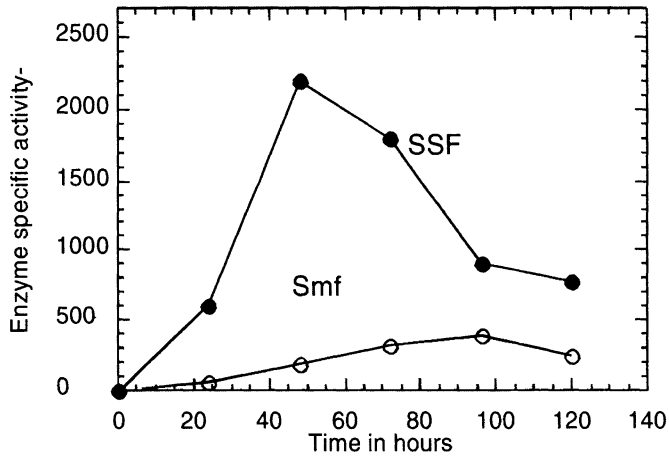

Fig 2. Production of endoxylanase from *Bacillus pumilis*

–8.5. Fermentation was carried out for 120 h at 40°C and 200 rpm. For SSF, wheat bran was mixed with appropriate concentration of mineral salt solution and has 50% moisture and pH 8.5. SSF was carried out at 40°C for 120 h. Results are shown in Fig 2. Production of endoxylanases was maximum at 48 and 96 hour in SSF and SmF, respectively.

4.1. Multiple forms of xylanases

Streptomyces sp. B-12-2 produces five endoxylanases when grown on oat spelt xylan. The culture filtrate of *A. niger* was composed of 15, and *Trichoderma viride* of 13 xylanases. The most outstanding case regarding multiple forms of xylanases was production of more than 30 different protein bands separated by analytical electrofocusing from *P. chrysosporium* grown in Avicel. There are several reports regarding fungi and bacteria producing multiple forms of xylanases. *T. viride* and its derivative *T. reesei* produced three cellulase free β-1, 4-endoxylanases. Due to the complex structure of heteroxylans all of the xylosidic linkages in the substrates are not equally accessible to xylan degrading enzymes. Because of the this hydrolysis of xylan requires the action of multiple xylanases with overlapping but different specificities. Post-translational modifications such as glycosylation, proteolysis or both could contribute to multiple occurrence of proteins. Multiple xylanases can also be the product from different alleles of the same gene or from independent genes. (Kuno et al. 2000, Subramaniyian & Prema 2002)

5. PURIFICATION AND PROPERTIES OF XYLANASES

Column chromatographic techniques, mainly ion exchange and size exclusion are the generally utilised schemes for xylanase purification, but there are also reports of purification with hydrophobic interaction column chromatography. Also, electrophoretic homogeneity has been achieved but the yield and purification fold varies in different cases (Table 2). In all the cases, the culture supernatants are initially concentrated using precipitation or ultrafiltration techniques. Thermostable xylanases from thermophilic organisms was purified by the inclusion of one additional step of heating. Use of cellulose materials as the matrix in column chromatography is impaired by the fact that certain xylanases are having cellulose binding domains, which interacts with the normal elution process. Takahashi et al. (2000) purified a low molecular weight xylanase (23 kDa) from *Bacillus* sp. strain TAR-1 using CM Toyopearl 650 M column. This xylanase with optimum activity at 70°C had broad pH profile. Kimura et al. (2000) purified *Penicillium* sp xylanase with molecular weight 25 kDa, which was induced by xylan and repressed by glucose.

6. STRUCTURE OF XYLANASES

The three-dimensional structure of family 10 and 11 endoxylanases has been determined from bacteria, actinomycetes and fungi. (Fujimoto et al. 2000) The endoxylanase 1BCX from *B. circulans* showed the features of Family 11 (Teleman et al. 2002). It had the catalytic domain folds into two β sheets (A and B), constituted by β strands and one short a helix. Endoxylanases of family 10 and 11 differ in the catalytic activities due to their difference in their tertiary structure. The family 11 endoxylanases are smaller well packed molecules of β-pleated sheets and catalytic groups present in the cleft that accommodate a chain of five to seven xylopyranosyl residues. In alkaline enzymes acid/base catalyst at position 100 is asparagine and aspartic acid for acidic pH enzymes. The overall structure of the catalytic domain of family 10 xylanase is an eight -folded barrel. The substrate binds to the shallow groove on the bottom of the 'bowl'. The (α/β) barrel appears to be the structure of two other endoxylanases of family 10. The substrate binding sites of the family 10 endoxylanases are not deep cleft as the substrate binding sites of family 11 endoxylanases and result in the lower substrate specificity of family 10 endoxylanases. (Subramaniyian & Prema 2002)

In the catalytic sites, especially *B. circulans* xylanase has two proximal carboxylates, Glu 172 and Glu 78, which act as an acid catalyst and nucleophil respectively. The abnormally high p*K*a of Glu 172, the character that enabled Glu172 to act as acid catalyst is resulting from the electrostatic interactions with

neighbouring groups like the Arg 112. Endo-1,4-β xylanase of the F10 xylanases is having a cylindrical [(α)/ (β)] barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the barrel and there are five xylopyranose binding sites The high molecular weight F10 xylanases tend to form low DP oligosaccharides. Xylanase cex from *Cellulomonas fimi* has a catalytic (N-terminus) region and a cellulose-binding domain (C-terminus), the former resembling the head and the latter the tail of a tadpole structure (Subramaniyian & Prema 2002).

Xylanases possess three to five subsites for binding the xylopyranose rings in the vicinity of the catalytic site. It was observed that *T. reesei* Xyn2 had five pyranose binding sites and three were found in Xyn I. The subsites for binding xylopyranose residues were tyrosine and not tryptophan that was essential for substrate binding in most glycosides.

7. CARBOHYDRATE BINDING MODULES (CBMS)

Most of the plant cell wall hydrolysing enzymes typically comprise a catalytic module and one or more carbohydrate binding modules (CBMs) that bind to plant cell wall polysaccharide (Hachem et al. 2000). CBMs allow alignment of the soluble enzyme with the insoluble polysaccharide to increase enzyme concentration at the point of attack and they are not essential for hydrolysis of the substrate. Binding of CBMs to insoluble substrates was significantly enhanced by the presence of Na^+ and Ca^{2+} ions. The CBMs are classified into different families based on their primary structure. Many of the modules in this classification system are not functionally characterised and their precise roles in hemicellulose hydrolysis are not yet fully understood. Of the different families of CBMs (more than ten), family 4 include thermostable *Rodothermus marinus* xylanase CBM with affinity for both insoluble xylan and amorphous cellulose. CBMs have also been reported to display additional functions such as substrate disruption and sequestering and feeding of single polysaccharide chains into active site of the catalytic modules. CBMs have been grouped into 23 different families, many of which are further divided into subfamilies (Bolam et al. 2001, Subramaniyian & Prema 2002).

8. MODE OF ACTION OF XYLANASES

Several models have been proposed to explain the mechanism of xylanase action. Generally hydrolysis may result either in the retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate. This suggests the involvement of one or two chemical transition states. Glycosyl transfer usually results in nucleophilic substitution at the saturated carbon of the anomeric centre and takes place with either retention or inversion of the anomeric configuration. Most of the polysaccharide hydrolyzing enzymes like cellulases and xylanases are known to hydrolyse their substrates with the retention of the C1 anomeric configuration. There is the involvement of double displacement mechanism for the anomeric retention of product. The double displacement mechanism involves the following features.

An acid catalyst protonates the substrate and a carboxyl group of the enzyme positioned on a covalent glycosyl enzyme intermediate with this carboxylate in which the anomeric configuration of the sugar is opposite to that of the substrate. This covalent intermediate is reached from both the directions through transition states involving oxo carbonium ions. Various non-covalent interactions provide most of the rate enhancement (Fig. 3). Based on the crystallographic study of xylopentaose binding to *Pseudomonas fluorescens* xylanase, enzyme mechanism has been proposed

Table 2 Characterisation of xylanases from differnet microorganisms.

Microorganisms	Mol. Wt.	Purification (KDa)	Yield (%) fold	Optimum pH and Temperature		Stabilities at[d]			pI	Km (mg/ml)	Vmax (mmol / min / mg)
				pH	Temp	pH (h)	Temp. (h)				
Acrophialophora nainiana	22	0.98	1.6	7.0	55	-	60 (1)	-	16 - 40.91	-	
Aspergillus awamori	39	-	-	5.5-6	55	-	-	5.7-6.7	1.0	10000	
	23	-	-	5.0	50	-	-	3.7	0.33	3333	
	26	-	-	4.0	45-50	-	-	3.3-3.5	0.09	455	
A. nidulans	34	24	7.5	6	56	4.0-6.7	56	3.4	0.97	1091	
A. sojae	32.7	8.2	9	5.0	60	5-8 (24)	50 (10 minutes)		3.5	-	-
	35.5	4.6	5	5.5	50	5-8 (24)	35 (10 minutes)		3.75	-	-
Aureobasidium pullulans Y-2311-1	25	5.8	10.3	4.8	54	4.5	50	9.4	7.6	2650	
Aureobasidium pullulans ATCC	21	38	6.3	3-4.5	35	-	-	-	2.93	866	
Cephalosporium sp.strain RYM-202	35	17.3	9.9	7.5-8.0	50	-	-	6.3	5.26	118.4	
	24	22.9	15.0	7.5-8.0	50	-	-	4.4	4.16	145.2	
Erwinia chrysanthemi	42	19.9	3.12	5.5	55	4-7	35	8.8	-	-	
Humicola insolens		6.0	-	-	6-6.65	55-60	-	-	9.0	-	-
	21	-	-	6-6.55	5-60	-	-	7.7	-	-	
Penicillium purpurogenum	33	15.8	5.7	7.0	60	6.0-7.5 (24)	40 (3)	8.6	-	-	
	23	5	4.3	3.5	50	4.5-5.5 (24)	40 (3)	5.9	-	-	
Trichoderma longibrachiatum	37.7	55.8	5.1	5-6	45	5	-	-	10.14	4025	

Microorganisms	Mol. Wt.	Purification (%) fold	Yield (KDa)	Optimum pH and Temperature		Stabilities at[d]				pI	Km (mg/ml)	Vmax (mmol / min / mg)
				pH	Temp	pH (h)		Temp. (h)				
Trichoderma viride		22	16	12.5	5	53		-	-	9.3	4.5	160
Trichoderma harzianun		20	7.5	-	5.0	50		-	40	-	0.58	0.106
BACTERIA												
Aeromonas caviae ME1		20	-	-	7	50		3.0-4.0	6.5-8	7.1	9.4	4330
Bacillus amyloliquefaciens	18.5-19.6	7.3	53.9	6.8-7.0		80	9	50	10.1	-	-	
B. circulans WL-12	85	-	-	5.5-7		-	-	-	4.5	8	-	50
Bacillus sp. Strain SPS-0	99	36	25	6.0	7	5	-		70 (4)0.7	145		
Bacillus sp. W1 JCM2888	21.5	124.6	25	6		65	4.5-10	-	8.54.5	-	74	
	49.5	9.6	2.6	7.9		70	4.5-70	-	3.70.95	-	74	
Bacillus sp. strain 41-1	(36)	36	3.6	15.3		9	50	-	- 5.3	3.3	1100	75
Bacillus sp. strain TAR-1	40	-	-	6		75	-	-	4.1	-	-	76
Bacillus sp. strain K-1	23	-	-	5.5		60	12	50	-	-	-	77
B. stearothermophilus T-643		38.9	46	6.5		75	-	70 (14.5 $^{1/2}$)	9	1.63	288	27
Streptomyces T-7	20.643	41.3	6.7	4.5-5.5		60	5.0 (144)	37 (264)	7.8	10	7600	78
Streptomyces sp.	50	48	33	5.5-6.5		60-65	5.5-6.5	55	7.1	9.1[a]	-	79
	25	2.85	2	5.0-6.0		60-65	5.0-6.0	55 10.06	-	-		
	25	3.6	8	5.0-6.0		60-65	5.0-6.0	55 10.26	11.2[a]	-		
Thermotoga maritima	217	54	6.5	85		95 (12$^{1/2}$)						
Thermotoga thermarum	266[b]	22.5	16.2	6		80	-	-	-	0.36	1.18	80
	35[c]	1.9	1.5	7		90-100	-	-	-	0.24	19.5	

a. Km estimated on xylotetrose. b. Dimer of 105 kDa and 150 kDa. c. Monomer
d. The stability in hours was given in bracket. Numbers preceding $^{1/2}$ represents the half-life time.

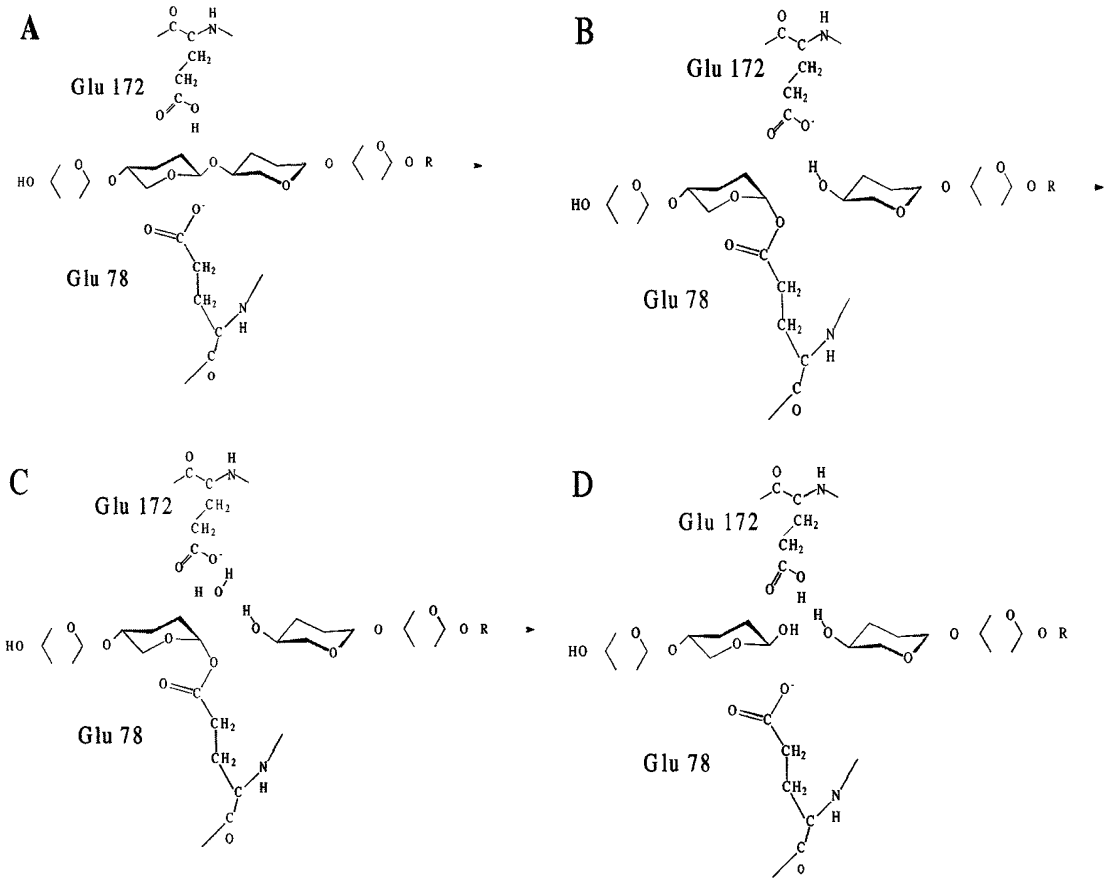

Fig. 3. Reaction mechanism by *Bacillus circulans* xylanase (1XNB). A) The helical xylan structure is positioned in the trough formed between Tyr 65 and Tyr 69. Glu 172 is the acid/base catalyst and Glu 78 is the nucleophile. B) The glycone in bound to Glu 78. This intermediate is retained during transglycosylation reactions. C) Water displaces the nucleophile. D) Dissociation and diffusion of the glycone (xylobiose) allow movement of the enzyme to a new position on the substrate. Xylanases of family 11 exhibit a random endo-mechanism rather than progressive cleavage. This is because the aglycone is released in step B and the glycone in D.

as follows:

(1) Xylan is recognised and bound by xylanase as a left-handed three fold helix

(2) The xylosyl residue at subsite -1 is distorted and pulled down toward the catalytic residues, and the glycosidic bond is strained and broken to form the enzyme-substrate covalent intermediate

(3) The intermediate is attacked by an activated water molecule, following the classic retaining glycosyl hydrolase mechanism and the product is released.

Bacterial xylanases during xylan hydrolysis release xylobiose, xylotriose and xylotetraose with formation of xylose during prolonged incubation. Fungal Xylanases exhibited synergistic interactions on different xylan substrates with different combinations of enzymes. A mixture of xylanases is more effective than single xylanase for hydrolysing different hemicellulosic substrates.

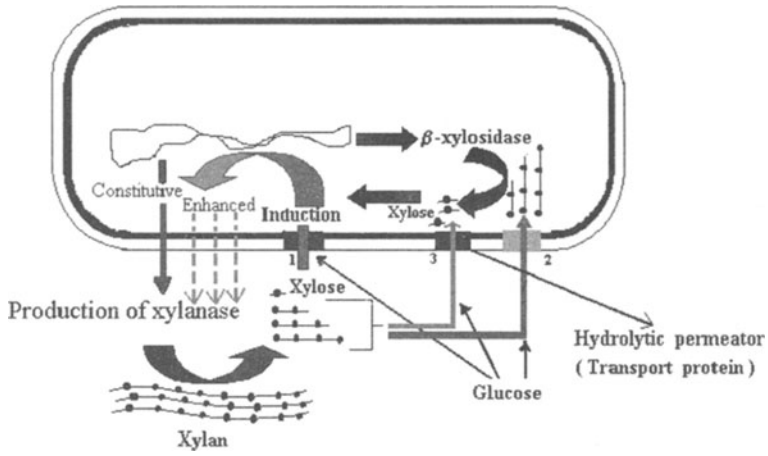

Fig. 4 Hypothetical model for xylanase gene regulation in bacteria. 1. Xylose monomers can be easily transported through the cell membrane which induces the enhanced xylanase synthesis. 2. The action of constitutively produced xylanases results in xylooligosaccharides eg. xylotriose, the transportation of which in to the cell later cause the enhanced synthesis. 3. The hydrolytic permeator can result in the transportation-coupled hydrolysis of xylooligomers from the constitutive xylanase action. All the cases could be affected by the presence of glucose. (Subramaniyian 2000)

9. XYLANASE GENE REGULATION

Xylanase production mainly occurs as constitutive enzyme production. The endo enzyme attacks xylan- the large heteropolysaccharide, which cannot enter to cell matrix and the resultant products are small molecular weight xylose, xylobiose, xylotriose and other oligosaccahrides. These smaller molecules could enter into the microbial cells for further degradation and stimulate the xylanase production by different methods. Entry of xylose is effected easily and it induces xylanase production. The larger xylooligosaccharides pose problem in transportation and there are two plausible explanations for the inductive xylooligosaccharides pose problem in transportation and there are two plausible explanations for the inductive role of larger molecules.

One of the explanations is that the xylo-oligomers are directly transported into the cell matrix where they are degraded by the intracellular β-xylosidase, which releases the xylose residues in an exo-action. The other possibility is that the oligomers are hydrolysed to monomers during their transportation through the cell membrane into cell matrix by the action of hydrolytic transporter having exo β-1,4- bond cleaving proteins like the β-xylosidases. Most of the microorganisms have β-xylosidases with transferase activities also. In both the ways the resulting xylose molecules enhance the production of xylanase. However, there are reports, where the xylose molecules repress the xylanase production where the

inducer may be yet another derivative from the xylan hydrolysates. If glucose is present in the growth medium, it imparts catabolic repression at the transcriptional level.

10. GENE CLONING OF XYLANASES

There are several reports regarding the genetic manipulation on xylanase producing microorganisms. Bacterial and fungal xylanase genes have been cloned and overexpressed in *E. coli, Bacillus* and yeast systems (Araki et al. 2000). *B. pumilus* β-xylosidase (xynB) gene and *T. reesei* β-xylanase-2 (xyn2) genes were cloned to *S. cerevisiae*. Genomic DNA from *B. circulans* Teri-42 was cloned in *E. coli* DH5-alpha using plasmid pUC19 resulted in 1- fold increase. The increase in expression was observed in when the same was cloned in *B. subtilis* with recombinant plasmid pBA7. *Bacillus* sp. strain NG-27 xylanase (47 kDa) active at 70^0 C and pH 8.4 was cloned in *E. coli* using shot gun library method (Subramaniyian & Prema 2002).

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Inulinases

18

Chandran Sandhya and Ashok Pandey

1. INTRODUCTION

Inulin, a linear β (2–1) linked fructose polymer, serves as a storage polysaccharide in the underground organs of several plants of the Asteraceae, including *Vernonia herbacea*, Jerusalem artichoke (*Helianthus tuberosus*) and chicory (*Cichorium endivia*) (Vandamme & Derycke 1983). It is insoluble in water and due to variation in chain length; its molecular weight varies between ± 3500 – 5500. It is non-toxic and almost totally degraded by colonic bacteria, but undigested by gastric or intestinal enzymes. Recently, inulin received a great interest as it is a relatively inexpensive and abundant substrate for the production of fructose rich syrups (Pandey et al. 1999a), which has beneficial effects in diabetic patients, increases the iron absorption in children, has high sweetening capacity so it can be used in the diet of obese persons (Roberfroid & Delzenne 1998). It also stimulates calcium absorption in postmenopausal women (Heuvel et al. 2000), prevents colon cancer (Rowland et al. 1998), used as dietary fibers because of its fat like texture (Roberfroid & Delzenne 1998). It also serves as a potential feedstock for fuel ethanol (Ohta et al. 1993). Since chemical hydrolysis of inulin to fructose displays several drawbacks, like undesirable colouring of the inulin hydrolysate, change in taste and aroma etc, much attention is now being paid to the use of inulinase for enzyme hydrolysis.

Inulinase is a non-specific β-fructosidase that releases fructose molecules from inulin. Inulin is depolymerised by two types of inulinase: exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) and endoinulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7). Endoinulinase are specific for inulin and hydrolyse the internal β-2,1-fructofuranosidic linkage to yield inulotriose, tetraose and pentaose as the main products whereas exoinulinases split terminal fructose units in sucrose, raffinose and inulin. The complete hydrolysis of inulin by exoinulinases yield 95% fructose syrup under optimized condition (Kumiko et al. 1999). Novo Industries has developed a commercial preparation of inulinase enzyme from *A. ficuum* (Novozym 230) (Carniti et al. 1991)

Due to inulin depolymerising ability, inulinases can be used for fructose and ethanol production (Kim & Rhee 1990, Pandey et al. 1999a, Vullo et al. 1991). They also find application in the production of inulo-oligosaccharides, which have macro-phage activating activity and lipid removing activity (Yakota et al. 1995, Yun et al. 1997, Park & Baratti 1992) as well as products such as gluconic acid, pullulan and acetone-butanol.

2. SOURCES OF INULINASES

Inulin hydrolysing enzymes are found in plants, filamentous fungi and bacteria. Microbial inulinases finds potential applications in reducing production costs and improving syrup quality compared with high-fructose corn syrup produced from starch (Pandey et al. 1999a, Ettalibi & Baratti 1987). Storey

and Schafhauser (1994) termed microbial inulinases as an important class of industrial enzymes. Table 1 lists the microorganisms commonly employed for inulinase production. Among filamentous fungi, *Aspergillus* and *Penicillium* species are major producers of inulinase (Onodera & Shiomi 1992, Nakamura et al. 1978a, Wenling et al. 1997, Gupta et al. 1994a, b). Several species of yeast are also considered a good choice for inulinase production, as enzyme from them can hydrolyse both inulin and sucrose (Roquette-Freres 1989, Selvakumar & Pandey 1998a, 1999a). There are also reports on production of inulinases by different bacteria, e.g. *Acetobacter* sp, *Arthrobacter* sp, *Staphylococcus* sp etc (Roquette-Freres 1989, Elyachioui et al. 1992, Selvakumar & Pandey 1997, 1998a, b, 1999a, b).

Table 1 Microorganisms employed for inulinases production

Microorganism	Nature of enzyme	References
Fungi		
Aspergillus aureus	Extracellular	Gupta et al. 1994 b
Aspergillus awamori	Extracellular	Kulminskaya et al. 2003
Aspergillus ficuum	Extracellular	Carniti et al. 1991, Tai-Boong et al. 1999, Jing et al. 2003, Ettalibi & Baratti 2001
Aspergillus fischeri	Extracellular	Gupta et al. 1994b
Fusarium sp.	Extracellular	Roquette-Freres 1989
Fusarium oxysporum	Extracellular, Intracellular	Gupta et al. 1988
Penicillium sp	Extracellular	Wenling et al. 1997
P. purpurogenum	Extracellular	Sharma et al. 2003
Rhizopus sp	Extracellular	Ohta et al. 2002
Streptomyces sp.	Extracellular	Gill et al. 2003
Bacteria		
Acetobacter sp.	Extracellular	Roquette-Freres 1989
Arthrobacter sp.	Extracellular	Elyachioui et al. 1992
Bacillus sp.	Extracellular, Intracellular	Allais et al. 1986
Escherichia coli	Intracellular	Yun et al. 1997
Flavobacterium mulivorum	Intracellular	Allais et al. 1986
Staphylococcus sp	Intracellular	Selvakumar & Pandey 1997, 1998a, b, 1999a, b
Yeast		
Candida sp.	Extracellular	Roquette-Freres 1989
Kluyveromyces marxianus	Extracellular	Selvakumar & Pandey, 1998b, 1999b

3. PRODUCTION OF INULINASES

The industrial application of inulinases is viable only when it is available in large quantities at competitive market prices. Hence, there is need for a microorganism producing high titres of the enzyme. Also, suitable fermentation technique has to be developed using cheaply available substrates.

3.1. Isolation and screening of inulinase producers

The increasing potential of inulinase application prompts screening for newer inulinase producing microorganisms, which can meet the conditions favourable to the industrial applications. The most useful

plate technique for screening inulinase producing microorganisms is based on the capability of carbohydrate assimilation (Yakota et al. 1991), but comparison of inulinase production by different strains is not possible with this technique. Another technique is based on the production of halos, which can be shown by precipitating the carbon source (inulin) by leaving the plates at 5°C for 7 days (Vullo et al. 1991). However, this method is tedious and time consuming process for large scale screening programmes. Castro et al. (1995) developed a plate assay based on radial diffusion on petri dishes containing inulin dye labelled with remazol brilliant blue as substrate. This procedure helps in specific, reliable and rapid inulinase estimation.

Passador et al. (1996) used a microtitre reader system for the screening of yeast belonging to the genera of *Kluyveromyces*, *Candida*, *Debaryomyces* and *Schizisaccharo-myces*. Four strains belonging to *K. marxianus* were found suitable for Inulinase synthesis. Viswanathan & Kulkarni (1996) isolated several fungal and yeast strains from dahlia rhizosphere belonging to *Aspergillus*, *Penicillium*, *Sporotrichum*, *Cladosporium* and *Streptomyces* sp. Wenling et al. (1997) isolated a high inulinase yielding species of *Penicillium*, designated as 91-4 from soil. Most microbial inulinases are exo-acting enzymes. Nakamura et al. (1978a) first reported that a strain of *Aspergillus niger* excreted two distinct inulin-hydrolysing enzymes, endo- and exoinulinase. *A. ficuum*, one of the industrially important fungi, also excreted endoinulinase as well as exoinulinase (Moussa 1987). *Clostridium* strains were isolated from sugar beet pulp from a sugar refinery, soil around a Jerusalem artichoke, fresh cow excrement, and mud from a tropical pond in a botanical garden. These strains were identified as new strains of *C. theromosuccinogenes* (Drent et al. 1991). *Staphylococcus* sp was isolated from rhizosphere soil samples around the dahlia tubers (Selvakumar & Pandey 1998a).

3.2. Submerged fermentation (SmF)

Among various substrates employed for inulinase production in SmF, inulin has been found most suitable in comparison to other substrates (Jing et al. 2003, Pandey et al. 1999b, Lam & Grootwassnik 1990, Kim et al. 1997). Several pure carbon sources have also been used for inulinase production (Table 2). If the microorganism exhibited inulinase activity coupled with invertase activity, sucrose may serve better source for enzyme production (Rouwenhorst et al. 1988, Nakamura et al. 1994). A 2% concentration of inulin has generally been found as most suitable. With higher inulin concentrations, accumulation of free reducing sugars at the initial hours of fermentation probably causes catabolite repression and consequently less

Table 2 Pure substrates used for inulinase production

Microorganism	Substrate	Concentration (%)
C. thermoautotrophicum, K. marxianus	Glucose	1-2
K. marxianus, K. fragilis	Fructose	1
A. niger, K. marxianus and several other yeasts strains	Sucrose	0.2-5
K. fragalis, C. thermoautotrophicum	Lactose	1
C. thermoautotrophicum	Maltose	1
F. oxysporum	Fructan	3
F. oxysporum	Fructosan	3
A. niger, K. marxianus, C. kefyr,	Inulin	0.5-2
Staphylococcus sp, Pseudomonas sp, K. marxianus	Caproyl-inulin	0.50
K. marxianus	Cholesteryl-inulin	0.5

enzyme activity is obtained (Jing et al. 2003). The effect of process parameters on inulinase production by *A. niger* were studied by Poorna & Kulkarni (1995) who suggested that inulinase production was probably inducible and subject to catabolite repression. However, reasonable amounts of enzyme could be produced in the absence of inducer. Chemically modified inulins could also be used as substrates for inulinase production (Derycke & Vandamme 1984, Fontana et al. 1994).

In any media for microbial cultivation, presence of adequate quantity of nitrogen is of prime importance. It could be supplemented in organic or inorganic form. Yeast extract, corn steep liquor, peptone, meat extract, beef extract, etc have been commonly employed as N-source for inulinase production (Toyohiko 1997, Gill et al. 2003, Nakamura et al. 1978a, Xiao et al. 1988, Wei et al. 1998). Influence of inorganic nitrogen sources on enzyme production has also been described by several workers (Pandey et al. 1999b, Nakamura et al. 1997). With ammonium salts, inulinase activity was very low (Gill et al. 2003) but with nitrates, marked activity was observed. In *Fusarium oxysporum* increased production of inulinase was observed with $NaNO_3$ (Gupta et al. 1990) whereas it exerted an inhibitory effect on inulinase production in *K. fragilis* (Choi et al. 1984).

Inorganic ions also influenced microbial inulinase production. Mg^{2+} and Zn^{2+} favoured inulinase production by *Aspergillis ficcum*. However, K^+, Ca^{2+}, Cu^{2+}, Fe^{2+} and Mn^{2+} inhibited inulinase production (Jing et al. 2003)

pH of the fermentation medium, growth temperature and agitation are important factors that determine enzyme yield. Several reports are available on inulinase production with acidic media (Nakamura et al. 1997, Ongen-Bayasal & Sukan 1996). Lower pH values offer advantageous for industrial fructose syrup production, as it prevent microbial contamination. However, in *Staphylococcus* sp, maximum enzyme activity was observed at pH 7.0 (Pandey et al. 1999b). *Flavobacterium* and *Arthrobacter* sp. showed maximum production at pH 8.0 and 7.0, respectively (Allais et al. 1986, Elyachioui et al. 1992).

Most of the reports on inulinase production describe mesophilic range as the suitable temperature (Nakamura et al. 1994, Ongen-Bayasal & Sukan 1996, Wei et al. 1998, Selvakumar & Pandey 1999b). However, thermophilic nature of inulinase producing bacteria has also been described (Kim et al. 1997).

3.3. Solid-state fermentation (SSF)

SSF can also be effective for inulinase production. Selvakumar & Pandey (1998b, 1999b) screened several substrates for inulinase production. Among these, wheat bran was found to the best. Roots and tubers of *Compositae* and *Graminae* also appear to be good sources for inulinase production. Chicory roots (Gupta et al. 1989), Dahlia rhizosphere (Wenling et al. 1997), Jerusalem artichoke (Pessoa et al. 1996) have widely been used for inulinase production. Each microbe-substrate system is unique and must be considered in terms of chemical composition and physical properties of the substrate, the growth characteristics and physiology of the organism to be cultured and nature of the product.

The optimum moisture content should be determined based on the desired product and the conditions of cultivation. Significant levels of inulinase activity were observed in substrates with 65% initial moisture content in both *Staphylococcus* sp. and *Kluyveromyces marxianus* cultures (Selvakumar & Pandey 1999b).

3.4. Immobilisation as a means for inulinase production

The application of immobilized microbial cells as an enzyme source eliminates the need for enzyme purification and in some cases the enzymes are more stable within the cell, their natural environment.

The method of immobilization and the mechanical properties of the matrix are important factors affecting the long-term stability of the biocatalysts. Significant enzyme yields and operational stability of the biocatalysts indicate the potential possibility for continuous enzyme production and continuous inulin hydrolysis by membrane-immobilized cells of thermophilic *Bacillus* sp. 11 on a large scale in a fluidized-bed reactor (Uzunova et al. 2002). The continuous production of fructose from inulin requires the use of inulinase in an immobilized form (Wenling et al. 1997). The use of barium alginate was adequate to immobilize *K. marxianus* cells with inulinase activity. The use of glutaraldehyde as a hardening agent offered greater mechanical resistance and durability and did not affect enzymic activity substantially. Temperature, an important factor for any enzymic activity, had a permeabilization effect on yeast cells and did not affect the enzyme activity within a large interval in the immobilized system. The cellular concentration affected the speed of hydrolysis, immobilized cell efficiency and gel resistance. The size of the beads, and therefore, the surface of contact influenced system efficiency (Barranco-Florido et al. 2001).

3.5.Improvement in inulinase production

In order to make production process economically feasible, it is imperative to improve the genetic characters in the microorganism so that their product formation characters are altered to desired level. Radiation such as ultra-violet (UV) and chemical such as ethylmethanosulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine can be used for the mutagenesis (Bourgi et al. 1986, Viswanthan & Kulkarni 1995, Nakamura et al. 1995, Skowronek & Fiedurek 2003). A mutant strain of *A. niger* van Teighem, generated by an exposure to UV for 15 minutes produced 3 times increased productivity (Viswanthan & Kulkarni 1995). Nakamura et al. (1995) also reported higher enzyme titres from a mutant strain of *A. niger* 817. Treatment of *K. fragilis* LG with 0.5% ethylmethanosulfonate resulted in mutants with enhanced activity of inulinase synthesis and one of them, D9, produced 1.5 times the amount of enzyme than the parent strain and secreted three times amount in to the medium (Bourgi et al. 1986). Thonart et al. (1988) mutated the strain of *K. fragilis* (ATCC 12424) using nitrosoguanidine and obtained 50 mutants. They found that most important improvement was intracellular inulinase activity. Conidia of *A. niger* 13/36, an active producer of inulinase, were subjected to mutagenesis with both UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), and the products were analysed for inulinase activity. The most active mutants grown under stress conditions showed significantly higher inulinase activity (about 1·2-4·5-fold), when compared with the parent strain (Skowronek & Fiedurek 2003).

4. INULINASE ASSAY

Colorimetric method employing inulin as substrate is the widely used assay procedure for estimating inulinase activity. To 2 ml 0.2% inulin solution, 2 ml acetate buffer (pH 4.6) and 0.5 ml enzyme is added. The reaction mixture is incubated at 50^{0}C for 20 min and then stopped by putting the tubes in boiling water for 10 min. The liberated reducing sugar is estimated by DNS method (Miller 1959). One enzyme unit (IU) is defined as the amount of enzyme, which produces 1 μMol of fructose under the assay condition.

Assay procedure specific for endoinulinases in the presence of exoinulinases utilizes a high molecular weight fraction of chicory inulin dyed with an azo-dye as substrate.

5. PURIFICATION AND CHARACTERIZATION OF INULINASES

Microbial extracellular inulinases can be purified by centrifugation/ultra-filtration, salt or solvent precipitation, followed by column chromatography (Wenling et al. 1997, Barthomeuf et al. 1991, Gupta

et al. 1994a, b, Gupta et al. 1988, Pandey et al. 1999a, Arand et al. 2002). Inulinases of intracellular nature need cell wall destruction followed by similar procedures as above. Table 3 provides an insight into various purification steps for inulinase from different sources. A crude inulinase preparation from *Aspergillus* sp. was dialysed for 48 h and the lypholysed enzyme powder was loaded onto a CM-Sepharose column for ion exchange chromatography. This step was selective for separating two forms of the enzyme. After high-performance gel permeation chromatography, endo-, and exo-inulinases were obtained in 5 and 13.9 % yields, respectively (Azhari et al. 1989). *K. marxianus* inulinase was extra-cellular in nature, which was fractionated from the fermented broth using acetone, yielding over 81% enzyme activity (Ku & Hang 1994). Vullo et al. (1991) also used acetone (40%, v/v) for partial purification of inulinase [precipitation after ammonium sulphate fractionation (85%)] from a bacterial source. About 70% of the total activity was extra-cellular in nature and all but 9% of the remainder was easily recoverable from cell soluble fractions. Jing et al. (2003) developed a new and convenient method to separate the inulinases by native-polyacrylamide gel electrophoresis (PAGE). Eight protein bands were obtained. Three bands were identified as exoinulinase and two bands were endoinulinase using TLC and HPLC.

Table 3 Purification of microbial inulinases

Microorganism	Purification steps	References
Aspergillus awamori	Ultrafiltration, Sephadex G-50 chromatography, DEAE-Sephadex chromatography, DEAE-5PW chromatography, Mono S chromatography, Phenyl-Sepharose chromatography	Arand et al. 2002
Penicillium sp	Ammonium sulphate precipitation, ion exchange chromatography on Wenling et al. 1997 DEAE-Sepharose and DEAE-Sephacel, ultra-filtration and Sephadex G-100 gel filtration, PAGE	
Rhizopus sp	DEAE- cellulofine A-500 and Sephacryl S-200 HP chromatography and SDS PAGE	Ohta et al. 2002
Kluyveromyces fragilis	Ethanol precipitation and chromatography on Sephadex G-200, DEAE-cellulose and CM-cellulose	Yun et al. 1997
Kluyveromces marxianus	DEAE-Trisacryl Plus and Superose 6HR column chromatography	Kushi et al. 2000

Characterisation of various forms of inulinases from microorganisms has been extensively reviewed by Pandey et al. (1999a). Xiao et al. (1989) reported that endoinulinase from *C. pannorum* was a glycoprotein and had an isoelectric point at pH 3.8. The enzyme was active on inulin but not on levan or sucrose and catalyzed the production of inulotriose, inulotetraose and inulopentaose. Extra- and intra-cellular inulinase from a bacterial strain of *C. acetobutylicum* showed pH and temperature optima as 5.5 and 47^{0}C. Extra-cellular inulinase showed high specificity for long chain inulofractions (Looten et al. 1987). Kim & Rhee (1989) characterised the immobilized inulinase from *A. ficuum*. The immobilized enzyme exhibited 23% of initial enzyme activity and was best active at pH 4.5.

Exoinulinase from *A. awamori* has a molecular mass of 69±1 kDa, with a pI of 4.4 and pH optimum of 4.5. The enzyme hydrolysed inulin and levan releasing fructose. The values of Michaelis constants Km and Vmax in the hydrolysis of inulin are 0.003 ± 0.0001 mM and 175 ± 5μmol min^{-1} mg^{-1}. The amino acid

sequence indicated that the protein belongs to glycoside hydrolase family 32 (Arand et al. 2002). Baron et al. (1996) studied immobilization of a fungal inulinase-I and bacterial inulinase-II using controlled-pore silica. Immobilized enzymes showed different pattern of inulin monomerization as well as different pH and temperature optima. Kulminskaya et al. (2003) applied ^{1}H-NMR analysis to investigate the hydrolytic activity of *A. awamori* inulinase. The obtained NMR signals and deduced metabolite pattern revealed that the enzyme cleaves off only fructose from inulin and does not possess transglycosylating activity.

From the literature, it can be seen that there has not been much difference in several properties such as pH and temperature optima of inulinases obtained from different microbial sources, i.e. fungi, yeasts and bacteria. The molecular weight ranges from 50kDa to 3000kDa (Table 4). Several fungal strains show pH optima between 4.5-7.0; for yeast strains, it is between 4.4-6.5 and for bacterial strains between 4.8-7.0. In case of temperature optima, it is in mesophilic as well as thermophilic range for fungal inulinases. Bacterial and yeasts inulinases generally show higher temperature optima (Table 4).

Table 4 Some properties of microbial inulinases

Microorganism	Forms	Mol.wt kDa	pH optima	Temperature optima	References
Penicillium sp	EI	60	4.5	50	Wenling et al. 1997
	EII	65			
F. oxysporum	Extra	300	6.5	37	Gupta et al. 1988
	Intra	300	5.8	30	
A. niger	P-IA	70	5.0	40	Nakamura et al. 1994
	P-IB	68	5.0	40	
A. awamori	Exo	69	4.5	-	Arand et al. 2002
C. pannorum	Endo	58	6.0-7.0	50	Xiao et al. 1989
B. subtilis	-	-	6.0-7.0	45-50	Vullo et al. 1991
C. acetobutylicum	Extra		5.5	47	Looten et al. 1987
K. fragilis	-	250	-	55	Gupta et al. 1994b
K. marxianus	-	-	4.4	55	Ku and Hang 1994

6. CONCLUSIONS AND PERSPECTIVES

The above discussions clearly indicate the importance of microbial inulinases. Environmental and nutritional factors have profound influence on inulinase production by fermentation techniques. The enzyme market demands price reduction and increased performance. Although most of the reports on inulinase synthesis is by submerged fermentation, solid-state fermentation offers a potential technique for inulinase production because of its advantages over submerged fermentation. Improvement in genetic characters of the microorganism leads to high enzyme titres. Immobilisation also provides an effective means for inulinase biosynthesis. Ample information is available on purification and characterization of these enzymes. Industrial application of enzyme requires ability to adapt to harsh conditions, hence future research interest should take place in the direction of enzyme engineering. Molecular approaches will also help in achieving desirable characteristics of the enzyme.

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Phytase

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1. INTRODUCTION

Recent market trends have clearly shown that enzymes have emerged as big feed supplements. Feed enzymes (protease, xylanase, phytase, and amylase) are the newest segment of animal nutrition market, which is growing fast. Several major animal nutrition companies are getting involved in the area of making dietary enzymes. One of these, phytase (myo-inositol hexakisphosphate phosphohydrolase, EC.3.1.3.8) catalyses the release of phosphate from phytate (myo-inositol hexakisphosphate), which is the main form of phosphorus predominantly occurring in cereal grains, legumes and oilseeds. Hydrolysis of phytic acid (phytate) to myo-inositol and phosphoric acid is considered an important metabolic process in several bio-systems. Increasingly demanding recent regulations world-over on controlling the agricultural pollution, particularly phosphorus pollution that limit the phosphorus content in manure, have intensified the phytase research. Environmental pollution due to the high-phosphate manure has resulted in the accumulation of phosphorus at various locations, especially in water bodies (Pandey et al. 2001a, Nampoothiri et al. 2002).

The current commercial feed supplement is a recombinant *Aspergillus niger* (formerly called as *A. ficuum*) phytase (Woodzinski & Ullah 1996) or phytase produced by *A. oryzae* i.e., Phytase Novo (Novo Nordisk 1995). BASF, which has marketing agreement with Dutch enzyme producer company Gist-Brocades, is the leading marketer of phytase worldwide. The trade name given to the feed enzyme containing phytase is 'Natuphos'. BASF has been collaborating since early 1990s with Royal Gist-brocades, a leader in feed enzyme technology in Europe, to market feed enzymes worldwide. Since then, Natuphos has been introduced in Europe, Asia, Canada, and recently in United States. The enzyme potentially increases phosphorus availability in the feed by as much as 30 %, allowing producers to reduce inorganic phosphorus supplementation by up to 17% (BASF-Animal nutrition Partner, 1996). 'Allzyme' is the trade name given by the Alltech, which has established a manufacturing facility in Mexico for the production of phytase (Anons 1999). Similarly 'Cenzyme' is a product from Cenzone, which is a unique blend of concentrated digestive enzymes including phytase from a fungal source (Cenzone 1999). 'Ronozyme' is the result of a joint development between F. Hoffmann-La Roche Ltd, Switzerland and Novozymes A/S (previously known as Novo Nordisk), Denmark. Roche provides a full complement of enzyme products for both pig and poultry diets under the brand names Ronozyme and Roxazyme. Natuphos (BASF), Ronozyme (Roche), Allzyme (Alltech) are some of the histidine acid phosphatases commercially produced and marketed in large scale coupled with over expression of phy

A gene. Danisco animal nutrition has launched a yeast phytase by name phyzyme XP that shows an efficacy of around 0.33 per ton of broiler feed. For its ideal role as an animal feed additive, efforts are now targeted towards improving its thermostability and pH optimum.

2. PHYTIC ACID

Phytic acid is the major organic phosphate component of soil. Phytate (myo-inositol 1, 2, 3,4,5,6 hexakis dihydrogen phosphate) is the storage form of phosphorus in plants and it represents 50-85% of total phosphorus. Phytate rapidly accumulates in the seeds during the ripening period. It is stored in the leguminous seeds and oil seeds in the globoid crystals within the protein bodies (Erdman 1979). In cereal grains such as rice and wheat, it is found in the bran fraction such as aluerone layer and pericarp whereas in corn, it is seen in the endosperm (O'Dell et al. 1972). Besides being a storage product of phosphorus, it is also believed to act as a natural antioxidant and a store of high-energy phosphoryl groups. Some of the beneficial effects include its role as a second messenger in cell signalling (Sasakawa et al. 1995), in ATP metabolism (Safrany et al. 1999), DNA repair (Hanakahi et al. 2000), RNA export from the nucleus (York et al. 1999), controlling cancer and preventing effects of heart disease, and diabetes (Shamsudddin & Vucenik 1999).

Phytic acid was first recognized as calcium/magnesium salt of organic phosphates by Pfeffer in 1872 in the subcellular particles of the wheat endosperm. In 1903 Posternak was the first to describe about phytic acid. Anderson in 1914, proposed the structure of phytic acid, which was confirmed finally by NMR spectroscopy in 1969 (Johnson & Tate 1969).

Dietary phytate (myo-inositol hexakisphosphate) has received much investigative attention as an antinutrient (Bolland et al. 1975, Common 1940). However, monogastric animals are unable to metabolize phytic acid and it is largely excreted in their manure. Hence, the presence of phytic acid in animal feeds for chickens and pigs are generally undesirable. Many animal experiments have documented the nutritional implications of phytate by binding dietary macro and microelements (Ca, Mg, Fe, Zn, Cu, Mn, Mo and Co), thus reducing their solubility and bioavailability (Erdman & Poneros-Schneier, 1989). The effect of phytic acid on mineral bioavailability is influenced by pH, the amount of phytic acid, mineral concentration, association/configuration of phytic acid with dietary protein/fiber/starch, heat treatment, prefeed processing of the diet and the presence of other metal ions in a diet (Graf 1986). Another hypothesis with regard to the negative effect of phytic acid on protein utilization is the possible inhibition of digestive enzymes such as trypsin and pepsin (Graf 1986, Nair & Duvnjak 1991). Furthermore, researchers demonstrated that amylases of different origin were strongly inhibited by phytic acid (Sharma et al. 1978). Phosphates pollution of fresh and ground water could lead to eutrophication with severe biological consequences such as cyanobacterial bloom, hypoxia, death of fish, production of nitrous oxide- a potent green house gas, etc. (Mallin, 2000).

3. PHYTASE – THE HYDROLYTIC ENZYME

Phytase is classified as the family of histidine acid phosphatases (Peddington et al. 1993) and is found primarily in microorganisms and plants. Phytase catalyzes the hydrolysis of phosphate from phytic acid to inorganic phosphate and myo-inositol phosphate derivatives. In addition, the inositol phosphates of various degrees of dephosphorylation from inositol hexakisphosphate are generated as intermediates or in some cases, as end products (Holub 1982). Myo-inositol and its isomers or biochemical derivatives

are broadly distributed in higher plants, microorganisms, and mammalian tissues and cells in which they provide important biological functions (Liu et al. 1998). Indeed, myoinositol is the primary form for nutritional and metabolic functions.

Phytase is an acid phosphohydrolase, which hydrolyzes phosphomonoester bonds from phytate, thereby liberating inorganic phosphates. Two classes of phytase are recognized based on the position of first phosphate hydrolyzed namely, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26) (Cosgrove 1980). 3-phytase (myoinositol-hexakisphosphate 3-phosphohydrolases) is mainly of microbial origin whereas 6-phytase is derived from plants. Yet another class of phytase, GmPhy (EC 3.1.3.2) has recently been isolated from the cotyledons of germinating soybeans (Hegeman & Grabau 2001). Most of the phytases are histidine acid phosphatases; however, alkaline phytases have also been identified. All phytases seldom have a similar structure and the cleavage of phosphate groups from the phytic acid is also not by the same mechanism.

Phytase was discovered by Suzuki et al. from rice bran when they isolated inositol and orthophosphoric acid as the reaction product from phytic acid in 1907 (Nagai & Funahashi 1962). The sequence of hydrolyses of phytase was first established by Tomlinson and Ballou in 1962.

4. SOURCES OF PHYTASE

4.1. Plant sources

Phytase has been reported in wheat, maize, corn seeds, faba beans, mung beans, lettuces, rye, and oil seeds. The highest activity is found in wheat, rye, and barley (Greiner & Konietzny 1997, Konietzny et al. 1995, Sandberg & Svanberg 1991). Phytase from germinating seeds (Gibson & Ullah 1988, Konietzny et al. 1995) and pollen (Scott & Loewus 1986) have been purified and characterized. A number of investigations have shown that phytase activity markedly increases with a concomitant reduction of IP6 levels during pollen and seed germination (Gibson & Ullah 1988, Lin et al. 1987). Alkaline phytases have also been reported to be present in the pollen of *Lilium longiflorum* (Scott & Loewus 1986), *Typha latifolia* (Hara et al. 1985), and in legume seeds (Scott 1991). They found that pea and bean phytases had the highest activities at 37°C. Honke et al. (1999) showed that phytase activities of lentils, peas and faba beans were within a range of 37–55°C. Phytase is also associated with the root cell wall and with mucilage in apical root zones. Kemme et al. (1999) have shown that plant phytases are effective in the digestion of phytate in pigs.

4.2. Animal sources

Mc Collum & Hart in 1908 reported the presence of phytase in the liver of calf. In 1937, Patwardhan detected phytate hydrolysis in rat intestine. Cooper & Gowing in 1983 detected phytase activity in the small intestine of mammals. Phytase was also reported in the erythrocytes of chicken (Martin & Luque 1985) and intestinal mucosa of rat (Yang et al. 1991).

4.3. Microbial sources

Several fungal, bacterial and yeast strains have been reported as the source of phytase (Table 1).

4.3.1. Fungal sources

Fungal cultures have most widely been used for the production of phytase (Pandey et al. 2001a, Liu et al. 1998) as they generally result high yields and acid tolerance for feed production (Kim et al. 1999b).

Table 1. Sources of phytase

Micro organism	References
Bacteria	
Bacillus sp.	Choi et al. (1999), Shimizu (1992), Yoon et al. (1996)
B. subtilis	Kerovuo et al. (2000)
B. amyloliquefaciens	Kim et al. (1999a), Ha et al. (1999)
Enterobacter sp.	Yoon et al. (1996)
Escherichia coli	Sunitha et al. (1999)
Klebsiella sp.	Shah & Parekh (1990)
K. anogenes	Tambe et al. (1994)
K. oxytoca	Jareonkitmongkol et al. (1997)
K. terrigena	Greiner et al. (1997)
Lactobacillus amylovorus	Sreeramulu et al. (1996)
Pseudomonas sp.	Richardson & Hadobas (1997)
Treponema sp.	Yanke et al. (1998)
Fungi	
Aspergillus sp.	Kim et al. (1999b)
A. carbonarius	Al-ashesh & Duvnjak (1995)
A. carneus	Ghareib (1990)
A. ficuum	Ebune et al. (1995), Howson & Davis (1983)
A. fumigatus	Rodriguez et al. (2000)
A. niger	Ahmad et al. (2000), Papagianni et al. (2000)
A. oryzae	Shimizu (1993)
A. terreus	Mitchell et al. (1997)
Penicillium sp.	Iwama and Sawada (1999)
Mucor racemosus	Bogar et al. (2003a)
Trichoderma reesei	Nasi et al. (1999)
Thermoascus auranticus	Nampoothiri et al. (2004)
Yeast	
Arxula adeninivorans	Sano et al. (1999)
Hansenula polymorpha	Mayer et al. (1999)
Rhodotorula gracilis	Bindu et al. (1998)
Schwanniomyces castellii	Segueilha et al. (1992)
S. castelli	Lambrechts et al. (1993)
S. occidentalis	Nakamura et al. (1999)

More than 200 fungal species have been reported for its production out of which *Aspergillus* sp. is the most common. *Mucor* (Bogar et al. 2003a), *Rhizopus* (Howson & Davis 1983) and *Penicillium* (Iwama & Sawada 1999) are the other phytase producing species. Howson & Davis (1983) examined 84 fungi from 25 species for the production of extracellular phytase capable of hydrolysing phytate, out of which *A. ficuum* NRRL 3135 showed the highest activity. *A. oryzae* (Shimizu 1992), *A. niger* (Volfova et al. 1994, Ahmad et al. 2000), *A. fumigatus* (Mullaney 2000), *A. carbonarius* (Al-Asheh & Duvnjak 1994), *A. terreus* (Mitchell et al. 1997) are some phytase producing species of *Aspergillus*. *Rhizopus* sp. namely *R. oryzae, R. oligosporus* and *R. stolonifer* have also been found to produce phytase (Howson & Davis 1983). *Trichoderma reesei* was also reported to produce phytase (Nasi et al. 1999). Thermophilic fungi such as *Thermomyces lanuginosus* and *Sporotrichum thermophile* were reported to produce phytase at 65 and 45°C, respectively (Berka et al. 1998, Ghosh 1997).

4.3.2. Bacterial sources

Not many bacterial strains have been used for phytase production as generally enzyme yields are low (Pandey et al. 2001a, Wodzinski & Ullah 1996) and function only at neutral pH (Liu et al. 1998). However, species of *Klebsiella* (Shah & Parekh 1990), *Bacillus* (Kim et al. 1999a), *E.coli* (Greiner et al. 1993), *Pseudomonas* sp. (Richardson & Hadobas 1997), *Treponema* sp. (Yanke et al. 1998) have been reported to produce the enzyme. Genetically modified strains of *Escherichia coli, Bacillus subtilis, B. amyloliquefaciens* and *Klebsiella* sp. are some of the potent phytase producers. A bacterial strain capable of producing a thermo-acid-tolerant phytase was isolated from soil around haystacks and designated as strain PH01 (Popanich et al. 2003). Another thermostable extracellular phytase was obtained from *Bacillus* sp DS11 isolated from the Korean cattle shed soil (Kim et al. 1998a). Lactic acid bacteria have also been reported as the source of phytase (Angelis et al. 2003).

Phytase activity could be extra- or intracellular. When localization of phytase activity of *Enterobacter* sp. 4 was determined, 81.7% of the enzyme activity was found in the extracellular fraction, 4.4% of the activity was detected in the periplasmic space, and the remaining activity was in the intracellular and cell-bound fraction (Yoon et al. 1996). A cytoplasmic phytase was reported from *Klebsiella terrigena* (Greiner et al. 1997).

4.3.3. Yeast sources

Only a few yeast strains have been reported to produce phytase, e.g. *Pichia spartinae* and *P. rhodanensis* (Nakamura et al. 2000), *P. anamola* (Vohra & Satyanarayana 2002), *Schwanniomyces castellii, S. occidentalis* (Nakamura et al. 1999), (Segueilha et al. 1992), *Arxula adenenivorans* (Sano et al.1999), *Rhodotorula gracilis* (Bindu et al. 1998), etc.

5. PRODUCTION OF PHYTASE

Though phytase can be obtained from various sources as described above, their commercial need for the animal feed industry is generally met by production through microbial sources. Both submerged (SmF) as well as solid-state fermentation (SSF) have been employed for phytase production (Pandey et al. 2000, 2001a, b). Nature of the substrate, availability of nutrients, type of strain, culture conditions are the critical factors affecting yield and should be critically considered for selecting a particular production technique. Culture conditions and genotype of the strain can affect the mass transfer rates. In SmF, filamentous fungus is exposed to hydrodynamic forces, while in SSF the surface of the solid matrix supports its growth.

Most of SSF studies have employed the strains of *Aspergillus* sp. as the producing organism and among these *A. ficuum* has been the most widely used. Often agro-industrial residues have been used as the substrate (Pandey et al. 1999, 2000, Pandey & Soccol 2000). These include canola meal, coconut oil cake, wheat bran, etc (Bogar et al. 2003a, Bogar et al. 2003b, Sabu et al. 2002, Sabu et al. 2003, Ebune et al. 1995, Al-asheh & Duvnjak 1994). The strains used belonged to *Aspergillus, Mucor* and *Rhizopus* sp. Depending up on the nature of the substrate, initial moisture content of substrate could be between 53-64%. Supplementation of the medium with additional carbon source such as glucose at lower concentrations (6g/l) and surfactants such as Na-Oleate or Tween-80 could be useful for enzyme synthesis. Among these, a strain of *M. racemosus* gave the maximum yield (14.51 IU/ g dry matter phytase activity) on coconut oil cake (Bogar et al. 2003a). Phytase production was increased to 26 IU/ g dry matter when coconut oil cake was supplemented with glucose, casein and ammonium sulphate.

Use of inert materials has been considered useful in SSF processes as it offers specific advantages in down stream processing (Pandey et al. 2000). This was considered as a useful point by Gautam et al. (2002) who used polystyrene beads for phytase production by a fungal strain and reported potential benefits of using polystyrene beads over conventional substrates such as wheat bran.

Papagianni et al. (2000) studied the phytase production using *A. niger* strain in SmF and SSF. In both the cases addition of slow releasing organic phosphate source enhanced the enzyme production. The solid substrate contained a combination of wheat bran and soybean meal whereas SmF medium contained corn starch, glucose, peptone and salts such as KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4. The pH of the medium was 5.0 – 5.3 initially, but the addition of wheat bran resulted in 5.5 – 5.8. For a bacterial culture of *Enterobacter* sp., which produced phytase, optimum pH was 5.5 (Yoon et al. 1996).

SmF has been used for phytase production using mostly bacterial or yeast cultures, although fungal strains too have been successfully employed. Nampoothiri et al. (2004) studied thermostable phytase production by *Thermoascus auranticus* in SmF, wheat bran was used as a carbon source supplemented with different mono-, di-, and polysaccharides such as glucose, sucrose, starch, etc. Addition of glucose and starch was found to be useful for enzyme production; addition of Tween 20 (2%) also resulted in higher enzyme titres. Sreeramulu et al. (1996) used several lactic acid bacteria (*Lactobacillus* and *Streptococcus* sp) and found *L. amylovorus* B4552 as the best strain, which produced 125–146 units phytase/ml in a glucose medium supplemented with inorganic phosphorous. A genetically modified *B. subtilis* produced phytase (2 U/ml) and the yield was 100-fold higher than the wild type *B. amyloliquefaciens* DS11 (Kim et al. 1999a). Another *Bacillus* sp strain KHU-10 produced extracellular phytase (0.2 U/ml) in a maltose, peptone and beef extract medium after 4 days of incubation (Choi et al. 1999).

Phytase production using yeast culture increased with pH and dilution rate in a continuous culture using *S. castellii*, while yield decreased when the phosphate content was increased. An efficient process for the low-cost production of phytase was developed by Mayer et al. (1999) using *Hansenula polymorpha*, where glucose or glucose syrups were used as main carbon source instead of glycerol. Exceptionally high enzyme titers were obtained (up to 13.5 g/l) with phytase representing over 97% of the total accumulated protein.

Apparently, medium composition and fungal morphology greatly affects phytase production in SmF. Sunitha et al. (1999) optimized the medium components for recombinant phytase production by *E. coli* using response surface methodology. The combined effect of the medium components: tryptone, yeast extract and NaCl was well understood by using the experimental design (Central Composite Design) and it was also found that addition of glucose to medium greatly influenced phytase production. The optimized medium with glucose resulted in phytase activity of 2250 U/ml. Another statistical optimization of medium components was carried out using response surface methodology to enhance the phytase production using a yeast strain *Pichia anomala* (Vohra & Satyanarayana 2002).

5.1. Heterologous gene expression for phytase production

Attempts have been made for the cost-effective enhanced production of phytase through various biotechnological means such as gene cloning by recombinant DNA techniques. Of course, in practice obtaining the recombinant protein is not easy as it sounds, still satisfactory yields of the recombinant protein have been achieved. A DNA fragment (1.4–kbp) coding region of gene for phytase was inserted into the expression vector pPICZalpha A and expressed in *Pichia pastoris*, which resulted in a high level functional expression of extra-cellular phytase (r-Afp) (Rodriguez et al. 2000). Similarly, *A. niger* phytase gene (phyA) was expressed in *S. cerevisiae*. Here the expression vector used was pYES2. The expressed phytase had a molecular weight of 120kDa (due to heavy glycosylation) and was thermostable. The gene phyA from *A. niger var. awamorii* ALKO243 encoding phytase was cloned and sequenced and the re-introduction resulted in over production of phytase (Piddington et al. 1993). A second phytase gene (phyB) from *A. niger* NRRL 3135 was cloned and was found to contain four exons (Ehrlich et al. 1993).

Gist-Brocades cloned multiple copies of *A. niger* NRRL 3135 phyA gene into their PluGBug system that yielded high levels of phytase and the product is being marketed as Natuphos (Van Dijck 1999). Novo Nordisk is producing a commercial phytase under the trade name Biofeed phytase (Ronozyme P). This product is a result of the phytase gene cloned from *Peniophora lycii* (a basidiomycete) being over-expressed in *A. niger*. The phyA gene from the thermophilic fungus *Thermomyces lanuginosus* was inserted into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter and the recombinant gene product obtained retained its activity at 75°C. The phytase gene (phyC) was cloned from *B. subtilis* VTT E-68013 genome library and the sequence did not show any homology to the known phytases or phosphatases, but exhibited phytase activity (Kerovuo et al. 1998). The gene coding for phytase from *Bacillus* sp. DS11was cloned in *E. coli* and molecular mass of the recombinant protein was estimated to be 41.80kDa (Kim et al. 1998b).

New concepts in plant biotechnology using transgenic plant engineering for industrial enzyme production have been employed for phytase production also (White-lam 1995, Day 1996). The phyA gene from *A. ficuum* coding for (441 aminoacid length) phytase was expressed in *Nicotiana tabacum* (tobacco) leaves. The temperature optima of the recombinant protein remained the same while the pH optima shifted from 5 to 4. Ullah et al. (2002) cloned the phytase gene from *A. ficuum* and expressed in alfa-alfa plants. The expressed enzyme was purified, which exhibited all the properties of fungal phytase. The phytase gene from *A. niger* NRRL 3135 has been expressed in soybean, but the recombinant phytase had a lower molecular weight than the native fungal enzyme.

Several attempts have also been made to clone and express fungal phytase in animals (Mullaney et al. 2000). The phytase gene app A from *E. coli* was cloned into mouse and regulated for expression in salivary glands. The salivary phytase from the transgenic mouse reduced the fecal phosphorus by 11% (Golovan et al. 2001).

6. PURIFICATION OF PHYTASE

Generally phytases are purified by typical salt or solvent precipitation followed by chromatography such as ion exchange and gel filtration. Ultrafiltration could also be used in combination with chromatography. An extracellular phytase from *B. subtilis* (natto) N-7 was purified 322-fold to homogeneity by ultrafiltration and a combination of sephadex G-100 and DEAE – Sepharose CL- 6B column chromatography (Shimizu 1992). The extra-cellular phytase from *Bacillus* sp. DS11 was purified to homogeneity by acetone precipitation and phenyl-sepharose, and Superose 12 column chromatography (Kim et al. 1998a). *E. coli* phytase was purified using ammonium sulfate precipitation (25 to 80% saturation), followed by CM-sepharose CL6B, DEAE-sepharose CL6B, phenyl sepharose CL4B and MonoS HR, 5/5 chromatography (Greiner et al. 1993). Golovan et al. (2000) purified a phytase from *E. coli*, which was further separated by chromatofocusing into two isoforms of identical size with isoelectric points of 6.5 and 6.3. Tambe et al. (1994) purified phytase from *K. aerogenes* using ion exchange chromatography on DE-52 followed by gel filtration on sephadex G-200 column. Another phytase from *Klebsiella oxytoca* MO -3 was purified 400-fold and partially characterized.

Nagashima et al. (1999) purified a phytase from *A. niger* SK-57 to homogeneity in four steps by using ion-exchange chromatography (two types), gel filtration and chromatofocusing. SDS-PAGE of this purified sample gave a single stained band at a molecular mass of approximately 60 kDa. An acid phosphatase was purified from *A. ficcum* NRRL 3135 by gel filtration and ion- exchange dextran chromatography (Irving et al. 1974). Pasamontes et al. (1997) concentrated *A. fumigatus* phytase over-expressed in *A. niger* NW205 approximately 50-fold by ultrafiltration followed by ion-exchange chromatography. Acetone fractionation (60% v/v), gelfiltration on G-100, followed by DEAE-cellulose chromatography was the technique used for the purification of phytase from *A. oryzae* NRRL 1998 (Wang et al. 1980). Gharieb, (1990) successfully purified phytase from *A. carneus*, 43–fold from the culture filtrate by acetone precipitation, gel filtration through sephadex G-75 and ion-exchange chromatography on DEAE-cellulose. Partially purified phytase was obtained from *A. adeninivorans* by sephadex G50 filtration and/or DEAE chromatography (Sano et al., 1999).

A three-stage purification method including anion and gel filtration chromatography was used to concentrate the phytase from *S. castellii* (Segueilha et al. 1992, Lambrechts et al. 1993). A phytase from *Candida krusei* Wz-001 was purified to electrophoretic homogeneity by ion-exchange chromatography, hydrophobic interaction chromatography and gel filtration. Superdex 75pg column and phenyl Sepharose HP column were used for this (Quan et al. 2002).

7. BIOCHEMICAL CHARACTERIZATION AND CATALYTIC PROPERTIES

Properties of enzymes are important in determining their potential use in industrial applications. Phytase is an ester-hydrolyzing enzyme with an estimated molecular mass of 35-700 kDa depending upon the sources of origin (Table 2). The distinct molecular forms of phytases obtained from different sources exhibited differences in physicochemical properties such as the Michaelis constant (K_m), optimal pH, and the thermostability.

Table.2. Characteristics of purified phytases from different sources

Source	Optimal pH	Optimal Temperature (°C)	Molecular weight (kDa)	Km (mM)	Reference
Bacteria					
Bacillus subtilis	7.0	55	36.5	0.04	Powar & Jaganathan 1982
Escherichia coli	4.5	55	42	0.13	Greiner et al. 1993
Klebsiella aerogenes	4.5-5.2	60	700	0.11	Tambe et al. 1994
Pseudomonas sp	5.5	40	-	0.016	Irving & Cosgrove 1971
Fungi					
Aspergillus niger 92	5.0	55	100	0.44	Dvorokava et al. 1997
Aspergillus oryzae	5.5 (4.5)	50	120-140	-	Shimizu, 1993
Penicillium caseoicolum	3.0	45	60-81	-	Amano Pharmaceuticals 1995
Rhizopus oligosporus	4.5	55	-	0.15	Sutardi & Buckle 1988
Yeast					
Schwanniomyces castellii	4-5	75-80	490	0.038	Segueilha et al. 1992
Arxula adeninivorans	4.5	75-80	-	0.25	Sano et al. 1999
Pichia anomala	4.0	60	64	0.20	Vohra & Satyanarayana 2002

7.1. Temperature

The temperature optimum for phytases falls in the range of 25 – 80°C. Optimum temperature could vary depending up on the source such as between 50-60°C for phytase from *A. carbonarius* (Al-asheh & Duvnjak 1994), *Klebsiella aerogenes* (Tambe et al. 1994), *Enterobacter* sp.4 (Yoon et al. 1996), *Selenomonas ruminantium* (Yanke et al. 1999) and 70-80°C from *Bacillus* sp. DS11 (Kim et al. 1998a), *S. castellii* (Segueilha et al. 1992), *A. adeninivorans* (Sano et al., 1999), *Pichia rhodanensis* and *P. spartinae* (Nakamura et al. 2000). However, phytases from *Aerobacter aerogenes* and *Candida krusei* Wz-001 showed optimal temperature as 25 and 40°C, respectively (Greaves et al. 1967, Quan et al. 2002).

Among the thermophilic fungi, *Thermomyces lanuginosus* had an optimum phytase activity at 65°C (Berka et al. 1998) and *Sporotrichum thermophile* at 45°C (Ghosh 1997). Phytase of mesophilic fungal sp. *A. fumigatus* and *A. niger* NRRL 3135 showed optimum activity at 37°C (Pasamontes et al. 1997) and at 55°C (Howson & Davis 1983), respectively. It is interesting to note that phytases from mesophiles generally showed temperature optima in the thermophilic range.

Thermostability of phytase is considered to be an important and useful criterion for its industrial application. Thermostable phytase from different sources have shown different degree of heat stability and half-life at elevated temperature (80°C and above) (Pandey et al. 2001a, Rodriguez et al. 2000, Kim et al. 1999a). Phytase produced by *Thermoascus auranticus* was thermostable whose temperature optima was 55°C. However, 80% of the activity still remained when temperature was shifted to 70°C (Nampoothiri et al. 2004). An extremely thermostable phytase from *A. fumigatus* has been reported by Pasamontes et al.

(1997), which was able to withstand temperatures up to 100°C over a period of 20 min with a loss of only 10% of the initial enzymatic activity. This was claimed to be the first such highly thermostable phytase.

7.2. pH

Most phytases are active within the pH range 4.5 – 6.0 and the stability of enzyme decreases dramatically by pH values lower than 3 or higher than 7.5. Phytases from fungal origin show pH optimum between 4.5 and 5.5, and those of bacterial origin have a pH optimum at 6.5 to 7.5. The phytase of *Aerobacter aerogenes* (Greaves et al. 1967), *Pseudomonas* sp. (Irving & Cosgrove 1971), *E. coli* (Greiner et al. 1993), *Lactobacillus amylovorus* (Sreeramulu et al. 1996) showed optimum pH in the range between 4 and 5.5. *Enterobacter* sp.4 (Yoon et al. 1996) and *Bacillus* sp. DS11 (Kim et al. 1998a) exhibited phytase activity with pH optimum in the neutral range (7.0 to 7.5). The mesophilic strain *A. niger* NRRL 3135 produced two different phytase designated as Phy A and Phy B, one with pH optima at 5.5 and the other with 2.0, respectively (Howson & Davis 1983). Phytase from *A. carbonarius* produced in SSF had pH optima of 4.7 (Al-asheh & Duvnjak 1994), *R. oligosporus* had a pH of 4.5 (Sutardi & Buckle, 1988). Yeast phytases were found to have an optimal pH of 4 to 5 when measured at optimal temperature of 50 to 60°C, while at 37°C, many strains produced another phytase with the optimum pH of 3 to 4 (Quan et al. 2002).

7.3. Metal ions

The requirement for metal ions for phytase activity differs depending upon the sources. The extracellular phytase actively purified from *B. subtilis* (natto) N-77 was greatly inhibited by EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+} (Shimizu 1992). Kerovuo et al. (1998) purified a phytase from *B. subtilis* strain VTTE-68013, which required calcium for its activity and/or stability and was readily inhibited by EDTA. Kerovuo et al. (2000) studied the metal ion requirement of a *B. subtilis* phytase. Removal of metal ions from the enzyme by EDTA resulted in complete inactivation of the enzyme. The loss of enzyme activity was most likely due to a conformational change, as the *circular dichroism* spectra of holo enzyme and metal-depleted enzyme were different. The enzyme was able to restore the active conformation partially when incubated in the presence of calcium whereas only minor reactivity was detected with other divalent metal ions and their combinations. Hence, it was concluded that for the active conformation of *B. subtilis* phytase calcium is essential. Phytase of *Bacillus* sp. DS11 was strongly inhibited by EDTA, Cd^{2+} and Mn^{2+} and moderately inhibited by Hg^{2+}, Mg^{2+}, Ba^{2+} and Cu^{2+} (Kim et al. 1998a) while the phytase activity of *Selenomonas ruminantium* was strongly inhibited by the presence of ions such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} (Yanke et al. 1999). In both the cases, the metal ion concentration in the reaction mixture was same (5 mM). This pattern of sensitivity was similar to that of *E. coli* and *K. terrigena* (Greiner et al. 1993, Greiner et al. 1997) and *A. ficuum* (Ullah & Cummins 1988). A partially purified phytase from *Klebsiella oxytoca* MO-3 was strongly inhibited by NaF, Zn^{2+}, Fe^{2+} and Cu^{2+}, but not by EDTA or N-ethylmaleimide (Jareonkitmongkol et al. 1997).

The extracellular phytase from the mesophilic strain of *A. niger* was inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+}, Cd^{2+} ions and was activated by Ca^{2+}, Mg^{2+} and Mn^{2+} ions (Dvorakova et al. 1997). Phytase of *S. castelli* was strongly inhibited by 5 mM Zn^{2+} and Cu^{2+} ions while the presence of 5 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} resulted in slight inhibition only. The cations Zn^{2+} and Cu^{2+} (0.5 mM) caused around 50% inhibition of activity (Segueilha et al. 1992). The phytase activity of *Candida krusei* Wz-001 was completely inhibited by Zn^{2+} and strongly inibited by Mg^{2+}. No significant inhibition was observed in the presence

of Ba^{2+} and Pb^{2+}. Increasing the concentration of Fe^{2+} (to 5 mM) resulted in the recovery of phytase activity. EDTA and oxalate did not have any inhibitory effect on the purified enzyme and was inhibited by SH reagents such as iodoacetate and *p*-chloro mercuribenzoate (*p*CMB), but stimulated by 2-mercaptoethanol and dithiothreitol (DTT). This showed that SH groups participate in the active site of the enzyme (Quan et al. 2002). The enzyme was sensitive to the presence of serine-specific regents such as phenyl methyl sulfonyl fluoride (PMSF).

7.4. Substrate specificity and kinetics

Phytases usually show broad substrate specificity, with the highest affinity for phytate (Vohra & Satyanarayana 2002). The substrate specificity for phytase may vary due to the differences in the molecular characteristics of enzymes purified from different sources (Liu et al. 1998). By testing with phytate, it is possible to distinguish phytase from acid phosphatase, which is incapable of degrading phytate (Konietzny et al. 1995). Phytase from *Bacillus* sp. DS11 was very specific for phytate and had little or no activity on phosphate esters such as *p*-nitrophenyl phosphate, ATP, ADP, AMP, β-glycerophosphate, sodium pyrophosphosphate and α-napthyl phosphate (Kim et al. 1998a). Phytase from *Pseudomonas* sp. did not show activity towards inorganic pyrophosphate, β-glycerophosphate, ADP or AMP and activity against *p*-nitrophenyl phosphate was 14% of that on phytate (Irving & Cosgrove 1971). Phytases of *A. niger, A. terreus* CBS, and *E. coli* were rather specific for phytic acid while that of *A. fumigatus, E. nidulans*, and *M. thermophila* exhibited a broad substrate specificity (Wyss et al. 1999). A broad substrate specificity was reported for phytases of *S. castelli.*

Generally, the rate of enzymatic hydrolysis is expressed as Michaelis-Menten Kinetics, i.e. the liberation of phosphate (Pi) by phytase is dependent on substrate concentration used. Kinetic parameters for dephosphorylation of phytate by phytase have been studied widely. The Michaelis constant (K_m) for the hydrolysis of sodium phytate by mung bean phytase was found to be 0.65 mM and was similar to the enzyme from *B. subtilis* (0.5 mM) (Shimizu 1992), canola seed (0.36 mM) (Houde et al. 1990), cotton seed (0.37 mM) (Valikhanov et al. 1981) and spelt (0.4 mM) (Konietzny et al. 1995). Comparatively lower K_m values have been determined for phytases from *A. ficuum* (40 mM) (Ullah & Gibson 1987) and *S. castellii* (38 mM) (Segueilha et al. 1992).

It is generally recognized that inorganic phosphates cause product inhibition (competitive inhibition) of phytate hydrolysis (Greiner et al. 1993, Howson & Davis et al. 1983). The substrate inhibition could be observed at the concentration of phytate higher than 1.2 mM for *A. ficuum* phytase (Ullah 1988) and similar inhibitions for maize root and soybean phytases were found at 300 mM and 20 mM, respectively (Hubel et al. 1996). It has also been observed that the presence of substrate along with the enzyme prevents thermal denaturation of the enzyme at temperatures above optimal temperature.

7.5. Molecular characteristics

7.5.1.Molecular weight

Phytases are high molecular-weight proteins ranging from 40-500 kDa. It was a monomer such as from *Selenomonas ruminantium* with 46 kDa (Yanke et al. 1999), *B. subtilis* with 35.6 kDa (Powar & Jaganathan, 1982), 36 kDa (Shimizu 1992), from *C. krusei* Wz-001 with 330 kDa (Quan et al. 2002) and tetramer from *Schwanniomyces castelli* with 490 kDa (Segueilha et al. 1992).

7.5.2. Sequencing and structural properties

The catalytic characteristics as well as the substrate binding properties of an enzyme can be understood only through the studies carried out at its molecular level. Various methods such as X-ray diffraction / scattering techniques, crystallographic analysis, chemical sequencing etc. have been exploited for the better understanding of the catalytic sites and the underlying amino acid sequences of phytase.

Based on chemical sequencing, the primary structure of phytase from *A. ficuum* was elucidated by Ullah (1988) and Ullah & Dischinger (1993). *A. ficuum* phytase contained 594 amino acids residues, which consisted of 37% non-polar, 42% polar, 11.5% acidic and 9.5% basic amino acids. The enzyme contained more acidic than basic residues. The secondary structure of *A.ficuum* (*niger*) contained 17.3% α-helices, 29% β-sheet, 32.6% turns and 24.7% coils (Ullah et al. 1994). It has been suggested that tryptophan is essential for the phytase activity from *A. ficuum*. It has also been demonstrated that the disulfide bonds in the active center are crucial for maintaining its structural geometry, and enzyme loses complete activity after the reduction of disulfide bonds by β-mercaptoethanol. This implies that these disulfide bridges are necessary for the phytase activity (Ullah & Mullaney 1996). The phytase gene (phy) of *A. niger* was cloned and characterized by van Hartingsveldt et al. (1993) whose translated product resulted in a peptide sequence containing 10 potential glycosylation sites. It has also been demonstrated that a certain region of the cloned gene increased the phytase activity over ten fold higher. Phytase of *Emericilla nidulans* comprised 463 amino acids, while that from *T. thermophilus* contained 466 aminoacids.

Amino acid sequence of a phytase from *B. subtilis* consisited of 383 residues which were neither homologous to the sequences of other phytases nor to any known phosphatases. The crystal structure of *E. coli* phytase has been determined by a two-wavelength anomalous diffraction method using exceptionally strong anomalous scattering of tungsten. Ha et al. (1999) performed preliminary X-ray crystallographic analysis of a novel phytase from a *B. amyloliquejaciens* strain using the hanging-drop vapour-diffusion method. The enzyme exhibited thermal stability, which was strongly dependent on calcium ions.

8. PHYTASE ASSAY

Phytase activity can be detected by several assay procedures such as Fiske and Subbarow (1925), Harland and Harland (1980), Heinonen and Lahti (1981), Shimizu (1992), Engelen et al. (1994), Kilmer et al. (1994), Ames (1966). The most common method to detect phytase activity is by measuring the phosphate (P_i) liberated by the action of the enzyme. The hydrolyzed inorganic phosphate is measured by the method based on colorimetric measurements of phosphomolybdate.

Bae et al. (1999) proposed a plate assay for detecting phytase in anaerobic microorganisms that uses a two step counter-staining procedure, first with an aqueous cobalt chloride solution and second by an aqueous ammonium molybdate/ammonium vanadate solution. This staining technique has also proven to be useful in the detection of phytase activity *in situ* in polyacrylamide gels. Phytase activity is also measured based on the determination of inositol formed by the hydrolysis of phytic acid by using high-performance liquid chromatography (HPLC) with a refractive index (RI) detector method (Matthaus et al. 1995).

The assay developed by Harland & Harland (1980) is one of the most commonly employed methods. The reaction mixture consists of 1 ml 0.1 M $MgSO_4.7H_2O$, 2.4 ml 6.82 mM phytic acid prepared in 0.2M

actetate buffer (pH 5.15) and 0.6 ml diluted crude enzyme solution to make the total reaction volume of four ml. The reaction is carried out at 55°C for 60 min in a temperature controlled water bath. After incubation, one ml of the reaction mixture is transferred to a test tube containing 0.5 ml 10% trichloroacetic acid to stop the reaction and diluted with one ml distilled water. Taussky schoor reagent (2.5 ml) is added to it and blue colour developed is read spectrophotometrically at 660 nm. One unit of phytase is defined as the amount of enzyme required to release one μ mol of inorganic phosphate per minute under assay conditions.

9. CONCLUSIONS AND PERSPECTIVES

Research on phytase has made significant progress during the last decade. Phytases from microbial sources certainly offer better techno-economical feasibility for their production and application. Fungal cultures as native microbes and genetically modified bacterial cultures are currently being used as producer organisms for the industrial applications. The ability of any given phytase to hydrolyze the antinutrient phytic acid in the digestive tract is determined by its enzymatic properties, such as catalytic efficiency, substrate specificity, temperature stability, pH optima and resistance to proteolysis. However, our knowledge on phytase has yet to yield a solution to meet its enormous nutritional and environmental demand. More research is needed to discover new phytases and engineering them to develop desired characteristics for specific purposes. Developing more cost effective process for its commercial production needs to be addressed further.

Acknowledgements

A grant from Department of Biotechnology, New Delhi for phytase research is gratefully acknowledged.

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Tannase

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1. INTRODUCTION

1.1 Tannase and its mechanism

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly known as tannase catalyses the hydrolysis of ester and depside bonds (Fig 1) in hydrolysable tannins. Tannins are phenolic compounds with molecular weights ranging from 500 - 3000 Daltons and can be found in a variety of plants (Swain & Bate-Smith 1962). Hydrolysable tannins such as tannic acid (Fig 1) consist of a central carbohydrate core, usually glucose, to which a number of gallic acid or ellagic acid molecules are esterified (Skene & Brooker 1995). The esterase and depsidase activities of tannase originate from two different isoenzymes of which the enzyme is composed. Tannase activity is found to be sequential, whereby cleavage of depside bonds occurs first, followed by the hydrolysis of the ester bonds (Beverini & Metche 1990). The hydrolysing pathway of tannic acid as catalysed by tannase was elucidated by Iibuchi et al. (1972). They found that the substrate was completely

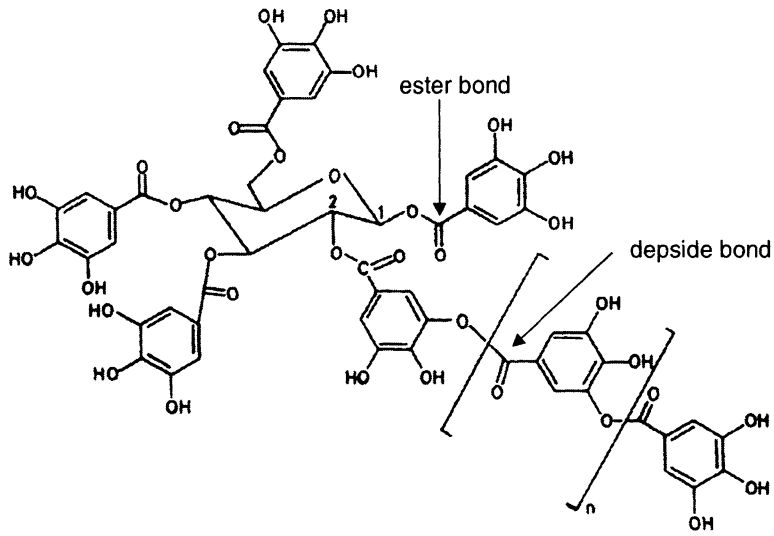

Figure 1. Chemical structure of tannic acid with the ester and depside bonds indicated. "n" is the number of galloyl groups (gallic acid moieties), which can vary from 0 – 4 (adapted from Scott and Eagleson 1988).

converted to glucose and gallic acid via four intermediate compounds, these being various galloyl esters of glucose. Additionally, Deschamps et al. (1983) observed the formation of di- and trigallic acids as intermediates. The action of tannase on other substrates (than tannic acid) such as chebulinic acid, methylgallate and digallic acid is well known (Lekha & Lonsane 1997).

1.2 Historical developments

Tannase was discovered by Scheele in 1786 (Yamada et al. 1968a). The first extensive studies on its properties, sources, applications, reaction mechanism and specificity were conducted by workers such as Fernback, Pottevin, Dykerhoff & Ambruster and Thom & Raper at the start of the twentieth century (Lekha & Lonsane 1997). These studies moreover showed that tannase was an inducible enzyme and could be synthesised in solid-state fermentation by filamentous fungi such as *Aspergillus* and *Penicillium*. The application of tannase for the manufacture of gallic acid from tannin-containing materials was soon recognised. According to Lekha & Lonsane (1997), in their overview of the historical highlights on tannase research, Toth demonstrated in 1944 that tannase consisted of an esterase and depsidase.

In the 1960's, tannase was characterised and purified from plant and fungal source by Madhavakrishna & Bose and Dhar & Bose, respectively (Lekha & Lonsane 1997). More purification and characterisation studies on *Aspergillus* tannase were conducted and reported by Japanese workers (Iibuchi et al. 1968; Yamada et al. 1968a) at the end of the 1960's. One of these workers (Iibuchi et al. 1967) also developed a spectrophotometric assay for the determination of tannase activity, which was formerly based on titration. In the early seventies, there was a trend in patenting potential applications for the use of tannase in (cold) tea, grape juice and wine production. It was found that tannase had the ability to deesterify precipitable, undesirable polyphenols in these beverages.

Around the 1980's many studies on the sources, assay, applications, immobilisation, purification and characterisation of tannase were conducted. It was found that filamentous fungi were not the only sources of tannase but it was produced also by animals (Lekha & Lonsane 1997) and bacterial sources (Deschamps et al. 1983). Later on methods were developed to determine/detect tannase activity using gas chromatography (Jean et al. 1981) and polyacrylamide gels (Aoki et al. 1979). There was a further focus on immobilisation and tannase applications for the production of instant tea and antioxidant propyl gallate (Lekha & Lonsane 1997). Beverini & Metche (1990) purified the two isoenzymes of tannase, containing the esterase and depsidase activities, respectively.

From the nineties onwards, there has been a strong focus on the production of tannase by bacterial strains (Kumar et al. 1999; Mondal et al. 2000; Mondal & Pati 2000; Osawa et al. 2000; Ayed & Hamdi 2002). Several studies demonstrated the potential advantages of solid-state fermentation (SSF) over submerged fermentation (SmF) for tannase production (Lekha & Lonsane 1994; Kar & Banerjee 2000; Aguilar et al. 2001a; Aguilar et al. 2002). SSF was therefore more often used, for instance by Van de Lagemaat & Pyle (2001) who developed a continuous SSF process for the production of fungal tannase. In addition, the induction and repression mechanisms were studied and compared in SSF and SmF (Aguilar et al. 2001b). Hatamoto et al. (1996) were the first to clone, sequence and amplify the tannase gene leading to elevated levels of tannase activity in *Aspergillus oryzae* transformants. Various studies on tannase applications (García-Conesa et al. 2001; Sharma & Gupta 2003), assay development (Sharma et al. 2000; Mondal et al. 2001; Nishitani & Osawa 2003), immobilisation (Abdel-Naby et al. 1999; Boadi & Neufeld 2001; Sharma et al. 2002), purification (Gupta et al. 1997), characterisation (Barthomeuf et

al. 1994; Farias et al. 1994; Skene & Brooker 1995) and productivity optimisation (Hadi et al. 1994; Kar & Banerjee 2000; Seth & Chand 2000) were also conducted during the last 10-15 years.

1.3 Applications, supply and demand

Tannase finds its use in the manufacture of instant tea, acorn wine and the gallic acid-containing drug trimethoprim (Bajpai & Patil 1996; Aguilar et al. 1999; Boadi & Neufeld 2001). In the past, it has also been applied as a probe for the determination of the structure of unknown gallic acid esters (Haslam & Tanner 1970). The enzyme has potential uses in the treatment of tannery effluents, propyl gallate production, stabilisation of grape wine, pre-treatment of tannin-containing animal feed and the clarification of beer, fruit juices and coffee-flavoured drinks (Bradoo et al. 1997; Lekha & Lonsane 1997). However, few of these potential applications have been commercially exploited mainly due to comparatively high costs of the enzyme. The latter is the consequence of the relatively low yields obtained in fermentation and a lack of sufficient knowledge about tannase properties, optimal production, and large-scale industrial application. Commercial tannase is currently available from few manufacturers, e.g. Kikkoman (Japan), Biocon (India) and Jülich (Germany). Kikkoman owns several patents on tannase production (Okamura & Yuasa 1987; Okamura et al. 1988) in which tannase production with submerged fermentation of an *A. oryzae* is described. Biocon manufactures their tannase by SSF of an *A. niger* strain on wheat bran (personal communication K. Prakash, Biocon 2003).

2. SOURCES

Tannin acyl hydrolase is mainly produced by the filamentous fungi *Aspergillus* and *Penicillium* (Yamada et al. 1968a; Bradoo et al. 1996) but other fungal species, bacteria, yeast and plants are also known to synthesise the enzyme (Aoki et al. 1976; Haslam 1981; Deschamps et al. 1983; Skene & Brooker 1995). Among the non-microbial sources, many tannin-rich plant materials (Haslam 1981) and the rumen mucosa of cattle (Lekha & Lonsane 1997) were found to be a source of tannase. However, it is not always clear whether the detected tannase was synthesised by these higher organisms or by their colonising microorganisms (Skene & Brooker 1995). Table 1 provides an overview of microorganisms known to produce the enzyme. To the best of our knowledge, the commercial sources of tannase currently available are synthesised by *Aspergillus* strains (Okamura & Yuasa 1987; Okamura et al. 1988; Lekha & Lonsane 1997; personal communication K. Prakash, Biocon 2003).

3. PRODUCTION

3.1 Modes of production and bioreactors

Tannase production has been carried out in liquid-surface, submerged (SmF) and solid-state fermentation (SSF). SmF has for a long time been the most common technique for tannase synthesis with commercial tannase often being manufactured using this method (Okamura & Yuasa 1987; Okamura et al. 1988). Although, SSF has been described potentially useful for enzyme production in SSF (Pandey1992, Pandey et al. 1999, 2000, 2001), both liquid-surface and SSF are less attractive alternatives as their processes and control are less developed and these are relatively understudied (Lekha & Lonsane 1994; 1997; Chatterjee et al. 1996; Kar et al. 1998; Kar & Banerjee 2000; Aguilar et al. 2001a; Aguilar et al. 2001b; Van de Lagemaat & Pyle 2001; Aguilar et al. 2002, Pourrat et al. 1982).

In terms of productivity, location and stability of the enzyme, SmF is not necessarily advantageous over the other modes of production. Comparative studies by Lekha & Lonsane (1994) and Aguilar et al.

Table 1. Microorganisms reported to produce tannase (partly adapted from Lekha & Lonsane 1997)

Microorganism	Reference
Fungi	
Ascochyta biochemica	Lekha & Lonsane 1997
A. boltshauseri	Lekha & Lonsane 1997
A. pisi	Lekha & Lonsane 1997
A. viciae	Lekha & Lonsane 1997
A. aculeatus	Banerjee et al. 2001
A. aureus	Bajpai & Patil 1996
A. awamori	Bradoo et al. 1996; Seth & Chand 2000
A. carneus	Lekha & Lonsane 1997
A. fischerii	Bajpai & Patil 1996
A. flaviceps	Lekha & Lonsane 1997
A. flavus	Yamada et al. 1968a
A. fumigatus	Lekha & Lonsane 1997
A. japonicus	Bradoo et al. 1996
A. leuchensis inui	Lekha & Lonsane 1997
A. nidulans	Lekha & Lonsane 1997
A. niger	Haslam & Stangroom 1966; Pourrat et al. 1982; Barthomeuf et al. 1994; Lekha & Lonsane 1994; Bajpai & Patil 1996; Bradoo et al. 1996; Lekha & Lonsane 1997; Bhat et al. 1998; Sharma et al. 2000; Van de Lagemaat et al. 2000; Aguilar et al. 2001a
A. oryzae	Iibuchi et al. 1967; Beverini & Metche 1990; Bajpai & Patil 1996; Bradoo et al. 1996; Hatamoto et al. 1996
A. parasiticus	Bajpai & Patil 1996
A. phoenicis	Van de Lagemaat et al. 2000
A. rugulosus	Bradoo et al. 1996
A. tamari	Lekha & Lonsane 1997
A. tereus	Bajpai & Patil 1996
A. ustus	Lekha & Lonsane 1997
Chaetomium lobosum	Lekha & Lonsane 1997
Cryphonectria parasitica	Farias et al. 1994
F. oxysporium	Bradoo et al. 1996
F. solani	Bajpai & Patil 1996; Bradoo et al. 1996
Helicostylum sp.	Bradoo et al. 1996
Mucor pranii	Lekha & Lonsane 1997
Myrothecium verrucaria	Lekha & Lonsane 1997
Neurospora sp.	Lekha & Lonsane 1997

Microorganism	Reference
Penicillium acrellanum	Bradoo et al. 1996
P. carylophilum	Bradoo et al. 1996
P. charlesii	Bradoo et al. 1996
P. chrysogenum	Rajakumar & Nandy 1983; Bajpai & Patil 1996; Bradoo et al. 1996
P. citrinium	Bradoo et al. 1996
P. commune	Van de Lagemaat et al. 2000
P. digitatum	Bradoo et al. 1996
P. fellutanum	Lekha & Lonsane 1997
P. glabrum (frequentans)	Van de Lagemaat et al. 2000
P. islandium	Lekha & Lonsane 1997
P. notatum	Lekha & Lonsane 1997
P. variable	Lekha & Lonsane 1997
Rhizopus oryzae	Hadi et al. 1994; Chatterjee et al. 1996; Kar & Banerjee 2000
Syncephalastrum racemosum	Bradoo et al. 1996
Trichoderma hamatum	Bradoo et al. 1996
T. harzianum	Bradoo et al. 1996
T. viride	Bajpai & Patil 1996; Bradoo et al. 1996
Trichothecium roseum	Lekha & Lonsane 1997
Bacteria	
Achromobacter sp.	Bhat et al. 1998
Bacillus cereus	Mondal et al. 2001
B. licheniformis	Mondal et al. 2000
B. polymyxa	Deschamps et al. 1983
B. pumilis	Deschamps et al. 1983
Citrobacter freundii	Kumar et al. 1999
Corynebacterium sp.	Deschamps et al. 1983
Klebsiella pneumoniae	Deschamps et al. 1983
Lactobacillus paraplantarum	Osawa et al. 2000
L. pentosus	Osawa et al. 2000
L. plantarum	Osawa et al. 2000; Ayed & Hamdi 2002
Lonepinella koalarum	Nishitani & Osawa 2003
Pseudomonas solanacearum	Bhat et al. 1998
Selenomonas ruminantium	Skene & Brooker 1995
Streptococcus gallolyticus	Nishitani & Osawa 2003
Yeast	
Candida sp. K1	Aoki et al. 1976
Debaryomyces hansenii	Bhat et al. 1998
Pichia spp.	Bhat et al. 1998

(2001a) indicated that the productivity of an *Aspergillus* sp strain was significantly higher (over 2.5 times) in SSF than SmF. This could be attributed to the absence of proteolytic activity in SSF cultures (Aguilar et al. 2002). Lekha & Lonsane (1994) also found that the enzyme was completely secreted out extracellularly in SSF while it was partly intracellular in SmF. This made the downstream processing expensive and enzyme from SSF showed improved thermal and pH stability. Studies of Kar et al. (1998) and Kar & Banerjee (2000) also demonstrated a higher tannase productivity and tannin conversion, respectively in SSF as compared to SmF. Currently, at least one commercial manufacturer employs SSF for tannase production (personal communication K. Prakash, Biocon 2003). These indicate a shift from SmF to SSF for tannase production. A few studies conducted on liquid-surface fermentation suggest that this mode of production is not suitable for tannase synthesis due to the low productivity obtained and intracellular character of the enzyme.

In SmF, flask cultivation has been mostly applied for laboratory scale tannase studies (Mondal et al. 2000; Ayed & Hamdi 2002). For a few laboratory studies but moreover for industrial production submerged fermenters were employed (Pourrat et al. 1982; Deschamps et al. 1983; Barthomeuf et al. 1994; Seth & Chand 2000). SSF studies have reported the use of Petri dishes (Chatterjee et al. 1996; Kar & Banerjee 2000), Erlenmeyer flasks (Lekha & Lonsane 1994), column reactors (Aguilar et al. 2002) and a continuous solid-state fermenter (Van de Lagemaat & Pyle 2001). The latter consisted of an inclined, rotating, baffled cylinder with continuous feeding and sampling devices at both ends of the reactor and was built with the aim to operate with a non-inoculated feed. Modified solid-state fermentation (MSSF) was conducted in a laboratory scale bioreactor containing a perforated float carrying the inoculated solid substrate (Kar et al. 1999). Surface liquid culture was carried out in Erlenmeyer flasks (Pourrat et al. 1982; Lekha & Lonsane 1994).

3.2 Fermentation conditions

Fermentation conditions for tannase production have been studied extensively for SmF and to a lesser extend for SSF. Optimal conditions can vary considerably depending on the microorganism of choice and type of fermentation. Incubation temperatures in the range of 25-40°C have been employed with most processes being conducted at around 30 °C (Yamada et al. 1968a; Rajakumar & Nandy 1983; Barthomeuf et al. 1994; Lekha & Lonsane 1997). Certain bacteria and yeast were cultivated at 35-40°C (Aoki et al. 1976; Deschamps et al. 1983) while some SSF were conducted around 25°C (Lekha & Lonsane 1997). The optimum (initial) pH for tannase production has been between 4.5-7.0 (Yamada et al. 1968a; Pourrat et al. 1982; Barthomeuf et al. 1994; Seth & Chand 2000) and 5.0-6.5 (Chatterjee et al. 1996; Lekha & Lonsane 1997) for SmF and SSF, respectively. Inoculation was carried out with spore or mycelial inocula, harvested from a liquid or solid medium, often containing the inducer tannic acid to increase productivity in the subsequent culture (Yamada et al. 1968a; Aoki et al. 1976; Pourrat et al. 1982; Hadi et al. 1994). Oxygen appears to be an essential requirement for the biosynthesis of tannase (Yamada et al. 1968a; Aoki et al. 1976; Pourrat et al. 1982; Barthomeuf et al. 1994) presumably since insufficient oxygen inhibits growth and thereby tannase production. However, tannase synthesis by *Lactobacillus plantarum* was optimal in the absence of oxygen (Ayed & Hamdi 2002). Barthomeuf et al. (1994) suggested dissolved oxygen concentrations of 30-40 % during SmF of an *Aspergillus* sp strain. Excessive oxygen caused tannin oxidation resulting in reduced tannase synthesis. The moisture content, a critical parameter during SSF, was favourable at 62 and 72 % for an *Aspergillus* sp and *Rhizopus*

sp, respectively, cultivated on wheat bran (Chatterjee et al. 1996; Lekha & Lonsane 1997). Kar et al. (1998) claimed that a moisture level of 93 % was optimal for tannase synthesis by their *R. oryzae* strain.

3.3 Substrates

Both defined (synthetic) and undefined media have been employed for tannase production. For SmF; defined media containing tannic acid as the main carbon source were used in most cases. Tannic acid concentrations ranged from 1-10 % (w v^{-1}) and were found to substantially affect tannase productivity (Yamada et al. 1968a; Aoki et al. 1976; Lekha & Lonsane 1997). However, high tannin concentrations were reported to inhibit microbial growth (Bajpai & Patil 1997; Lekha & Lonsane 1997). Optimal concentrations for growth and enzyme synthesis varied depending on the microorganism and fermentation conditions used. Additional carbon sources such as glucose and sucrose were employed in concentrations of 0.06-7 % (w v^{-1}) (Hadi et al. 1994; Aguilar et al. 2001a; Van de Lagemaat & Pyle 2001). The latter were presumably used to promote initial growth of the microorganism as tannic acid is harder to metabolise than simple sugars. Nitrogen sources often employed in defined media include sodium nitrate, ammonium chloride, ammonium di-hydrogen phosphate, ammonium oxalate, ammonium sulphate, monosodium glutamate and glutamic acid (Yamada et al. 1968a; Aoki et al. 1976; Lekha & Lonsane 1997). Most reported salts and trace elements in defined media were Na_2HPO_4, KCl, K_2HPO_4, $KHPO_4$, $MgSO_4$, $FeSO_4$, $AlCl_3$ (Yamada et al. 1968a; Aoki et al. 1976; Lekha & Lonsane 1997). Some workers used undefined media during SmF, including compounds such as chestnut extract, tannin extract from gallnut and Czapek-Dox (Deschamps et al. 1983; Rajakumar & Nandy 1983; Barthomeuf et al. 1994).

During SSF, natural substrates such as wheat bran enriched with a tannin-source were mostly applied (Chatterjee et al. 1996; Lekha & Lonsane 1997). Several workers applied an inert carrier, such as polyurethane foam, impregnated with a tannic acid-containing medium (Van de Lagemaat & Pyle 2001; Aguilar et al. 2002).

3.4 Induction and inhibition of enzyme synthesis

Tannase is reported to be an inducible enzyme produced in the presence of certain tannins or their degradation products (Lekha & Lonsane 1997). Low constitutive levels of the enzyme were measured in the absence of tannic acid and tannase production increased significantly when tannic acid was added to the culture medium (Bradoo et al. 1997; Aguilar et al. 2001a). Enzyme formation was also found to increase with increasing tannic acid concentrations (Lekha & Lonsane 1997; Aguilar et al. 2001b). It is therefore thought that tannase production was induced by tannic acid or some of its derivatives but the exact regulatory mechanism is unknown. Lekha & Lonsane (1997) questioned the inductive role of tannic acid due to its large molecular size and reactivity and suggested that gallic acid acted as an inducer. However, other workers demonstrated that gallic acid did not induce tannase formation and in some cases even showed end-product repression (Bradoo et al. 1997; Aguilar et al. 2001b). In a study of Bajpai & Patil (1997), both tannic acid and gallic acid could function as an inducer. These contradictions indicate that the regulatory mechanism may vary for different microorganisms and fermentation conditions. Apart from tannic and gallic acid, methyl gallate has also been reported to possess inductive properties (Bajpai & Patil 1997).

Inhibition of tannase formation by catabolite repression or feedback inhibition is hardly observed. According to Lekha & Lonsane (1997) both the presence of glucose or sucrose was not found to repress enzyme

synthesis. However, Bradoo et al. (1997) and Aguilar et al. (2001a) showed end-product repression on tannase with gallic acid and glucose, respectively.

3.5 Tannase location and downstream processing

In microorganisms tannase can be produced intracellularly, secreted by the cell or both, depending on the type of organism and fermentation conditions employed (Lekha & Lonsane 1994; Bajpai & Patil 1997). When produced in SmF, tannase is generally intracellular in most fungi (Yamada et al. 1968a; Pourrat et al. 1982; Rajakumar & Nandy 1983) and extracellular in yeast and bacteria (Aoki et al. 1976; Deschamps et al. 1983; Kumar et al. 1999; Mondal & Pati 2000). Two studies using *Aspergillus* demonstrated that tannase was intracellular at the start of liquid cultivation but was secreted into the medium with the progress of the fermentation (Barthomeuf et al. 1994; Lekha & Lonsane 1994). In SSF, the enzyme was mostly extracellular (Lekha & Lonsane 1994; Chatterjee et al. 1996; Kar & Banerjee 2000; Aguilar et al. 2001b).

It is evident that the location of tannase does have its implications for recovery of the enzyme from the fermentation broth or solid substrate mass. A SSF study by Van de Lagemaat & Pyle (2001) has anticipated on tannase recovery from the solid substrate by selecting an inert carrier impregnated with a tannin-containing liquid medium. Crude extracellular tannase could simply be obtained through pressing of the polyurethane foam inert carrier. For SmF, downstream processing has mainly focussed on tannase recovery from the biomass, as the enzyme is mostly membrane-bound. However, information on this topic is rather scarce presumably due to the lack of described industrial production processes. Centrifugation and filtration can be employed for the separation of biomass from the fermentation broth (Lekha & Lonsane 1997). Physical disruption of the biomass by sonication, grinding or frosting and defrosting may be required to release the intracellular enzyme (Rajakumar & Nandy 1983; Barthomeuf et al. 1994). Barthomeuf et al. (1994) added concanavalin A to defrosted broth to facilitate desorption of tannase from its binding site. Methods for the further purification of tannase are described in Section 5.

3.6 Kinetics and modelling of enzyme synthesis

The kinetics of tannase biosynthesis and the modelling thereof has not been studied except by Van de Lagemaat (2001). A mathematical growth model for a batch SSF process for fungal tannase production was developed and tested experimentally. The unstructured model described the uptake and growth kinetics of *Penicillium glabrum* in an impregnated polyurethane foam substrate system containing tannic acid as the main carbon source. Figure 2 presents a summary of the key biochemical processes that were believed to take place. The first stage involved the enzymatic conversion of tannic acid to gallic acid and glucose by tannase. The gallic acid and glucose produced on hydrolysis were used for the formation of fungal biomass (stage 2). The fungal cells secreted the tannase (stage 3), which catalysed the hydrolysis reaction in stage 1. As a means of reproduction, fungal spores were produced in stage 4.

From Figure 3 it can be observed that the overall model gave a reasonable fit to the experimental data. Note that the glucose concentrations were not determined experimentally. The biomass, tannase and spore production were described by logistic kinetics with a time delay between biomass production and tannase and spore formation. The induction mechanism of tannase was believed to be based on two separate processes. The presence of tannic acid or a combination of tannic acid and gallic acid in the growth medium induced the formation of intracellular and/or low, undetectable levels of extracellular tannase in the lag-phase. Subsequently, the cessation of glucose at 18 h of incubation raised the rate of

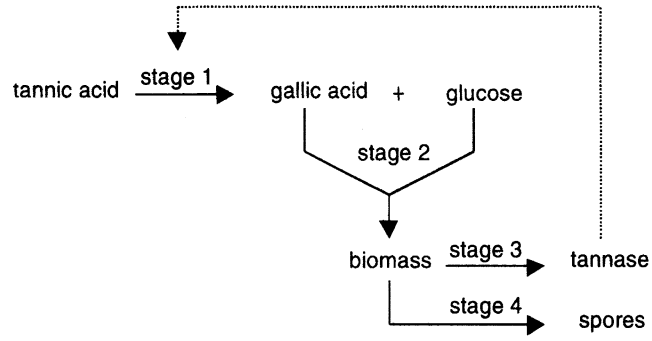

Figure 2. Major biochemical processes representing the uptake and growth kinetics of *Penicillium glabrum* in a batch polyurethane foam system.

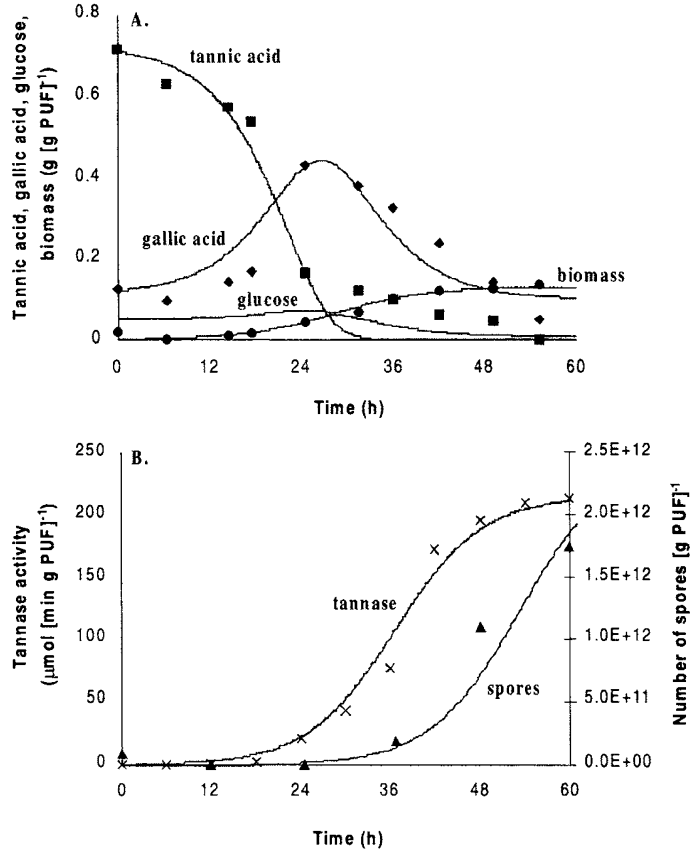

Figure 3. Model predictions and experimental values of the fermentation variables in a 5 % (w v^{-1}) tannic acid solid-state fermentation with *Penicillium glabrum*. Curves (—) of the predicted values are plotted for tannic acid, gallic acid, glucose and biomass in figure A and for tannase and spores in figure B. Experimental data are presented as mean data points for tannic acid (■), gallic acid (♦) and biomass (●) in figure A and for tannase (×) and spores (▲) in figure B.

extracellular tannase formation. Hydrolysis of tannic acid, and thus glucose and gallic acid formation, were reasonably well described with Michaelis-Menten kinetics with time-varying enzyme concentration. However, a more complex reaction mechanism was suspected. The latter and the assumption that gallic acid and glucose uptake took place simultaneously caused the predicted tannic and gallic acid concentrations to deviate slightly from the experimental results. The metabolism of gallic acid was shown to be growth limiting during the main growth phase and not tannase synthesis of tannase catalysis.

4. ASSAY

4.1 Methods for the determination of tannase activity

Qualitative determination of tannase activity in polyacrylamide gels has been described by Aoki et al. (1979). Native PAGE electrophoresis on a tannase-containing sample was followed by incubation of the polyacrylamide gel with tannic acid and subsequently quinine. Both compounds interacted to form a white and water insoluble complex. However, tannase was distinguishable, since the enzyme hydrolysed the substrate (tannic acid) into gallic acid and glucose not forming the complex. Another qualitative assay was developed by Osawa & Walsh (1993) who detected the presence of tannase in culture supernatants with a visual reading method. The latter was based on the coloration of gallic acid under the influence of oxygen.

Assays developed for the quantitative measurement of tannase activity include titration (Haslam & Stangroom 1966), colorimetric (Haslam & Tanner 1970; Iacazio et al. 2000; Mondal et al. 2001; Nishitani & Osawa 2003), UV-spectrophotometric (Iibuchi et al. 1967; Bajpai & Patil 1996) and chromatographic (Jean et al. 1981; Beverini & Metche 1990) methods. All methods are based on the estimation of the residual substrate (tannic acid, methyl gallate or *p*-nitrophenyl esters of gallic acid) or its hydrolysis products (gallic acid, glucose) formed due to enzyme action. The various assays have been reviewed (Lekha & Lonsane 1997), criticised and compared (Jean et al. 1981; Aguilar et al. 1999). Some methods present problems in determining an endpoint accurately (titration), others are not always specific (colorimetric) or limited to one substrate (spectrophotometric). The chromatographic methods are specific and reproducible but time-consuming and restricted to the availability of sophisticated equipment. The (modified) colorimetric rhodanine assay is very similar in terms of specificity and reproducibility but much easier and quicker to perform (Van de Lagemaat 2001). Both the HPLC method by Beverini & Metche (1990) and the (modified) colorimetric rhodanine assay (Sharma et al. 2000; Van de Lagemaat 2001) are well established and have, to our experience, given satisfactory results. Both assays are discussed in more detail below.

4.2 Determination of tannase activity by HPLC and the modified rhodanine assay

These methods for tannase determination consist of two steps; (1) enzymatic hydrolysis of tannic acid and (2) quantification of the gallic acid product. The first stage, as based on a method described by Beverini & Metche (1990), is identical for both assays and is carried out as follows: 50 µl (diluted) tannase-containing sample is added to 1.0 ml of a 0.3 mM solution of tannic acid in acetate buffer (0.1 M, pH 5.0). After incubation at 30 °C for 30 min, the hydrolysis reaction is stopped by addition of 0.2 ml HCl (2 M). A blank is produced by adding 50 µl of the tannase-containing sample to the same reaction mixture already containing the HCl. The quantity of gallic acid released during hydrolysis of tannic acid represents the tannase activity, which is expressed in µmol of gallic acid per ml sample and per minute. The above conditions are favourable for

tannase action (Section 6) and reaction times may be prolonged to produce sufficient gallic acid for detection.

The second step, the quantification of gallic acid in the reaction mixtures and blanks can be done with the colorimetric rhodanine reaction or by HPLC. The latter is also based on the method of Beverini & Metche (1990) and uses the following chromatography conditions: PhaseExtra cartridge column (250 × 4.6 mm) S5-ODS-2 (PhaseSep, Deeside, UK), isocratic elution (1.0 ml min^{-1}) with a methanol-water mixture (28 % [v v^{-1}] methanol) containing acetic acid (2 % [v v^{-1}]) and UV detection at 260 nm. 20 µl of the (diluted) sample is injected into the HPLC column. The quantity of gallic acid is measured after HPLC fractionation and determination of the area under the peak corresponding to gallic acid.

Quantification of gallic acid can also be achieved by the formation of a red gallic acid-rhodanine complex with a maximum absorbance at 518 nm (Inoue & Hagerman 1988). The rhodanine reaction as described by Sharma et al. (2000) was slightly modified by Van de Lagemaat (2001) to become as follows: to 100 ml of (diluted) sample, 150 ml of rhodanine solution (0.667 % [w v^{-1}] in methanol) is added and the mixtures are vortexed. After exactly 5 min of incubation, 2.25 ml of aqueous KOH solution (0.5 M) is added. 20 min afterwards, the absorbance is read at 520 nm. Reactions are carried out at room temperature. Rhodanine (2-thioxo-4-thiazolidinone) can be purchased from ICN (Thame, UK). The rhodanine reaction is specific to free gallic acid and does not take place with gallic acid esters, ellagic esters, or other phenolics. For both methods, standard curves can be obtained by determining the gallic acid content of standard solutions with concentrations ranging from 0.00–0.20 g l^{-1} gallic acid.

5. PURIFICATION

Tannases from various microorganisms have been purified, in which enzyme precipitation, followed by (repeated) ion-exchange and gel-filtration was the most commonly used method (Iibuchi et al. 1968; Yamada et al. 1968a; Aoki et al. 1976; Rajakumar & Nandy 1983). The starting material was culture filtrate or cell extract depending on the location of the enzyme (see also Section 3.5). Tannase concentration through precipitation was usually performed with ammonium sulphate or acetone (Yamada et al. 1968a; Beverini & Metche 1990) but other precipitants such as rivanol, polyethylene glycol, polyvinyl alcohol and dextran have also been used (Aoki et al. 1976; Gupta et al. 1997; Lekha & Lonsane 1997). The following stage, ion-exchange chromatography, was usually carried out at pH 5.0 with an anion exchanger as tannase is an acidic protein. Ion exchange resins used include DEAE-sephadex/cellulose (Yamada et al. 1968a; Rajakumar & Nandy 1983) and ECTEOLA-cellulose (Aoki et al. 1976). Tannase was eluted by gradient elution, i.e. by increasing the ionic strength of the mobile phase, which usually consisted of a buffer (e.g. acetate) solution containing a salt (e.g. sodium chloride). The final purification step comprised gel-filtration chromatography, whereby sephadex G-100 and G-200 were frequently employed stationary phases (Iibuchi et al. 1968; Yamada et al. 1968a; Rajakumar & Nandy 1983). Gel-filtration was mostly carried out at pH 5.0 and elution also took place with a buffer (e.g. acetate) solution sometimes containing a salt (e.g. sodium chloride).

Few workers have employed other techniques such as affinity chromatography and high-pressure size exclusion chromatography (Beverini & Metche 1990; Barthomeuf et al. 1994). Beverini & Metche (1990) performed their purification of tannase through acetone precipitation followed by repeated gel-filtration on sephadex G-50 and Biogel P-300 columns. Subsequent, affinity chromatography on Con-A-Ultrogel combined with elution with methyl-D-glucose led to the separation of the esterase and depsidase

activities of tannase. Barthomeuf et al. (1994) obtained their purified enzyme by repeated ultrafiltration with 200 000 and 100 000 Dalton threshold membranes, respectively. This was then followed by high-pressure size exclusion chromatography with a Protein-pak SW 300 column, which was isocratically eluted with a salt solution (acetate buffer and sodium chloride).

6. CHARACTERIZATION

The characterisation of tannases purified from various microorganisms has shown that their biochemical properties can vastly differ. In general, tannase is an acidic protein with a pH optimum of 5.5. However, enzyme activity has been reported in a broad pH range of 3.5 - 8.0 (Iibuchi et al. 1968; Aoki et al. 1976). The temperature optimum for tannase activity is generally around 30 °C (Iibuchi et al. 1968; Yamada et al. 1968a; Aoki et al. 1976; Rajakumar & Nandy 1983; Barthomeuf et al. 1994; Skene & Brooker 1995) but optima of 50-60°C (Mondal & Pati 2000; Banerjee et al. 2001) have also been stated.

Molecular weights from various purified tannases range from 59 000 – 300 000 Daltons and the reported number of subunits varies from 2 to 8 (Farias et al. 1994; Skene & Brooker 1995; Hatamoto et al. 1996; Lekha & Lonsane 1997). These differences can be attributed to the microbiological origin of tannase. Additionally, the described subunits have been defined differently by the various authors and molecular weights may vary depending on their glycosylation pattern. Hatamoto et al. (1996) carried out a very thorough structural study on tannase aided by cloning and sequencing of an *Aspergillus oryzae* tannase gene. They found that the gene codes for 588 amino acids with a molecular weight of approximately 64 000 Daltons. This tannase gene product is translated as a single polypeptide and subsequently cleaved during its post-translational modification into two subunits (molecular weight of about 30 000 Daltons) connected by disulphide bond(s). The native tannase consists of four pairs of these two subunits, thereby creating a hetero-octamer with a molecular weight of approximately 300 000 Daltons.

Tannase is a glycoprotein and a typical serine esterase, containing a serine in the active site of the enzyme (Rajakumar & Nandy 1983). Its relatively high carbohydrate content (25 - 66 % [w w^{-1}]) is suggested to protect its polypeptide backbone, which could then be less accessible to the tannin molecule. This could explain the resistance of tannase to the tannin effect, i.e. the formation of an insoluble complex of a tannin with a macromolecule such as tannase (Strumeyer & Malin 1970; Beverini & Metche 1990; Lekha & Lonsane 1997). The high solubility of tannases in water may also be explained by their high carbohydrate content (Yamada et al. 1968a).

Values of the Michaelis-constant (K_m) have been reported in the literature and mainly for the substrates tannic acid and methylgallate. Reported K_m values for tannic acid are 0.048 mM (Rajakumar & Nandy 1983), 0.05 mM (Yamada et al. 1968a), 0.95 mM (Farias et al. 1994) and 7.3 mM (Abdel-Naby et al. 1999) for fungal tannases from *Penicillium chrysogenum*, *Aspergillus flavus*, *Cryphonectria parasitica* and *Aspergillus oryzae*, respectively. For methylgallate K_m values of 0.3 mM (Sharma et al. 2002), 6.2 mM (Beverini & Metche 1990), 7.5 mM (Farias et al. 1994) and 8.6 mM (Yamada et al. 1968a) were found for *Aspergillus niger*, *Aspergillus oryzae*, *Cryphonectria parasitica* and *Aspergillus flavus* tannase, respectively. Several authors investigated relatively unknown substrates, thereby reporting a K_m of 5.1 mM for hamamelitannin (Farias et al. 1994) and 1.4 mM for glucose-1-gallate (Yamada et al. 1968a). From the above Michaelis constants can be observed that tannase has the highest affinity for tannic acid, which suggests this is the best substrate.

Tannase inhibition has been studied by various workers often with the purpose of understanding the enzyme mechanism. Phenyl methyl sulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP), both known to inhibit serine esterase, showed strong inhibition of the enzyme (Yamada et al. 1968b; Rajakumar & Nandy 1985; Barthomeuf et al. 1994), thereby suggesting that serine is involved in tannase activity. Iibuchi et al. (1972) found competitive inhibition by gallic acid and its analogues (e.g. monohydroxy benzoic acid) containing phenolic hydroxyl groups. It is therefore suggested that the binding site of tannase can react with any kind of phenolic hydroxyl but the substrate forming the enzyme-substrate complex has to be a gallic acid ester. Metals such as Ag^{+}, Ba^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+} and Hg^{2+} have also been found to act inhibitory (Iibuchi et al. 1968; Rajakumar & Nandy 1983; Barthomeuf et al. 1994; Abdel-Naby et al. 1999; Kar et al. 2003). Other reported inhibitory compounds are *o*-phenanthroline, EDTA, beta-mercaptoethanol, sodium lauryl sulphate, Tween 60, Triton X-100 and dimethyl sulphoxide (Kar et al. 2003)

7. CONCLUSIONS

This work has attempted to provide a brief overview of the knowledge gathered on tannase during the last 40 years and update this with the latest developments in the field. From the literature it can be seen that a great deal of work has been dedicated to the tannase mechanism, characterisation, purification, production (techniques), assays and microbial sources. A reasonable to good understanding of these topics was generated while other subject areas have received less attention and thus a large number of questions remain unanswered. The regulation of tannase biosynthesis, including the induction and inhibition mechanisms, has been studied by some workers and results are contradictory. Consequently, more scientific input is required to elucidate these mechanisms moreover as this may attribute to the so-needed higher tannase yields. The kinetics and modelling of tannase biosynthesis, also in relation to microbial growth has received little or no attention although this could help in understanding the mechanisms. Strain improvement is also a highly understudied area in tannase research and efforts should concentrate on obtaining improved tannase producers both through classical mutation and genetic modification. Furthermore, the applications and feasibility for commercial uses of the enzyme have been scarcely investigated in detail, which hardly stimulates industry to use the many potential tannase applications available. Trends in tannase research over the last 10-15 years comprise the expansion of bacterial tannase sources and the increased application of SSF. The advantages of SSF for tannase production, such as the high titres and enzyme stability, appear to be taken seriously by industry.

8. PERSPECTIVES

For many of the potential tannase applications mentioned above, the enzyme is currently too costly due to the relatively low titres obtained during its fermentative manufacturing. If no stronger specific focus on increasing tannase productivity develops in future research, expansion of the current applications cannot be expected. The developments concerning SSF could aid greatly in improving enzyme productivity. However, more effort is required in this field to further improve the yields and make the process more economically viable. Furthermore, extensive strain improvement programmes should be carried out.

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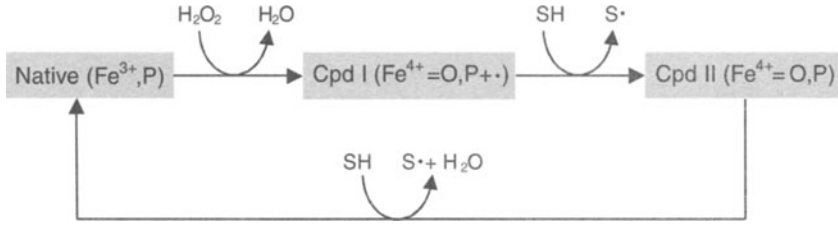
Peroxidases

Carlos G. Dosoretz and Gary Ward

1. INTRODUCTION

As early as 1810, it was observed that placing a fresh piece of horseradish root tissue into a solution of guaicum resulted in the development of an intense blue color. It was later determined that this was due to the action of peroxidase enzymes. Peroxidases (E.C. 1.11.1.7) are widely distributed in nature and are found in plants, microorganisms and animals, where they catalyze the oxidation of a variety of substrates utilizing H_2O_2 as an electron acceptor (Adam et al. 1999). They are typically named after either their sources, e.g., horseradish peroxidase (HRP) and soybean peroxidase (SBP) or their substrates, such as lignin peroxidase (LiP) or cytochrome c peroxidase (CcP).

The catalytic cycle of peroxidases (Ortiz de Montellano 1992) (Fig 1) begins with the reaction of native ferric enzyme (Native; Fe^{3+}, P (porphyrin)) with H_2O_2 yielding compound I (Cpd I) a complex of high valent oxo-iron and porphyrin cation radical (Fe^{4+}=O, $P^{\cdot+}$). One-electron-oxidation of a reducing substrate (SH) by Cpd I yields a radical cation (S•) and the one-electron-oxidized enzyme intermediate, Compound II (Cpd II; Fe^{4+}=O, P). A single one-electron oxidation of a second substrate molecule returns the enzyme to the native ferric state, completing the catalytic cycle:



Peroxidases are versatile biocatalysts, with an ever increasing number of applications (Colonna et al. 1999, May 1999, Veitch & Smith 2001). HRP is presently used in a large variety of analytical and diagnostic systems important in the clinical laboratory. Coupled enzyme assays have been developed for the determination of substances such as glucose, uric acid and cholesterol in biological fluids such as blood, plasma and urine. The assays can be manipulated to give products that can be easily monitored by colorimetric, fluorometric or chemiluminescent methods. Additional applications include chemical synthesis, immunoassays for detection of pesticides and treatment and detoxification of waste water containing phenols and anilines.

The U.S. market for HRP used in analytical and diagnostic clinical tests was estimated to be approximately $2 million in the year 2000 (Wolnak 2001). With the diagnostic enzyme market growing at about 5% per year, it is calculated that the market for peroxidase in the year 2009 will be approximately $3.2 million. Total annual world markets for HRP are $6.0 million, which in 2009 is estimated to grow to $9.4 million. These markets are expected to increase significantly if the present production cost of peroxidases is lowered as a result of new production technology.

2. SOURCES AND BIOLOGICAL FUNCTIONS

Peroxidases are ubiquitously found in plants, microorganisms and animals. They may be categorized into two superfamilies on the basis of sequence similarity, the mammalian heme peroxidase superfamily and the plant peroxidase superfamily (Dunford 1999, Welinder 1992) (Table 1). These two superfamilies do not include di-heme cytochrome C peroxidases (Fulop et al. 1995) or chloroperoxidase (Sundaramoorthy et al. 1995), an enzyme with some attributes characteristic of cytochrome P450. Although the arrangement of peroxidases into superfamilies was originally based on sequence structure, it has gained further support as crystal structures of enzymes from each class have been elucidated. Subsequently, it was concluded that structural topology is more highly conserved than amino acid sequences in the three classes. The plant peroxidase superfamily may be further categorized into three classes: intracellular peroxidases, secreted fungal peroxidases and secreted plant peroxidases, which will be the focus of the following sections.

Table 1. Heme peroxidases classification (Adapted from Dunford 1999).

Superfamily	Class	Examples
Mammalian	I. Halide peroxidases	Lactoperoxidase (LPO), thyroid peroxidase (TPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO)
	II. Prostaglandin synthases	Prostaglandin H synthase (PGHS)
Plant	I. Intracellular peroxidases	Cytochrome *c* peroxidase (CcP), ascorbate peroxidase (AsP)
	II. Secreted fungal peroxidases	Lignin peroxidases (LiP), manganese peroxidase (MnP), *Coprinus cinereus* peroxidase (CiP), *Arthromyces* peroxidase (ArP)
	III. Secreted plant peroxidases	Horseradish (HRP), peanut (PeP), soybean (SBP), barley (BaP) peroxidases
Catalase	I. Animal	Beef liver catalase and human erythrocyte catalase
	II. Bacterial	*E. coli*, *M. lysodeikticus* and *P. mirabilis* catalases

Class I plant-peroxidases include cytochrome *c* peroxidase (CcP) (Erman & Vitello 2002, Finzel et al. 1984) and ascorbate peroxidase (AsP) (Shigeoka et al. 2002). CcP detoxifies H_2O_2 formed in the intermembrane space of mitochondria during aerobic metabolism. It does so using ferro-cytochrome c in the intermembrane space as the electron donor (Bosshard et al. 1984). AsP is a H_2O_2–scavenging enzyme that is specific to plants and algae and is indispensable to protect chloroplasts, mitochondria and peroxisomes from damage by H_2O_2 and hydroxyl radicals (Shigeoka et al. 2002). Four different AsP

isozymes have been detected in at least four distinct cellular compartments, *i.e.* the stroma and thylakoid membrane of chloroplasts (Miyake et al. 1993), microbodies (mAsP) including peroxisomes and glyoxysomes (Bunkelmann & Trelease 1996, Yamaguchi et al. 1995), and the cytosol (cAsP) (de Leonardis et al. 2000, Ishikawa et al. 1996, Mittler & Zilinskas 1991). A fifth AsP isoenzyme has been found in mitochondria (de Leonardis et al. 2000), although its molecular characteristics remain unknown.

Class II plant-peroxidases include lignin peroxidase (LiP) and manganese peroxidase (MnP) (Tien & Kirk 1983, Glenn et al. 1983, Paszczynski et al. 1985, Kirk & Farrell 1987, Boominathan & Reddy 1991, Reddy & D'Souza 1993, Hatakka 1994) as well as *Coprinus cinereus* peroxidase (CiP) and *Arthromyces ramosus* peroxidase (ArP) (Akimoto et al. 1990, Kim et al. 1991, Kjalke et al. 1992, Baunsgaard et al. 1993, Fukuyama et al. 1995, Abelskov et al. 1997). The main role of class II peroxidases appears to be the degradation of lignin in wood (Hiner et al. 2002).

LiP is secreted as a series of glycosylated isozymes with pIs ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDA, with each isoenzyme containing 1 mol heme per mole of protein (Farrell et al. 1989, Gold & Alic 1993, Leisola et al. 1987). It possesses a higher redox potential and a lower pH optimum than that of any other isolated peroxidase or oxidase (Call & Mucke 1997, Hammel et al. 1986, Kersten et al. 1990). Due to its exceptionally high redox potential and low pH optimum, it is able to oxidize non-phenolic aromatic substrates, typically not oxidized by other peroxidases including the non-phenolic phenylpropanoid units of lignin (Hammel et al. 1986, Hatakka 1994, Kersten et al. 1990, ten Have et al. 1998).

MnP exists as a series of glycosylated isozymes with pI's ranging from 4.2 to 4.9 and molecular masses ranging from 45 to 47 kDa. To date, five isozymes have been detected in *P. chrysosporium* MP-1 (Kirk & Cullen 1998). In many fungi, MnP is thought to play a crucial role in the primary attack on lignin, because it generates Mn^{3+}, a strong diffusible oxidant able to penetrate the small 'molecular pores' between cellulose microfibrils, which precludes the action of LiP because of steric hindrances (Flournoy et al. 1993). Organic acids, such as oxalate (Kuan & Tien 1993), fumarate and malate (Hofrichter et al. 1999), which are also produced by white rot fungi, chelate Mn^{3+}. These stable complexes then deliver the oxidizing power. Although MnP does not oxidize non-phenolic lignin structures during normal turnover, these structures have been shown to be slowly co-oxidized when MnP peroxidizes unsaturated fatty acids (Jensen et al. 1996, Kapich et al. 1999). Lipid peroxidation has been suggested as the mechanism involved in the oxidation of the nonphenolic lignin structures by white rot fungi that do not produce LiP (see Ward et al. 2003).

Coprinus cinereus is an ink cap basidiomycete that, unlike other basidiomycetes, contains only a single peroxidase gene (Baunsgaard et al. 1993). The secreted peroxidase, CiP is an acidic protein (pI 3.5) consisting of a single polypeptide chain of 41.6 kDa molecular weight (Morita et al. 1988). Interestingly, CiP shows enzymatic properties similar to those of HRP-C and yet very different from those of fungal LiP and MnP when assayed at pH 7 and 5 (Morita et al. 1988) despite the fact that CiP is only 18% identical in amino acid sequence to HRP-C but 40-45% identical to these fungal peroxidases (Baunsgaard et al. 1993). The functional similarity to HRP-C also extends to a broad and rather high pH optimum for phenolic compounds (Kjalke et al. 1992). In chemiluminiscent oxidation of luminol, CiP was 100-fold more efficient catalyst than HRP-C (Akimoto et al. 1990, Kim et al. 1991). *Arthromyces ramosus* peroxidase (ArP) is secreted by the hypomycete *Arthromyces ramosus* (fungi imperfecti) and has been

shown to be essentially identical in both sequence and properties to CiP (Kjalke et al. 1992, Fukuyama et al. 1995). ArP has attracted a lot of attention recently due to its ease of production, high specific activity and broad substrate specificity (Abelskov et al. 1997).

Class III peroxidases consists of the secretory plant peroxidases, which have multiple tissue-specific functions: e.g., removal of H_2O_2 from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defense responses towards wounding; indole-3-acetic acid (IAA) catabolism; ethylene biosynthesis; and so on (Campa 1991). This class includes HRP (Veitch & Smith 2001) and SBP (Amisha Kamal & Behere 2002, Henriksen et al. 2001, McEldoon & Dordick 1996, Nissum et al. 1998). HRP is a glycoprotein possessing a molecular weight of 44,000 Da and exists as at least 15 different isozymes (Veitch & Smith 2001). Isoenzyme nomenclature reflects the variation in the isoelectric point (pI) from acidic (A group), through neutral (B and C) to basic (D and E). SBP is a relatively new peroxidase derived from soybean hull, where it is present at high concentration. This 37 kDa enzyme is acidic (pI 4.1) and has important characteristics that make it of much interest in biotechnology. SBP has a very high thermostability similar to that typically found in thermophilic microbial enzymes (McEldoon & Dordick 1996) and is stable over a broad pH range. It can oxidize not only phenolics, but also certain other nonphenolic aromatics, presumably on account of a high redox potential (McEldoon et al. 1995). Furthermore, the hull of the soybean is a byproduct of the soybean food industry and therefore could be cost effective for biotechnological applications.

3. PRODUCTION AND ISOLATION

3.1. Plant peroxidase-Class I: Intracellular peroxidases

3.1.1. Production of CcP

CcP can be isolated from aerobically grown yeast by the following procedure (Djavadi-Ohaniance et al. 1978): Pressed yeast (2 Kg) are lysed with ethyl acetate (600 ml) for five hours at room temperature. Subsequently, 1500 ml of 30% ammonium sulphate is added and the mixture is incubated at 4°C for 3 hours. The suspension is then centrifuged for 60 minutes at 10, 000 rpm. The ethyl acetate extract is then saturated with ammonium sulphate (480 g/L) and mixed with 20 g of celite. The precipitated enzyme is then collected on a celite 545 filter prepared by mixing 300g of celite 545 with 1.5 L saturated ammonium sulphate and filtering with suction through a Whatman No. 1 filter. The enzyme forms a colored layer on the top of the celite filter. This layer is collected and suspended in 250 ml of cold distilled water, dialyzed for 24 hours against four changes of 20 liters of cold water and clarified by centrifugation for 60 min at 10,000 rpm. The enzyme can then purified by DEAE cellulose chromatography using a linear gradient from 50 mM to 500 mM potassium phosphate buffer, pH 6.0. Further purification can be conducted by gel filtration on a Sephadex G-100 column using 50 mM potassium phosphate buffer, pH 6.0 as the mobile phase.

3.1.2. Production of AsP

cAsP may be isolated from komatsuna (*Brassica rapa*) leaves by the following procedure (Ishikawa et al. 1996): Komatsuna leaves are homogenized in 10 mM phosphate buffer, pH 7.0 containing 20% w/v sorbitol, 1 mM ascorbate, and 1% (w/v) polyvinylpyrrolidone (insolubles) using a pestle and mortar. The homogenate is then filtered and the filtrate centrifuged to remove particulate matter. The supernatant is then centrifuged at 10000 g for 30 min and the pellet suspended in the same buffer without

polyvinylpyrrolidone. The crude enzyme is then subjected to a series of purification stages. In the first stage, it is loaded onto an anion exchange column such as DEAE Sephacel equilibrated in 10 mM phosphate buffer, pH 7.0 containing 20% sorbitol, 1mM ascorbate and 1 mM EDTA (solution A) and proteins eluted with a linear NaCl gradient from 0-300 mM. Fractions containing AsP activity are pooled and subjected to hydrophobic chromatography using a Toyopearl column and eluted using a descending gradient of $(NH_4)_2SO_4$ from 30 to 0%. The active fractions are combined, concentrated by fractionation and applied onto a gel filtration column equilibrated with solution A. The purified enzyme is collected and concentrated. The same procedure can be employed to isolate AsP from the alga *Chlorella vulgaris* (Takeda et al. 1998)

3.2. Plant peroxidase-Class II: Secreted fungal peroxidases

3.2.1. Production of LiP and MnP

The production of LiP and MnP by white-rot fungi generally occurs at the onset of the secondary growth phase, when utilizable nutrients are depleted and primary fungal growth ceases (Bonnarme et al. 1991). These enzymes play a central role in lignin degradation. Carbon, nitrogen and manganese are critical nutritional variables in the production of LiP and MnP and indeed other ligninolytic enzymes by *Phanerochaete chrysosporium* and other white rot fungi (Bonnarme et al. 1991). Mn^{2+} is a specific effector that induces MnP and represses LiP. In addition, increasing the O_2 levels in culture has a strong activating effect on the production of ligninolytic enzymes (see Dosoretz et al. 2003).

3.2.1.1. Production of LiP

Liquid cultures of *P. chrysosporium* need to be concomitantly starved and exposed to a pure oxygen atmosphere in order to trigger LiP expression (Bar-Lev & Kirk 1981, Dosoretz et al. 1990, Leisola et al. 1984). Starving cultures without excess oxygen is not enough for triggering LiP synthesis, indicating that both effects need to be simultaneous (Bar-Lev & Kirk 1981, Dosoretz et al. 1990, Dosoretz & Grethlein 1991, Forney et al. 1982). Increasing oxygen availability leads to an increase in the titer of LiP even when atmospheric air is supplied and balanced (non-limiting) medium is employed (Dosoretz et al. 1993, Rothschild et al. 1995).

A multiplicity of LiP isozymes have been noted in different strains of *P. chrysosporium* (Asther et al. 1992, Boominathan et al. 1993, Farrell et al. 1989), as well as in other white-rot fungi (Johansson & Nyman 1993, Vares et al. 1994). *P. chrysosporium* LiP has been reported to be produced in several isoforms designated as isozymes H1, H2, H6, H8 and H10 partially differing in their physical characteristics, substrate specificity and stability (Farrell et al. 1989, Rothschild et al. 1997, Stewart et al. 1992).

A procedure for efficient production of LiP isozymes, (yielding mainly H1) from *P. chrysosporium* Burds BKM-F-1767 immobilized on cubes of polyurethane foam is as follows: (Dosoretz et al. 1993). The growth medium is that described by Tien and Kirk (1988), but containing 20 mM acetate buffer (pH 4.5) instead of dimethyl succinate. The initial glucose concentration is 56 mM (10 g/liter), and the nitrogen concentration, as di-ammonium tartrate, is 45 mM. Stationary cultures are inoculated with conidia, incubated for 2 days, homogenized and used to inoculate 250-ml flasks containing 81 ml medium (10% v/v) and 1.8 g polyurethane, of approx. 0.5 cm per side. The cubes are arranged such that the level of liquid is approximately one-fourth of the total height of the bed. Veratryl alcohol is added at the time of incubation (0.4 mM) and again after 48 h of incubation (2 mM). Flasks are then sealed

with rubber stoppers and flushed with pure O_2 for three min at the time of inoculation and then twice a day thereafter. The flasks are incubated with agitation (150 rpm) at 37°C. Generally, maximum activity is noticed on day four of growth. The extracellular fluid is then separated from the mycelium by filtration and concentrated by ultrafiltration using a 10-kDa type YM-10 membrane (Amicon, Danvers, Mass), centrifuged for 10 min at 20,000 × *g* and dialyzed against 10 mM sodium acetate (pH 6.0). LiP isozymes can then be purified by anion exchange HPLC using a MonoQ column (Pharmacia, Piscataway, N.J.) in a program which increases the concentration of sodium acetate buffer linearly over 40 min from 10 mM to 1M (Kirk et al. 1986). The flow rate is maintained at 1 ml/min and monitoring at 409 nm. Under these conditions, H1 is the first isoenzyme to elute (Dass & Reddy 1990).

Many studies on *P. chrysosporium* culturing conditions have been carried out in an attempt to optimize LiP production (Feijoo et al. 1995). Maximum activity and efficiency of LiP production by *P. chrysosporium* under different conditions are given in Table 2.

Table 2. Maximum activity and efficiency of LiP production by *P. chrysosporium* (Adapted from Feijoo et al. 1995)

Bioreactor	Reactor	Maximum volume (l)	Efficiency[a] activity (U)	References
Semi continuous, STR[b]	0.4	38	17	(Kirkpatrick & Palmer 1987)
Batch, STR	42	4284	20	(Janshekar & Fiechter 1988)
Batch or continuous, STR	7.6	5548	140	(Linko 1988)
Batch, STR	0.5	87.5	29	(Michel et al. 1990)
Continuous, PBR	0.4	2004	83	(Rogalski et al. 1992)
Continuous, STR	2.5	185	15	(Venkatadri & Irvine 1993)
HFR	1.5	285	24	
SMR	1.5	345	29	
Batch, STR	1.7	1135	103	(Bonnarme et al. 1993)
BCR	2.2	6904	628	
ALR	2.2	9900	900	
Semi-continuous, PBR[c]	(A1) 0.17	700	264	(Feijoo et al. 1995)
	(A2) 0.17	1462	578	
	(A3) 0.17	400	151	
	(B1) 0.17	3563	899	
	(B2) 0.17	6163	1492	

[a]Efficiency is defined as, $\varepsilon = \frac{U_o}{V_R T}$ where: U_o is overall activity, V_R is the volume of the bioreactor (l) and T is the total operation time (d). [b]STR, stirred tank reactor; ALR, airlift reactor; BCR, bubble column reactor; PBR, packed bed reactor; HFR, hollow fibre reactor; SMR, silicone membrane reactor. [c]Results of 5 studies with different recycling ratios (recycling flow:feed flow): A1 (no recycling flow), A3 (12:1), A2, B1 and B2 (2:1). In reactors A1, A2 and A3, oxygen was supplied by saturation of the feed whereas in reactors B1 and B2, oxygen was supplied directly. A residual glucose concentration was maintained during secondary metabolism in reactor B2.

3.2.1.2. Production of MnP

MnP was first described in *P. chrysosporium* (Kuwahara et al. 1984). Aside from starvation, the presence of Mn^{2+} is essential for MnP gene expression in *P.chrysosporium* (Brown et al. 1991) and *Dichromitus squalens* (Perie & Gold 1991). In fact, elevated manganese concentrations are beneficial for the production of MnP in many white-rot fungi (Bonnarme & Jeffries 1990).

A typical procedure for producing MnP isozymes from *P. chrysosporium* Burds BKM-F-1767 is as follows (Palma et al. 2000): The growth medium is Nitrogen limited BIII medium (Tien & Kirk 1988), with 10 g/L glucose, 0.4 mM veratryl alcohol, 235 μM Mn^{2+}, 0.5% sodium acetate buffer (pH 4.5). Stationary cultures are inoculated with conidia, incubated for 2 days, homogenized and used to inoculate 250-ml flasks containing 90 ml medium (10% v/v). The flasks are incubated with agitation (150 rpm) at 37°C and flushed with 100% with pure O_2 for 3 min at the time of inoculation and then once a day thereafter. Maximal MnP activity is typically witnessed after 5 days and may reach 900 U/L. MnP may be purified by strong anion exchange chromatography employing the same procedure described above for LiP.

A simple and rapid method for producing high amounts of MnP from the agaric white-rot fungus *Clitocybula dusenii* was recently described (Ziegenhagen & Hofrichter 2000). The fungus was immobilized on disc shaped supports composed of cellulose and polypropylene in 500 ml flasks. After being grown as stationary cultures for 11 days until the carrier surface was completely covered with mycelium, the carrier was transferred to 500 ml flasks containing 250 ml fresh medium and 300 μM Mn^{2+} (as $MnCl_2$) to stimulate MnP production. The carrier containing flasks were then incubated on a rotary shaker (160 rpm) at 24°C in the dark. After 6 days cultivation 1900 U/L activity was obtained. Subsequently, the culture medium was replaced with fresh medium and after a further 6 days a similar amount of MnP activity was obtained. After another cycle, 2800 U/L were obtained within 4 days and similar results were obtained after three further cycles.

MnP from *Phanerochaete chrysosporium* ME446 was recently produced in a membrane gradostat reactor in quasicontinuous regime (Govender et al. 2003). The fungus was immobilized by dircctly inoculating fungal spores onto the external surface of ultrafiltration capillary membranes by reverse filtration and then a nutrient gradient was induced across the developing biofilm. Repeated runs of the reactor lasted for approx. 40 d before excessive biofilm growth prevented continuation. A typical MnP productivity peak of approx. 209 U/L/d was witnessed after seven d.

3.3. Plant peroxidase-Class III: Secreted plant peroxidases

3.3.1. Production of HRP

Most methods for purification of peroxidase from horseradish roots involve ammonium sulphate fractionation followed by ion exchange and size-exclusion chromatography. A typical procedure is as follows (Shannon et al. 1966): Horseradish roots are cut into cubes and homogenized with a small volume of 0.1M K_2HPO_4 in a blender. The homogenate is filtered though cheesecloth and ammonium sulphate is added to 35 % saturation. The supernatant is collected and brought to 90 % saturation. After standing overnight, the precipitate is collected by centrifugation, redissolved in a minimum volume of 0.05M Tris buffer, pH 7.0, and dialyzed against 0.05 M Tris buffer, pH 8.0, containing 0.1 M KCl. The dialysate is centrifuged and the supernatant collected. HRP isozymes can then be purified from this

supernatant as follows (Shannon et al. 1966): After dialysis against 0.005M acetate, pH 4.4, the supernatant is injected into a CM-cellulose column, equilibrated with the same buffer. Isozymes may be separated by employing a linear gradient from 0.005M acetate to 0.1 M acetate, pH 4.4 and then to 0.25M acetate, pH 4.4. This procedure separates isozymes B, C, D and E. The fraction that does not adsorb to the column is dialyzed against 0.005M Tris buffer, pH 8.4 and separated on a DEAE-cellulose column using a linear gradient of 0-0.1M NaCl in 0.005M Tris buffer, pH 8.4. This yields isozymes A1, A2 and A3. An improved method for isolation of isoenzyme C (HRP-C), which is by far the most abundant and most commonly studied of HRP isozymes involves affinity chromatography (Reimann & Schonbaum 1978). This method is based on the differential activity of the HRP isozymes towards the aromatic compound, benzhydroxamic acid. HRP-C can be effectively separated from the other isozymes in the horseradish root extract in a single step with a high degree of purity as reflected by an RZ of 3.25.

3.3.2. Production of SBP

SBP can be extracted from soybean by ammonium sulphate fractionation followed by ion-exchange chromatography. The enzyme is located in the hull, which is a byproduct of the soybean food industry and thus offers a cheap cost effective source of peroxidase. A typical procedure is as follows (Van Haandel et al. 2000): Soybean hulls are incubated at 10% (w/v) in 0.1 M potassium phosphate buffer, pH 7.6 for one h at 0°C and then homogenized by vortexing for two min. The mixture obtained is then centrifuged for 10 min at 16000 × *g* and 4°C, and the obtained supernatant is collected. SBP can then be purified from the crude supernatant by weak anion exchange chromatography, for instance by using a DEAE sepharose column, with a linear gradient of 0-0.5 M KCl in 25 mM KH_2PO_4 buffer (Amisha Kamal & Behere 2002). In a recent report (Shakya et al. 1999), it was suggested that best separation of SBP using DEAE Sepharose was achieved at pH 6.0 using a 15 column volume sodium chloride salt gradient from 0 - 0.75 M and the purified protein has a RZ value of 2.5.

3.4 Production of recombinant peroxidases

There are a multitude of reports in the literature regarding the expression of recombinant peroxidases from the plant peroxidase superfamily. Recombinant HRP, MnP and LiP will be referred to in the current chapter as they are the most studied members of the two subclasses of extracellular peroxidases.

Recombinant HRP has been successfully expressed in a number of different host organisms including *E. coli* (Smith et al. 1992, Smith et al. 1990), Baculovirus (Hartmann & Ortiz de Montellano 1992) and *Saccharomyces cerevisiae* (Vlamis-Gardikas et al. 1992). Several independently synthesized HRP-C genes were used in these studies (Jayaraman et al. 1991, Ortlepp et al. 1989, Smith et al. 1990). A popular and low-cost method is refolding of recombinant HRP-C isolated from inclusion bodies of *E. coli* (Nagano et al. 1995, Smith et al. 1990). However, this method is inefficient since the procedure necessitates the controlled reoxidation of reduced denatured HRP-C solubilized from the inclusion bodies and refolding is dependent on both urea and Ca^{2+} concentrations. Yields of between 2-4 mg/L have been obtained (Smith et al. 1992, Smith et al. 1990). Expression in baculovirus results in production of active, but highly glycosylated recombinant HRP-C with yields of between 5-10 mg/L (Hartmann & Ortiz de Montellano 1992). Expression in *Saccharomyces cerevisiae* results in the production of hyperglycosylated secreted enzyme, with yields of around 50 μg/L being reported (Vlamis-Gardikas et al. 1992). A mutant of HRP-C has been identified by directed evolution techniques that exhibits some activity in *E. coli* without the need for refolding (Lin et al. 1999).

Several groups have investigated the recombinant expression of fungal peroxidases in different hosts, which could enable large scale production of such enzymes for biotechnological applications. The baculovirus expression system has been employed in insects for producing active LiPH8 (Johnson & Li 1991), LiP H2 (Johnson et al. 1992) and MnP (Pease et al. 1991). However, this system suffers from low yields and enzymes with only partial activity (60% for H8 and 77% for H2) were obtained. Since the procedure also has high production costs, it is not suitable for use on a large scale.

Expression of LiP H8 in *E. coli* resulted in production of the inactive apoproteins in inclusion bodies and the active enzyme was recovered after controlled *in vitro*, refolding albeit at low yields (Doyle & Smith 1996). LiP H2 has also been expressed in *E. coli* and after refolding, the active recombinant enzyme had spectral characteristics and kinetic properties identical to that of native enzyme isolated from *P. chrysosporium* (Nie et al. 1998). Similarly after refolding of inactive apoproteins, recombinant MnP expressed in *E. coli* exhibited rate values essentially the same as seen with wild type MnP (Whitwam & Tien 1996). However, the thermal stabilities of recombinant LiPH2, LiPH8, and MnPH4, which were expressed without glycosylation in *E coli*, were lower than those of corresponding native peroxidases isolated from *P. chrysosporium* indicating that glycosylation plays a crucial role in protein stability of native peroxidases (Nie et al. 1999).

LiP H8 has also been expressed in homologous expression systems in *P. chrysosporium* (Sollewijn Gelpke et al. 1999). The constitutively expressed glyceraldehyde phosphate dehydrogenase (*gpd*) promoter was used to drive the expression of the recombinant gene during primary metabolism, when it is normally not expressed. Despite the use of the strong promoter, production levels of recombinant LiP H8 proteins remained at comparable levels to those produced by the endogenous genes during secondary metabolism. A similar homologous expression system was employed for MnP in *P. chrysosporium* (Mayfield et al. 1994).

3.5 Peroxidase inhibition/inactivation

Many substrates referred to as mechanism-based suicide substrates result in the inactivation of peroxidases. For example, HRP mediates the one electron oxidation of azide ions forming azidyl free radicals which covalently bind to the δ-meso edge of the heme moiety, thus inactivating the enzyme (Ortiz de Montellano et al. 1988). LiP and the peroxidase from *Coprinus macrorhizu* are inactivated in the same manner (DePillis & Ortiz de Montellano 1989, Tatarko & Bumpus 1997). Similarly, cyanide is oxidized by peroxidases to cyanyl radical which covalently adds to the prosthetic group of the peroxidase yielding a δ-mesocyanoheme adduct (Chen et al. 2000). Additional mechanism-based suicide substrates include alkylhydrazines, cyclopropanone hydrate, nitromethane, which all similarly add to the δ-meso edge after being oxidized by peroxidases to radical intermediates (Ortiz de Montellano 1992).

Another mode of inactivation is attributed to the binding of phenoxy radicals or polyphenol products formed during oxidation of phenols (Aitken 1993, Aitken & Heck 1998, Baynton et al. 1994, Klibanov et al. 1983, Nakamoto & Machida 1992). Indirect evidence for such inactivation during oxidation of phenolic substrates includes the observation that the rate at which activity is lost increases with increasing phenol concentration (Baynton et al. 1994). In addition, removal efficiencies for phenolic substrates increase with increasing enzyme concentration or with decreasing phenolic substrate concentration under otherwise constant conditions (Klibanov et al. 1983).

Excess H_2O_2 will inactivate peroxidases when reducing substrate is absent or exhausted in a process referred to as H_2O_2-dependent inactivation. Of all the peroxidases, LiP appears to be the most sensitive to H_2O_2-dependent inactivation due to the high reactivity of LiP compound II with H_2O_2 yielding LiP compound III (Cai & Tien 1992, Cai & Tien 1989, Wariishi & Gold 1990, Wariishi et al. 1990).

L-cystine, dichromate, ethylenethiourea, hydroxylamine, sulfide, vanadate *p*-aminobenzoic acid and the divalent anions of Cd, Co, Cu, Fe, Mn, Ni and Pb have all been reported to inhibit HRP (Zollner 1993).

LiP catalyzed veratryl alcohol oxidation was reported to be inhibited by EDTA due to the reduction of the veratryl alcohol cation radical by EDTA (Shah et al. 1992). Ascorbic acid similarly reduces phenoxy radicals resulting from peroxidase-catalyzed oxidation of phenols and the concentration of the phenol remains essentially constant (Goodwin et al. 1995).

4. ACTIVITY ASSAYS OF PEROXIDASES

Determination of activity of peroxidases is based on the oxidation of a reducing substrate (electron donor) in the presence of a peroxide (electron acceptor). Due to the ability of peroxidases to catalyze the oxidation of a wide variety of substrates, many of which have strong absorption bands or give colored oxidation products, or emit fluorescence or luminescence, a broad range of activity assays are available. Owing to its wide application as a reporting enzyme in immunological (ELISA and immunoblotting) and coupled-enzymatic assays, reactions of HRP are the most widely studied and characterized and over 200 substrates have been used for its activity determination (see Silaghi-Dumitrescu 2002).

The activity reactions can be grouped into three major categories including (i) chromophoric reactions; (ii) fluorogenic reactions; (iii) chemiluminescent reactions. Procedure selection depends on sensitivity, interferences, background impurities, accuracy, substrate solubility and availability of equipment.

4.1. Chromophoric reactions

This kind of activity assay involves the use of a colorless substrate (chromogen) which forms a colored product (chromophore) upon oxidation by peroxidase in the presence of a peroxide electron acceptor. H_2O_2 is the most widely electron acceptor used for activity reactions. A schematic representation of the reaction is given in the equation below:

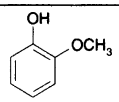
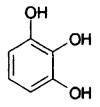
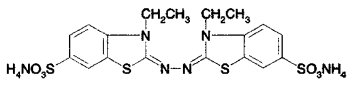
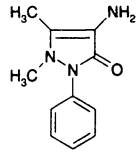
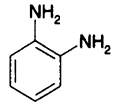
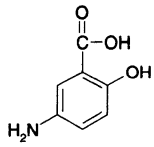
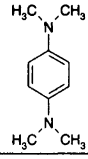
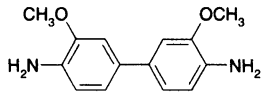
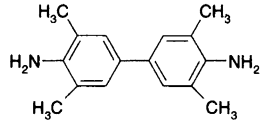
$$H_2O_2 + \text{Chromogen}_{(\text{reduced})} \xrightarrow{\text{Pox}} H_2O + \text{Chromophore}_{(\text{oxidized})}$$

Although less sensitive than fluorescent and chemiluminescent substrates, chromogenic substrates are widely used for determination of peroxidase activity in solution due to simplicity and cost (Porstmann et al. 1981). The chromogens employed can be divided into two major groups: phenolic compounds and aromatic amines (Table 3).

4.1.1. Phenolic substrates

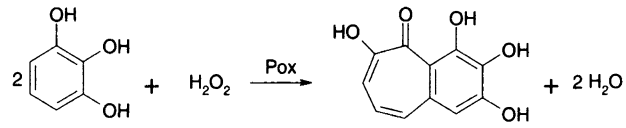
The HRP catalyzed oxidation of a phenolic substrate includes condensation reactions through generation of free radicals with the formation of a chromophore. Typical substrates of this category include guaiacol, pyrogallol and aminoantipyrene-phenol. The oxidation of pyrogallol leading to the formation of the reddish-brown purpurogalin with a maximum absorption at 420 nm has traditionally been used to express the activity of commercial peroxidases (Polis & Shmukler 1953). The assay is normally performed by

Table 3. **Most common chromogenic assays for peroxidases.**

Substrate Name	Chemical Structure	Product color (λ, nm)	ε* $(M.cm)^{-1}$	References
Guaiacol (2-Methoxyphenol)		Brown (470)	26600	Meahly & Chance 1955
Pyrogallol (1,2,3-Trihydroxybenzene)		Reddish-brown (420)	1800	Polis & Shmukler 1953
2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid (ABTS)		Green (410/650)	28000	Shindler et al. 1976
4-Amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one ampyrone (4-aminoantipyrene)		Red (510)	13900	Trinder 1966, Kayyali et al. 1991
o-Phenylendiamine (OPD)		Green (490) - stopped Orange (450) - continuous	10500	Boviard et al. 1982
5-Amino-2-hydroxybenzoic acid (5-Aminosalicylic acid, 5-ASA)		Red (550) - stopped Brown (450) - continuous		Ghose et al. 1978, Ellens & Gielkens 1980
N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD)		Blue (563/610)	12200	Van der Ouderaa et al. 1977
3,3'-Dimethoxy benzidine (o-dianisidine, DMB)		Brown (465)	21800	Avrameas & Guilbert 1972
3,5,3′5′-Tetramethyl benzidine (TMB)		Yellow (450) - stopped Blue (652) - continuous	39000	Avrameas & Guilbert 1972, Josephy et al. 1982

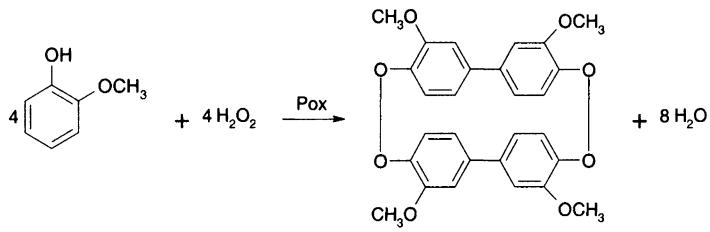
*Adapted from de Lauzon et al. 1999.

continuously monitoring the absorbance at 420 nm in a spectrophotometer. The major drawback of this assay is its relatively low sensitivity and the low photo-stability of the substrate:



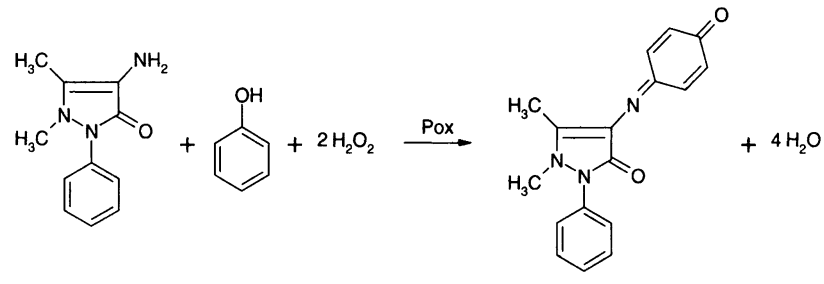
Pyrogallol Purpurogallin (λ = 420 nm)

Guaiacol is readily oxidized by peroxidases forming a tetramer product. The tetraguaiacol solution turns a red brown color absorbing at 436/470 nm but begins to fade a few minutes after its formation (Meahly & Chance 1955). Guaiacol is normally used in continuous spectrophotometric assays. Measurements must be made within one or two minutes after initiating the reaction:



Guaicol Tetraguaicol (λ = 436-470 nm)

The one-electron oxidation product of guaiacol was identified as the dimeric 3,3'-dimethoxy-4,4'-biphenoquinone also absorbing at 470 nm. Two moles of guaiacol are oxidized for each mole of peroxide reduced by the peroxidase (Doerge et al. 1997). The oxidation of a phenol in the presence of the heterocyclic amine substrate, 4-aminoantipyrine is another common and simple spectrophotometric procedure for the determination of peroxidase activity. The oxidation product is an intensely coloured red quinoneimine dye possessing a maximum absorbance at 510 nm (Trinder 1966, Kayyali et al. 1991). The use of several phenolic electron donors coupled to 4-aminoantipyrine have been reported, such as 2,4-dichlorophenol (Ishida et al. 1987), *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-m-toluidine, *N*,*N*-diethyl-aniline and *p*-hydroxybenzensulfonate (see Silaghi-Dumitrescu 2002). The formed dye is stable and this reaction can be applied for either stopped or continuous activity assay:



4-Aminoantipyrine Phenol Quinoneimine (λ = 510 nm)

The sulfonic dye ABTS is another commonly used substrate for determination of peroxidase activity. ABTS produces a soluble cation radical-end product that is green in color and can be continuously measured at 405-410 nm in a spectrophotometer (Shindler et al. 1976). However, the chromophoric product has been reported as being somewhat unstable at pH values above neutrality and in the presence of common biological buffers (McCoy-Messer & Bateman 1993). It is recommended that use of ABTS be confined to acetate buffers if a low pH is required, and phosphate or Tris buffers, if a pH near neutrality is required.

Due to the ability of peroxidase to oxidize a broad range of phenolic compounds random use of several other phenolic derivatives has been described in the literature including cresols and halophenols (see Silaghi-Dumitrescu 2002). Oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) to the corresponding *o*-quinone has also been reported as an assay for peroxidase activity (Mercer et al. 1996, Antonopoulos et al. 2001, Nappi & Vass 2001).

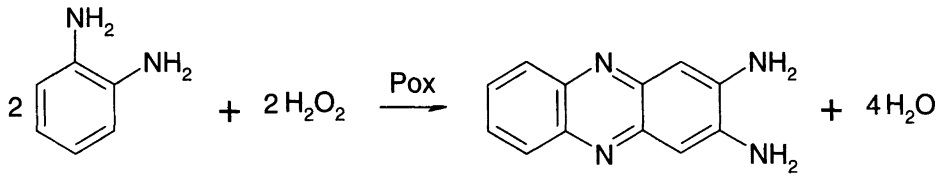
4.1.2. Aromatic amines

A second major group of substrates widely used for determination of peroxidase activity are aromatic amines, which are further subdivided into (i) monoaromatic amine derivatives, such as *o*-phenylenediamine (OPD), aminosalycilic acid (4-AS) and TMPD; (ii) benzidine derivatives such as dimethylbenzidine (DMB) and tetrametylbenzidine (TMB) (Karasyova et al. 2003).

4.1.2.1. Monoaromatic amines

Due to the strong activation of the benzene ring by the amino group of monoaromatic amines, these are very reactive substrates for peroxidases generating a series of diazonium coupling products and thus, more than one defined colored product may be expected. In the examples given below the most characteristic product is presented.

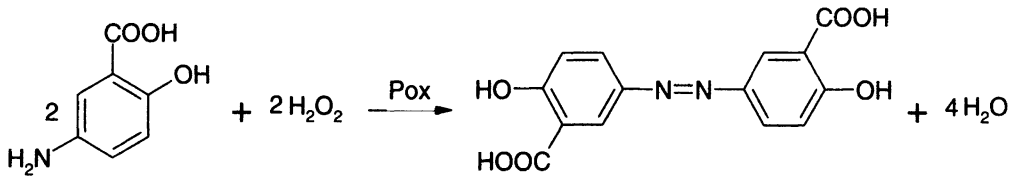
o-Phenylenediamine is a peroxidase substrate widely used for determination of peroxidase activity, in particular in ELISA procedures. The substrate produces a soluble end product, 2,3-diaminophenazine (DAP), that is orange-brown in color and can be measured spectrophotometrically at 450 nm in a continuous assay. Alternatively, the oxidation reaction may be stopped by addition of 3 M HCl or 3 M H_2SO_4 and read at 492 nm (Boviard et al. 1982, Tarcha et al. 1987, Hamilton et al. 1999):



OPD DAP (λ = 450 nm)

5-Aminosalicylic acid (5-ASA) is another common monoaromatic amino derivative used in peroxidase assays. This substrate produces a soluble end product that is brown in color and can be continuously

monitored spectrophotometrically at 450 nm. The reaction may also be stopped with 3M NaOH and read at 550 nm (Ghose et al. 1978, Ellens and Gielkens 1980, Howie & Thorsen 1981):



4-ASA

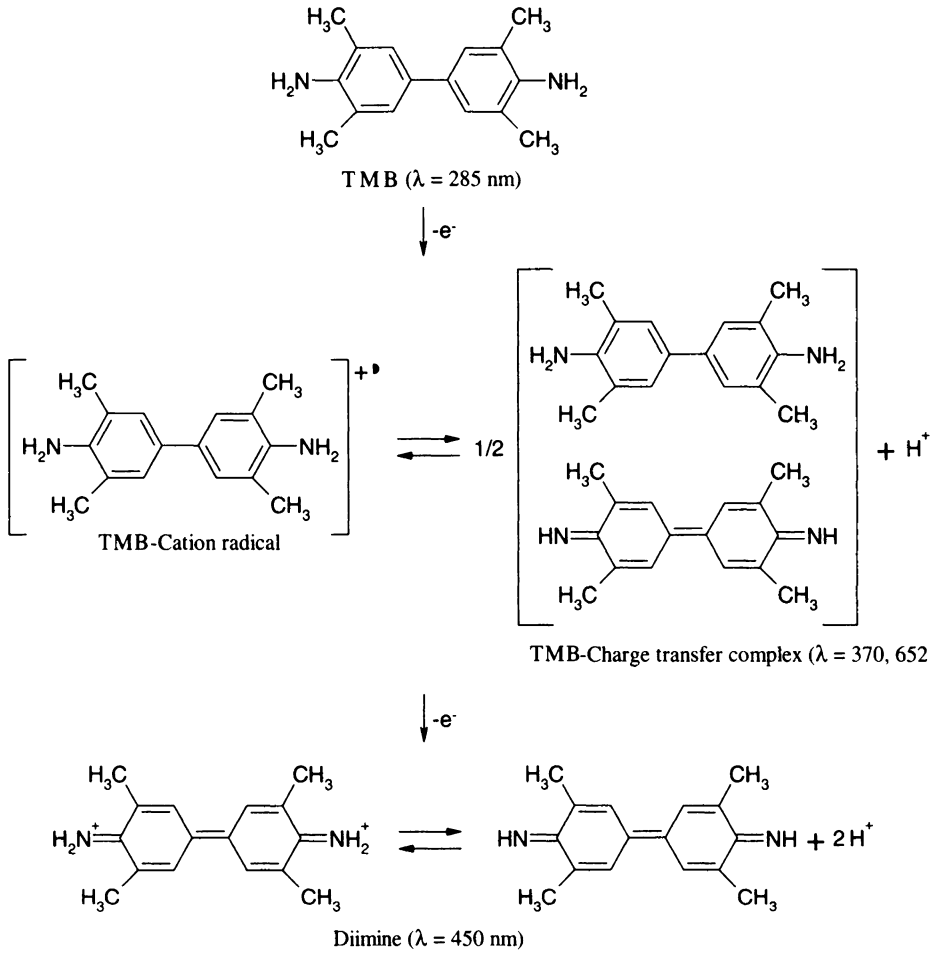
Benzoquinonediimine (λ = 450 nm)

TMPD is an easily oxidizable reducing substrate for peroxidases that undergoes one-electron oxidation by the heme peroxidase higher oxidation states (compounds I and II) producing a blue product exhibiting two absorbance maxima, at 563 and 610 nm. The stoichiometry of oxidation is 2 moles of TMPD oxidized per mole of peroxide reduced by the peroxidase (Van der Ouderaa et al. 1977, Kulmacz & Lands 1983, Kadima & Pickard 1990).

4.1.2.2. Benzidines

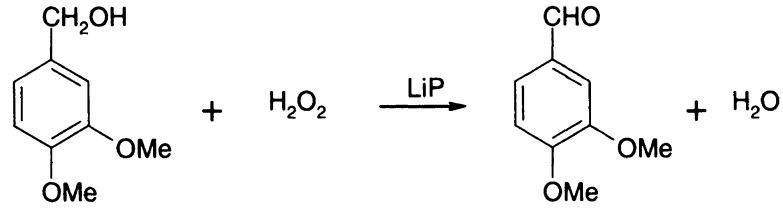
o-Dianisidine (DMB) is oxidized by peroxidase producing a brown chromophore that can be continuously monitored spectrophotometrically at 405 nm. The reaction may be stopped by lowering the pH through addition of either HCl or H_3PO_4 (Avrameas & Guilbert 1972, Claiborne & Fridovich 1979). Although DMB is widely used due to the high stability and extinction coefficient of the chromophoric end-product, necessary precautions must be taken when working with DMB due to its carcinogenicity.

Tetramethylbenzidine (TMB) is perhaps the most commonly used benzidine derivative for determination of peroxidase activity because it is an innocuous derivative of benzidine (Liem et al. 1979). The one-electron oxidation product of TMB by heme peroxidases is a cation free radical which is in equilibrium with a charge-transfer complex with maximum absorption bands at 370 and 655 nm, responsible for the blue color obtained during the course of TMB oxidation. The two electron oxidation of TMB yields a yellow diimine derivative with an absorbance maximum at 450 nm (Josephy et al. 1982, Gallati & Pracht 1985, Bally & Gribnau 1985, Porstmann et al. 1991, Marquez & Dunford 1997). The formation of the charge-transfer complex (blue) is used for continuous assays at either 370 or 655 nm and diimine formation (yellow) is used in acidic stopped assays, measured at 450 nm:



4.1.3. Activity of lignin degrading peroxidases

Due to its distinguishable ability to oxidize methoxyarenes, the most common, specific and sensitive assay for LiP activity is the spectrophotometric measurement (λ = 310 nm) of veratraldehyde (3,4-dimethoxybenzyl aldehyde) formation resulting from the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) in the presence of H_2O_2 (Tien & Kirk 1983, Tien & Kirk 1988):

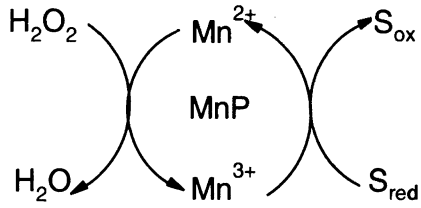


Veratryl alcohol

Veratraldehyde (λ = 310 nm)

The oxidation of azure B, involving absorbance measurements in the visible range, was suggested as an alternative method for measuring LiP activity. This method is claimed to be less susceptible to interferences caused by organic material absorbing in the near UV range when measuring activity in crude extracellular fluid media (Archibald 1992, Arora & Gill 2001, Ferreira-Leitão et al. 2003).

The activity assay of MnP is based on the oxidation of a phenolic reducing substrate in the presence of H_2O_2 and Mn^{2+}, according to the following scheme:



The use of buffers with chelating activity such as citrate, lactate, succinate or malonate has been reported to improve the reaction. The most commonly used substrates are phenol red and 2,6-dimethoxyphenol (DMP) (Kuwahara et al. 1984, Gold et al. 1989, Michel et al. 1992, Field et al. 1992, Heinfling et al. 1998). DMP is oxidized to the dimer cerulignon (3,3',5'5-tetramethoxy-*p,p'*diphenylquinone) which absorbs at 420 nm with an extinction coefficient of 49,600 $M^{-1}.cm^{-1}$ (Field et al. 1992). The oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino)benzoic acid in the presence of H_2O_2 and Mn^{2+} was also reported as a specific assay for MnP activity (Castillo et al. 1994). This reaction yields a deep purple-blue color with a broad absorption band with a peak at 590 nm. The extinction coefficient is high (53,000 $M^{-1}.cm^{-1}$), enabling the detection of low MnP activities.

4.2. Fluorogenic reactions

This kind of activity assay involves the use of a non-fluorescent substrate (fluorogen) which forms a fluorescent product (fluorophore) upon oxidation by peroxidase in the presence of a peroxide electron acceptor. A schematic representation of the reaction is given in the equation below:

$$H_2O_2 + \text{Fluorogen}_{(\text{reduced})} \xrightarrow{\text{Pox}} H_2O + \text{Fluorophore}_{(\text{oxidized})}$$

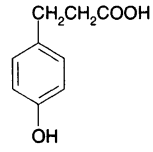
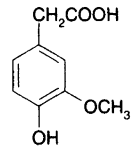
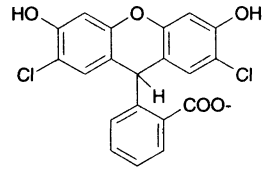
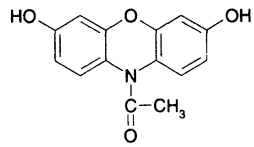
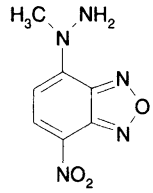
Because of its high sensitivity, the use of fluorometric techniques has become a widely adopted procedure for determination of peroxidase activity, in both immunological and enzymatic applications, and several commercial products are available (Table 4).

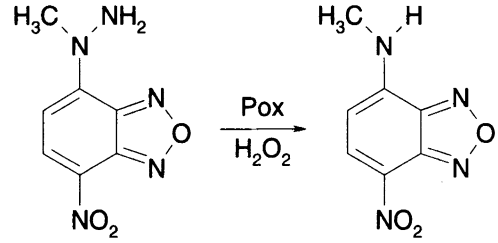
Three major families of fluorogenic substrates extensively reported for measurement of peroxidase activity are phenolic derivatives, aromatic amines and reduced forms of fluoresceins and rhodamines. The most extensively used fluorogenic phenolic substrates studied are *p*-hydroxyphenylacetic acid (*p*-HPA) (Ex. 318/Em. 406), homovanillic acid (HVA) (Ex. 317/Em. 420), *p*-hydroxyphenylpropionic acid (*p*-HPPA) (Ex. 320/Em. 406) and tyramine (Ex. 317/Em. 406) (Zaitsu & Ohkura 1980, Tuuminen et al.

1991). The fluorescent product obtained upon oxidation of HVA by peroxidase has been identified as the *o,o'*-biphenyl dimer (Guilbault et al. 1966, Foppoli et al. 2000). A major disadvantage in using some *p*-hydroxyphenylcarboxylic acids in continuous assays is the difference between the pH optima for peroxidase activity (acidic to neutral) and pH at which the products fluoresce (most of them alkaline) (Zaitsu & Ohkura 1980, Meyer et al. 2000).

Meyer et al. (2000) described a peroxidase activity assay based on the oxidation of the fluorogenic methylhydrazine derivative 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) to the flurorophoric product 4-(*N*-methylamino)-7-nitro-2,1,3- benzooxadiazole (MNBDA). Both the enzymatic reaction and fluorescence detection take place at pH 5.8:

Table 4. Most common fluorescent assays for peroxidases.

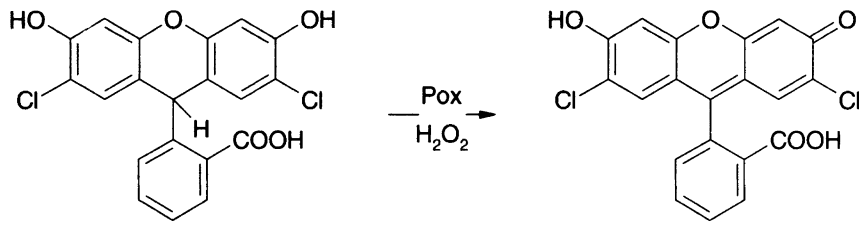
Substrate Name	Chemical Structure	Excitation/Emission (nm)	References
p-hydroxyphenyl propionic acid (*p*-HPPA)		320/406	Li and Townshend 1997
4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA)		317/420	Guilbault et al. 1966
2'-7'-dichlorodihydro fluorescein (DCFH)		498/ 522	Rota et al. 1999
10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red)		587/530	Zhou et al. 1997
4-(N-Methylhydrazino)-7-nitro-2,1,3-benzooxadizole (MNBDH)		470/547	Meyer et al. 2000



MNBDH MNBDA

The aromatic amines *o*-phenylenediamine (OPD) and *N,N'*-dicyanomethyl-*o*-phenylenediamine (DCM-OPA) (Ex. 360/Em. 454) have also been reported as fluorogenic substrates for peroxidase (Zaitsu & Ohkura 1980, Li et al. 1996). Li & Townsend (1997) reported the synthesis of four 3,4-dihydroquinoxalin-2(lH)-one derivatives as potential substrates for peroxidase, including: 3,4-dihydroquinoxalin-2(lH)-one (DHQ) (Ex. 350/Em. 413), 3-methyl-3,4-dihydro-quinoxalin-2(1H)-one (MDHQ) (Ex. 344/Em. 404), 3,4-dihydroquinoxalin-2(1H)-one-6-acid (DHQ-6-A) (Ex. 351/Em. 417) and 3-methyl-3,4-dihydroquinoxalin-2(1H)-one-6-acid (MDHQ-6-A) (Ex. 346/Em. 405).

Dihydrofluoresceins dihydrorhodamines and dihydroethidium (hydroethidine) are oxidized to highly fluorescent products by peroxidase. A sensitive assay for peroxidase activity involving the oxidation of 2',7'-dichlorofluorescin (DCFH) in the presence of thiocyanate has been reported by Proctor & Chan (1994). The two electron oxidation product of DCFH is the highly fluorescent compound 2'-7'-dichlorofluorescein (DCF) (Rota et al. 1999):

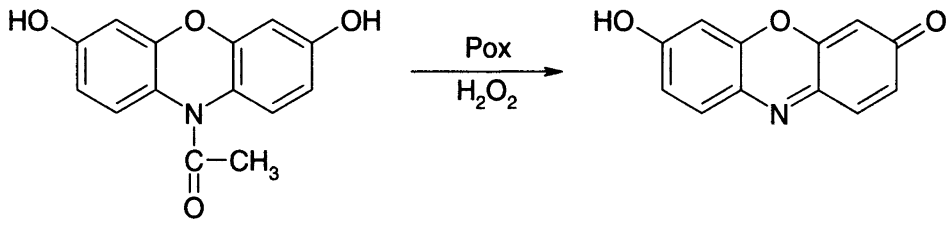


DCFH DCF

Because of the propensity of dihydrofluoresceins to autooxidation a commercial diacetate derivative, 2',7'-dichlorofluorescin diacetate (H_2DCFDA) has been developed (Molecular Probes, 2004). However, this assay requires prior cleavage of the diacetate precursor to the active fluorescein by an esterase, which makes the assay somewhat cumbersome for *in vitro* determination of peroxidases.

A widely commercially available fluorogenic substrate for peroxidase is 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), which is a sensitive and stable probe for H_2O_2. In the presence of HRP, the Amplex Red reagent is oxidized in a stoichiometric ratio of 1:1 with H_2O_2 to produce the highly fluorescent resorufin. Although resofurin itself is a substrate for peroxidase decomposing into a non-fluorescent polymeric product when the substrate:H_2O_2 ratio is greater than 1:1, this reaction is 30-fold

slower than resofurin formation by oxidation of Amplex Red (Zhou et al. 1997):

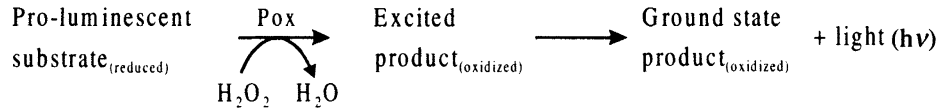


Amplex Red Resofurin

QuantaBlu™ (QB) is a fluorogenic substrate of undisclosed structure commercialized by Pierce Chemical Co (Rockford, IL) which is converted into a stable blue fluorescent product (Ex. 325/Em. 420) upon oxidation by peroxidase (Savage et al. 1998).

4.3. Chemiluminescent reactions

Chemiluminescent reactions for determination of peroxidase activity are based on the quantification of light emission (photons) obtained upon oxidation of a pro-chemiluminescent substrate by the enzyme. Typically, chemiluminescence reactions involve the production of an electronically excited species from a number of reactants which releases visible light when it reverts to its ground state energy. A schematic representation of the reaction is given below:

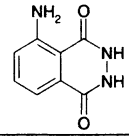
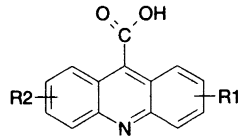
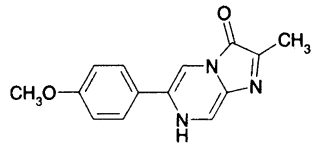


The initial flash of light occurs instantaneously. In order to increase the sensitivity of the detection in commercial applications, the resulting light is normally intensified and the signal duration increased by the addition of a chemiluminescent enhancer. Chemiluminescent determination of peroxidase activity has found broad application in immunobloting applications and several commercial products are available (Table 5).

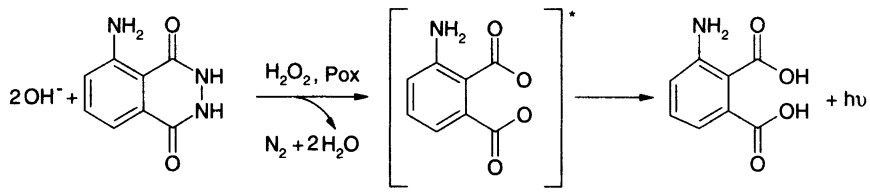
The two most commonly applied chemiluminescent reactions for detection of peroxidase activity are based on the oxidation of hydrazides such as luminol (3-aminophthalhydrazide) and a *N*-alkylacridancarboxylate nucleus such as acridan orange derivatives.

Luminol is oxidized by peroxidase at alkaline pH (10) forming an excited aminophthalate dianion (3-APA anion) accompanied by the release of nitrogen gas (N_2) (Olsson et al. 1979, Thorpe & Kricka 1986).

Table 5. **Most common chemiluminescent assays for peroxidases.**

Substrate Name	Chemical Structure	Light color (λ, nm)	References
3-aminophthalhydrazide (luminol)		Blue (425)	Thorpe & Kricka 1986
N-alkylacridancarboxylic acid derivatives		Blue (411)	Akhavan-Tafti et al. 1998
2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, hydrochloride (MCLA)		Blue (462)	Mitani et al. 1994

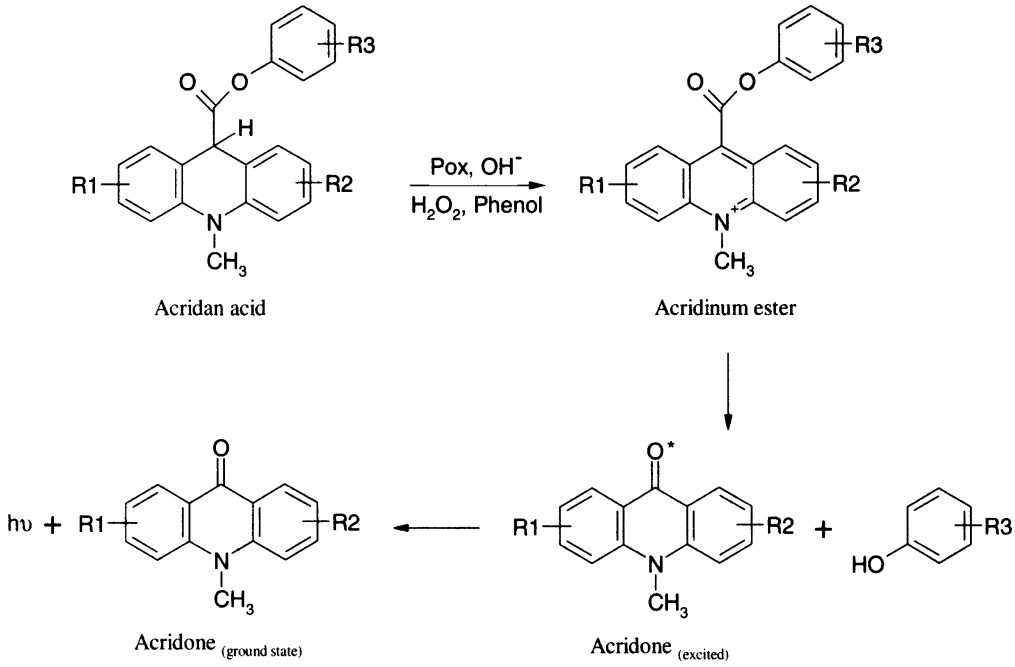
Upon relaxation to the ground state, 3-aminophthalate (3-APA) emits light (λ = 425 nm):



Enhanced chemiluminescence (ECL) can be obtained during luminal oxidation by addition of 6-hydroxybenzothiazole, phenylboronic acid derivatives, phenol and polyphenols, although the exact mechanism of enhancement is still unresolved (Thorpe et al. 1985, Ji & Kricka 1996, Nozaki et al. 1995, Ilyina et al. 2000).

N-alkylacridancarboxylic acid derivatives including esters, thioesters, and sulfonamides are oxidized by peroxidases to produce the corresponding acridinum ester intermediate. The reaction proceeds by two electron oxidation. The acridinum reacts with excess peroxide under slight alkaline pH to yield the corresponding singlet-excited acridone which spontaneously reverts to the ground state with release of blue luminescence. Phenolic compounds (e.g., phenol, *p*-phenylphenol) act as enhancers facilitating

high light intensity with extended duration (Akhavan-Tafti et al. 1995, 1998, http://www.lumigen.com/):



The kinetics of formation of the acridinum intermediate and light production seems to depend on the structure of the leaving groups (R3) and the substitution of the hydrogen ring (R1, R2) of the acridancarboxylate. Leaving groups such as a difluorophenoxy or trifluorophenoxy groups speed up the reaction, whereas substitution of one or more ring hydrogens on the acridancarboxylate nucleus by an alkyl or alkoxy group prolongs maximum light intensity. Leaving groups comprising substituted phenyl and naphthyl ester groups, aryl and alkyl thioesters with both electron-donating and electron-withdrawing, and sulfonylamino groups functionally generate chemiluminescence upon oxidation by peroxidases (Akhavan-Tafti et al. 1998).

Methyl-cypridine-luciferin analogue (MCLA) has been reported as a chemiluminescent substrate for HRP, emitting blue chemiluminescence (462 nm) upon oxidation at pH 5.5 (Mitani et al. 1994).

5. CONCLUSIONS

Although peroxidases are primarily intracellular enzymes with important roles in cellular processes and are widely distributed throughout plants, animals, and microorganisms, the intrinsic properties, structure and mode of action of the extracellular plant and fungal peroxidases of the plant peroxidase superfamily make them the most attractive for applications in enzyme technology.

Class II and class III-plant peroxidases are typically small heme glycoproteins of 30-60 kDa, that are able to oxidize a variety of substrates, ranging from simple to complex, by different type of reactions: peroxidative oxidation, oxidative, catalytic and hydroxylation. They are the most potent oxidizing enzymes in nature, forming an excited ferryl intermediate, possessing a redox potential in the range of +920-1100 mV.

Peroxidases have been and are widely used as reporting enzymes for immunological and clinical diagnosis, chemical detection and biosensors. Because of the wide range of chemicals that can be modified by the catalytic activity of these enzymes, several novel applications have been suggested, such as treatment of wastewater containing phenolics and synthesis of various aromatic chemicals, including valuable biopolymers with thermoresistant and photoresist properties in the area of photolithography and adhesives. The most notable candidates for these applications are HRP, CiP, ArP and SBP. To date no major commercial uses in foods and pharmaceuticals or chemical operations have been found for peroxidases.

The comparatively high cost of producing peroxidases has been the major reason for the lack of development of applications for industrial purposes. Improvement in fermentation processes, as well as development of recombinant enzymes with improved properties and large scale production at low cost are expected to open a new window for industrial applications of peroxidases.

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Chitinases

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1. INTRODUCTION

Chitin is the second most abundant organic compound in nature, after cellulose. It is a linear β-1,4-homopolymer of N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose), found naturally in the shells of crustaceans, insect exoskeletons, fungal cell walls, micro fauna and plankton. Approximately ten giga tonnes of chitin are estimated to be synthesized and degraded each year in the biosphere. The commercial sources of chitin are shrimp, crab and lobster wastes. It has been found that shrimp and crab processing waste contains 14-27% and 13-15% by dry weight of chitin respectively (No et al.1989). Chitooligosaccharides derived as a hydrolysis product of chitin are useful for agrochemical and medicinal purpose (Hirano 1996). In natural state, chitin is tightly bound with proteins, lipids, pigments and minerals such as calcium carbonate. Hence preparation of chitin and its oligomers involves demineralization and deproteinization of chitinous waste using strong acids and bases. These processes involve high cost, low yield, and corrosion problems due to acidic wastes, by which the cost of N-acetyl-D-glucosamine (monomer) or its oligomers are still prohibitive. The production of inexpensive chitinolytic enzymes is an important element in the utilization of chitinous waste that not only solves environmental problems but also reduces production costs. In the last two decades there has been a lot of focus on the production of N-acetyl-D-glucosamine through enzymatic hydrolysis of chitin.

Chitinases are a group of hydrolytic enzymes that catalyze depolymerisation of chitin. Chitin degradation can be initiated by chitinases (endochitinase) to oligo N-acetyl glucosamine (NAG) chains. The oligomers, tri-NAG and di-NAG are subsequently degraded to metabolizable NAG monomers by chitobiase. The monosaccharide released can then be metabolized by many organisms (Jeuniaux 1966).

1.1. Classification of chitinases

Although chitinolytic enzyme system is almost parallel to the cellulolytic enzyme complex, their classification is not well defined. According to the recommendation by International Union of Biochemistry and Molecular Biology (IUBMB) (1992), chitinolytic enzymes can be classified based on the mode of action (Patil et al. 2000).

1. Endochitinases - Random hydrolysis of the chain (EC 3.2.1.14)
2. Chitobiase - Hydrolysis of terminal non-reducing sugar (EC 3.2.1.29)
3. β-N-acetylglucosaminidase- Successive removal of sugar unit from the non reducing end (EC 3.2.1.52).

Thus, complete hydrolysis of chitin is performed by an enzymatic system consisting of three kinds of chitinases. It is believed that chitobiosidases hydrolyse chitin from the non-reducing ends and these enzymes, therefore, are described as *exo*-glycoside hydrolase.

Based on amino acid sequences of glycosyl hydrolases, Henrissat (1991) grouped chitinases and N-acetyl-hexosaminidases into three families- 18, 19 and 20. The families 18 and 19 comprised endochitinases from different sources such as viruses, bacteria, fungi, insects and plants. Plant chitinases are grouped mainly in family 19, while N-acetyl hexosaminidase (EC 3.2.1.30) from *Vibrio harveyi* and N-acetyl hexosaminidases (EC 3.2.1.52) from human and *Dictyostelium discoideum* are grouped in family 20.

1.2. History

Chitinase was first observed by Bernard in 1911, when he isolated a thermostable and diffusible chitinolytic fraction from orchid pulp. This study was supported by Karrer and Hoffman for the presence of chitinase in snail (Flach et al. 1992). In recent years, there has been a lot of research for enhanced production of chitinases from microorganisms. Advent of recombinant DNA technology has contributed much to the improved production of microbial chitinases. Ample reports are available on application of chitinases in improving insect and disease resistance of crops. The first such application became commercially available by mid 1990's.

1.3. Current production and demand for chitinases

In past several years, there has been steady increase in the demand of chitin and its derivatives for various industrial, clinical and pharmaceutical applications. The potential of developing enzymatic process for the production of chitooligosaccharides has attracted the attention of biotechnologists. Chitooligomers produced by enzymatic hydrolysis of chitin can be used in human health care. Chitinolytic enzymes are gaining importance for their biotechnological applications. Since the excessive use of chemical pesticides has caused serious environmental problems, demand for safer biocontrol agents such as chitinase is increasing. However, currently, the progress in the industrial production of chitinolytic enzymes is limited. Since, the cost of chitinase represents an important share of the total cost of bioconversion of chitin (due to high prices of commercially available chitinases), there is necessity to develop bioprocesses with reduction in the cost of the production. The success in employing chitinases for different purposes would largely depend on the supply of active preparations at reasonable cost.

Usukizyme, a commercial fungal cell wall lytic enzyme preparation from *Trichoderma viride* was developed at Usuki Bio Center, Japan. Microbial chitinases from different other sources (*Streptomyces* spp, *Serratia marcescens,* etc) are commercially available from Sigma. Approximate cost of one unit is US$ 30.5 (1,200 units/g solid, Sigma 2003).

1.4. Role and application of chitinases

The physiological function of chitinases depends on the source. They have important role in nutrition and parasitism in bacteria and have morphogenetic function in fungi, protozoa and invertebrates. In plants and vertebrates, chitinases are associated with defense mechanism (Gooday 1995). Chitinase activity in human serum has also been reported with possible role of defense against fungal pathogens (Escott et al. 1996).

Chitinases have many industrial and agricultural applications. The ability of chitinases to degrade chitin makes them valuable in the fields of pest control and pollution abatement. Chitinases are exploited for their use as a biocontrol agent (Lorito et al. 1993, Inbar & Chet 1991). They also have a critical role in generation of fungal protoplasts (Kelkar et al. 1990), mosquito control (Shaikh & Deshpande, 1993), production of bioactive chitooligosaccharides (Kobayashi et al. 1997) and degradation of fish waste (Tom & Carroad 1981). *In vitro* antifungal activity test using chitinase from *Trichoderma harzianum* showed extensive cell wall lysis of fungal phytopathogen *Colletotrichum gloeosporioides* (Fig 1). Enzymatic hydrolysis of the cell walls using chitinase preparation was also found to be effective in the recovery of tannase enzyme (Barthomeuf et al. 1994).

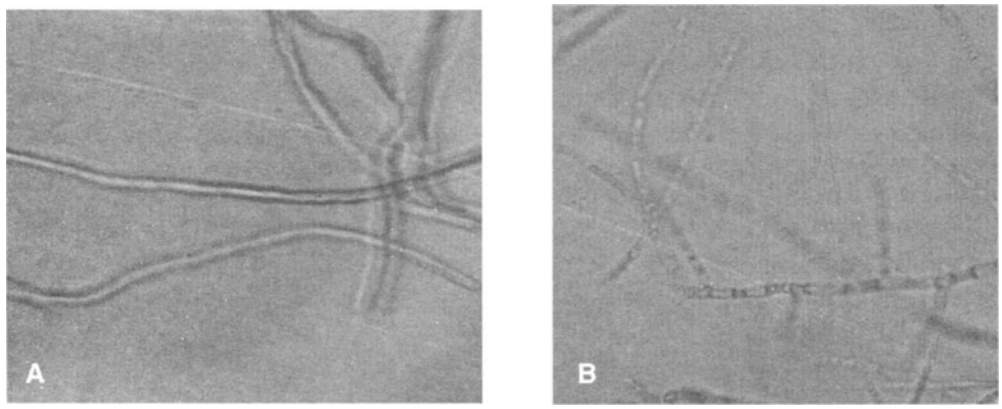

Fig. 1. Effect of chitinase from *T. harzianum* on morphology of *C. gloeosporioides* A: *C. gleosporidies* grown in heat inactivated chitinase B: Hyphal wall lysis of *C. gloeosporioides* grown in presence of chitinase from *Trichoderma harzianum.*

2. SOURCES OF CHITINASES

Due to the potential applications of chitinase, it is very important to study the organisms that can produce the enzyme. Chitinolytic organisms are those organisms capable of degrading chitin solely by hydrolysis of glycosidic bonds. Chitinases have been detected in bacteria, fungi and plants, and in the digestive systems of coelenterates, nematodes, polychaets, moluscs and arthropods. In the vertebrates, chitinases are secreted by the pancreas and digestive mucosa of insectivorous fishes, amphibians and reptiles as well as by the gastric mucosa of some insectivorous birds and mammals. Microorganisms are generally preferred to plant and animals as sources of industrial enzyme because their production cost is low and enzyme contents of microbes are more predictable and controllable. Another factor is easy availability of raw materials with constant composition for their cultivation. The ability of a microbial community to degrade chitin is important for the recycling of nitrogen in the soil. Chitinolytic microbes occur widely in nature and prevent the polysaccharide deposited from dead animals and fungi from accumulating in land and marine sediments. Among bacteria, chitinase producing organisms are in the genera of *Aeromonas, Bacillus* and *Serratia*. Among actinomycetes, chitinolytic organisms are in the genera of *Streptomyces* while in fungi the organisms occur in the genera *Trichoderma* and *Aspergillus*.

The major sources for thermophilic microbes producing chitinases are *Bacillus licheniformis* X-7u, *Bacillus* sp. BG-11, *Streptomyces thermoviolaceus*, *Thermococcus chitinophagus* (Haki & Rakshit 2003). Table 1 presents a list of some chitinase producing microorganisms.

Table 1 Chitinase producing microorganisms and their applications

Microorganism	Application	Reference
Bacteria		
Vibrio alginolyticus	Chito-oligosaccharide production	Murao et al 1992
Streptomyces kurssanovii	Chito-oligosaccharide production	Stoyachenko et al 1994
Serratia marcescens	Biocontrol agent	Ordentlich et al 1988
Serratia plymuthica	Biocontrol agent	Kalbe et al 1996
Bacillus circulans	Protoplast generation	Johnson et al 1979
Aeromonas cavie	Biocontrol agent	Inbar & Chet 1991
Streptomyces lydicus	Biocontrol agent	Mahadevan & Crawford 1997
Stenotrophomonas maltophilia	Biocontrol agent	Zhang & Yuen 2000
Paenibacillus illinoisensis	Biocontrol agent	Jung et al 2002
Fungi		
Trichoderma harzianum	Generation of fungal protoplast Biocontrol agent	Kumari & Panda 1992 Irene et al 1994
Myrothecium verrucaria	Biocontrol agent Mosquito control Single cell protein production	Vyas & Deshpande 1989 Mendosa et al 1996 Vyas & Deshpande 1991
Trichoderma reesei	Chito-oligosaccharide production	Usui et al 1990

3. PRODUCTION OF CHITINASES

Chitinases can be synthesised in the absence of substrate (constitutive enzyme) or in its presence (adaptative enzyme). However, addition of chitin to culture media greatly enhances enzyme production.

3.1. Screening and selection of microorganisms producing chitinases

Usually chitinases producing microorganisms are isolated from soils, chitinous wastes and marine environments. Screening and isolation is carried out on a chitin containing medium. Many strains of genus *Aspergillus, Penicillium* and *Trichoderma* have been isolated from soil and examined for their chitinases activity. Forty bacterial strains were isolated by Sabry (1992) from shrimp shell waste and tested for their ability to produce chitinase. A strain of *Aeromonas* sp, isolated from pond water, was found to be best chitinase producer, using colloidal crab shell chitin as substrate (Huang et al. 1996). Tagawa & Okazaki (1991) isolated microorganisms with lytic activity towards cell wall of *A. niger*. Gupta et al. (1995) screened and isolated a variety of *Streptomyces* species for chitinase production. Forty *T. harzianum* isolates were screened for extra cellular chitinase production in solid-state fermentation, using wheat bran as substrate (Nagy et al. 2003).

3.2. Fermentation

Most of the studies on chitinases production have been carried out in liquid or submerged fermentation (SmF), although some attempts have been made in more recent times on solid-state fermentation (SSF) too. Generally, presence of chitin in the production medium is useful for the production (Monreal & Reese 1969, Ulhoa & Peberdy 1993). Among different sources of chitin, colloidal chitin was found to be best for chitinase production (Bhushan 2000, Nampoothiri et al. 2003). Addition of carbon sources other than chitin reduced chitinase production but supported growth. In most cases, chitin concentration in the range of 1-1.5% was found to be suitable for chitinase production (Felse & Panda 2000).

Among the various pentoses and hexoses studied with *Streptomyces* species, arabinose doubled enzyme production while glucose repressed enzyme synthesis (Gupta et al. 1995). No chitinase production was observed when *Stachybotrys elegans* was grown on glucose, sucrose or N-acetyl glucosamine (Tweddell et al. 1994). Frandberg & Schnurer (1994) reported that production of chitinase by *B. pubuli* K1 grown on chitin was repressed by the addition of glucose, starch, β-glucan and glycerol.

Young et al. (1984) developed a comprehensive model for chitinase production during the growth of *S. marscescens* QMB 1466 on chitin, taking into account the rate of chitin hydrolysis in order to estimate the rate of bacterial growth. The equations constituting the proposed model were fitted to the experimental results from both continuous and batch fermentation to obtain parameters describing substrate yield, metabolic maintenance and enzyme yields.

Binod et al. (2003) used different substrates containing chitin for the production of chitinase in SSF, which included fungal cell walls, crab, prawn and shrimp shells. Utilization of these wastes is frequently important for economic and environmental reasons. Colloidal chitin based rice husk medium was found to be a potent substrate for chitinase production. Nagy et al. (2003) reported corn cob as an appropriate substrate for chitinase production in SSF. Kovacs et al. (2003) found that though high levels of chitinase were produced when *T. longibrachiatum* IMI 92027 was grown on different carbon sources other than chitin (showing enzyme to be constitutive), addition of chitin to SSF medium induced the production of chitinase considerably.

Nitrogen regulation is of wide significance in industrial microbiology since it affects the synthesis of enzymes involved in both primary and secondary metabolism. Ample reports are available on the effects of the supplementation of nitrogen sources on chitinase production. Kapat et al. (1996) observed that exclusion of urea from the production medium increased chitinase production. Peptone at a concentration of 3% was found to be the best nitrogen source for the production of chitinase by *Pseudomonas stulzeri* YPL-1 (Ho-Seong & Sang-Dal 1994). Sandhya et al. (2004) also reported that the supplementation of nitrogen sources (0.42%w/v) such as peptone and tryptone in the fermentation medium showed a marked increase in chitinase production by *T. harzianum*. Kovacs et al. (2003) observed that there was no significant effect of different nitrogen additives on the chitinase yield in wheat bran chitin medium in SSF. However, Nampoothiri et al. (2003) reported that supplementation of 2.0% (w/w) yeast extract to wheat bran based medium showed enhancement in chitinase yield by *T. harzianum*.

The presence of non-ionic surfactants such as Tween 20 enhanced chitinase production by *Alcaligens xylosoxydans* (Vaidya et al. 2001) but cationic and anionic surfactants were inhibitory. Addition of H_2O_2 to the cultivation medium promoted specific chitinase activity (Akimoto et al. 2000). H_2O_2 could pass

freely through the cell membrane and promotion of chitinase production may probably due to generated oxygen species. Chitinase synthesis in *T. harzianum* was found to be blocked by both 8-hydroxyquinoline and cycloheximide, inhibitors of RNA and protein synthesis respectively (Ulhoa & Peberdy 1991).

Studies have been carried out on the effect of process parameters such as pH of the medium, temperatue of fermentation, aeration and agitation, etc. on chitinase production in SmF. Chitinase production by *S. marcescens* using pre-treated chitin from crab and shrimp shells as substrate in shake flask was observed at a pH and temperature range of 6.0-8.4 and 25-30^{0}C, respectively. Maximum chitinase activity was predicted at 28^{0}C and pH 7.6 by response surface methodology (Cosio et al. 1982). The effect of initial pH of the culture medium on enzyme production by *Verticillium lecanii* ATCC 26854 in SmF showed that in acidic conditions, chitinolytic activity was higher than neutral and alkaline pH (Matsumoto et al. 2001) while *Alcaligenes xylosoxydans* showed optimum chitinase production at pH 8.0 (Vaidya et al. 2001). However, in case of SSF, generally agro-industrial residues are used as substrates and since they possess excellent buffering capacity (Pandey 1992, Pandey et al. 1999, 2000, 2001), generally there is no need to adjust the pH of the substrate/medium. This was supported by the results obtained by Nampoothiri et al. (2003). However, initial pH of shrimp shellfish waste medium significantly affected chitinase production in SSF by *Aspergillus* sp. S1-13 (Rattanakit et al. 2002).

In SSF, the particle size of substrate and moisture level plays crucial role in chitinase production. Maximum chitinase production by *Beauveria bassiana* in a wheat bran medium was obtained with an average particle size of <425 mm (Suresh & Chandrasekharan 1999). Reduction in particle size provides larger surface area for microbial growth but inter-particle porosity is less. In the case of larger particles, porosity is greater while surface area is less. Hence, an optimum particle size is needed for growth and enzyme production (Muniswaran & Charryalu 1994). The importance of substrate moisture and chitinase production by *Verticillium lecanii* ATCC 26854 in SSF has been discussed by Matsumoto et al. (2001) who found that 75% moisture level was needed for best activity by the culture. Maximum chitinase activity by *T. harzianum* was observed when the initial moisture content of the substrate was adjusted to about 65% (Nampoothiri et al. 2003). At lower and higher initial moisture levels, the metabolic activities of the culture and consequently the product synthesis was variously affected.

Chitinase production has been generally best in the mesophilic temperature range, i.e. 28-30^{0}C in SmF and SSF. For example, highest production of chitinase by *Bacillus pabuli* K1 in SmF and by *T. harzianum* in SSF was observed at 30^{0}C (Frandberg & Schnurer 1994, Nampoothiri et al. 2003). Studying the effect of aeration and agitation, Felse & Panda (1999) found aeration rate of 1.5l1 (-1) per minute and agitation rate of 224 rpm as the best combination for the production of extracellular chitinase by *T. harzianum* in batch mode. Khoury et al. (1997) observed best chitinase production by *Serratia marcescens* at mild agitation and aeration condition. Culturing of *Pencillium janthinellum* P9 in a 3-l bench top bioreactor showed that both agitation and aeration significantly influenced enzyme production. Highest level of chitinase production was obtained at an impeller speed of 500rpm and aeration rate of 1.5vvm (Massimiliano et al. 1998).

3.3. Improving production of chitinase using recombinant organisms

Although naturally occurring organisms provide a major source of chitinolytic enzymes, genetic improvement plays an important role in their biotechnological applications and several attempts have been made towards this. A non-pigmented chitinase and chitobiase over-producing mutant of *S. marcescens*

was obtained by UV irradiation and nitrosoguanidine treatment (Joshi et al. 1989). Kole & Altosaar (1985), using a non-pigmented, stable mutant of *S. marcescens* designated BL 40 showed about 167% increase in chitinase activity over the wild type strain under similar conditions.

Extensive literature is available on molecular cloning for chitinases either to increase biocontrol efficiency, to prepare highly active chitinase or even transgenic plants to increase pathogen resistance (Wiwat et al. 1996, Chen et al. 1997). Genes from mycoparasitic fungi was found to be a rich source for controlling disease in plants (Lorito et al. 1998). Terakawa et al. (1997) showed that a fungal chitinase gene from *Rhizopus oligosporus* conferred antifungal activity to transgenic tobacco. Chen et al. (1997) used recombinant *E. coli* strain harbouring a chitinase gene from *Aeromonas hydrophilia* for enhanced production of chitinase. The expression of chitinase was induced by IPTG and the cell growth rate was controlled by varying aeration rate and the concentration of nitrogen source in the medium. The co-transformation of *T. reesei* protoplasts with *Aphanocladium album* chitinase was reported. The 6.5 fold higher activity expressed in transformant was found to be useful in bioremediation and biocontrol (Deane et al. 1999).

3.4. Immobilization as an effective means for chitinase production

Immobilization of whole cells and protoplasts has proved to be very effective for chitinase production for longer periods of time when compared to cell free systems. However, the major disadvantage is the mixing and mass transfer limitations. Mass transfer limitation can be avoided by using two different columns for continuous production and separation of chitinase. O'Riordan et al. (1989) reported that co-immobilization of *Micromonospora chalcae* with chitin in calcium alginate maintained maximum chitinase activity for a long time when compared to free cells where chitinase activity reduced drastically after three days. Chitinase production by protoplasts of *Wasabia japonica* immobilized on double layered gel fibres were found to be more effective in producing chitinase than free cells of *W. japonica* (Tanaka et al. 1996).

4. ASSAY OF CHITINASE ACTIVITY

Different methods are available for the assay of chitinases. Optimal conditions for chitinase assay are determined depending on the source of chitinase. The specific determination of *endo*-chitinase activities employs chitin derivatives and is more cumbersome. The commonly used assays suffer important drawbacks, such as time consuming, need substrates that are not commercially available or when commercially available the substrate may be of variable quality. Moreover, none of these methods allow the direct determination of initial rates by time-course monitoring of the enzymatic reaction. Also, none of these substrates allow an unambiguous determination of the *endo* versus *exo* mode of action of these enzymes.

4.1. Viscometric assay makes use of the rate of decrease in viscosity as a function of enzyme concentration (Jeuniaux 1966). However, this method is not generally considered suitable for screening large number of samples.

4.2. Turbidometric assay depends on the measurement of the rate of decrease in light scattering that accompanies depolymerisation of a suspension of colloidal chitin. This method is suitable only for relatively high activities (Jeuniaux 1966).

4.3. Radiometric assay involves the radioactive counting of water-soluble oligosaccharides released from radio-labelled chitin (Cabib 1988)

4.4. Fluorometric assay gives reproducible results. This assay employs fluorogenic substrates (Yang & Hamaguchi 1980)

4.5. Dye linked assay- This method is based on the precipitability of the non-hydrolysed chitin by hydrochloric acid. A carboxymethyl-substituted soluble chitin covalently linked with Remazol Brilliant Violet 5R can be used for detection of chitinase activity (Wirth & Wolf 1990)

4.6. Chitinase activity can be determined after native or denaturing polyacrylamide electrophoresis (PAGE) by incorporating glycol chitin into the gel. Glycol chitin exhibits high affinity toward Calcofluor white M2R so lysis zones can be visualized by UV illumination as non-fluorescent dark bands in contrast to the fluorescent intact glycol chitin (Trudel & Asselin 1989)

4.7. Solid-state CP/MASS ^{13}C-NMR spectroscopy can detect oligomers produced during the initial phase of chitin hydrolysis. This method indicates which enzyme of the chitinase complex initiates the hydrolysis. Rajamohanan et al. (1996) used this method to monitor the time course hydrolysis of chitin by the chitinase mixture produced by *Myrothecium verrucaria*.

4.8. Assays based on measurement of end products- The increase in reducing activity that results from the depolymerization of chitin has been used as an assay for chitinases. Measurement of the rate of liberation of N-acetylglucosamine from chitin is probably the most widely used method for the assay of chitinases. The dinitrosalicylic acid reagent arrests the hydrolysis reaction and also helps to develop colour, which is read at 575 nm (Miller 1959).

Procedure:

Chitinase activity can be determined following the method of Jeuniaux (1966) using colloidal chitin as substrate. Colloidal chitin can be prepared by the method as described in Sandhya et al. (2004). The reaction mixture contains 0.5 ml of enzyme, 0.5 ml of 0.5% colloidal chitin and 1.0 ml of citrate phosphate buffer (pH 5.6). The mixture is kept in a water bath at 50^{0}C for 1 h. Then the reaction is arrested by addition of 3 ml dinitrosalicylic acid reagent followed by heating for 10 minutes. The coloured solution is then centrifuged at 10,000 x g for 5 minutes and absorption of the supernatant measured at 575nm against the control. One unit of enzyme is defined as the amount of enzyme which catalyses the release of 1μM of reducing sugar per min under assay conditions.

5. PURIFICATION AND CHARACTERIZATION OF CHITINASES

A variety of conventional techniques such as ammonium sulphate and alcohol precipitation, DEAE (diethylaminoethyl) cellulose and SP – sephadex chromatography, etc have been used for the purification of chitinases. Affinity chromatography is potentially the most powerful method for chitinase purification and has been used with varying degrees of success. A chitinase from autolysed culture filtrate of *Pencillium oxalicum* was purified by combination of ammonium sulphate precipitation, gel filtration and ion exchange chromatographies (Rodriguez et al. 1995). An extracellular chitinase secreted by *B. brevis* was purified to homogeneity by ammonium sulphate precipitation, phenyl-sepharose hydrophobic interaction chromatography and DEAE anion-exchange chromatography (Li et al. 2002). Chitinase

produced by *Vibrio* sp was purified 13.6-folds with a yield of 9.9% utilizing gel filtration on Bio-gel P-30, acetylated chitin column chromatography and gel filtration on Bio-gel P-150 (twice) (Takahashi et al. 1993).

Table 2 shows some of the biochemical properties of chitinase from different sources. Extracellular chitinase produced by *Acremonium obclavatum* had an apparent Km value of 0.9 U N-acetyl glucosamine (GlcNAc) equivalents and a Vmax of 23.5 U (GlcNAc)/mg protein (Gunaratna & Balasubramanian 1994). N- terminal amino acid sequence analysis showed that ten N-terminal amino acids- AVSNSKIIGY in a novel chitinase from *B. brevis* (Li et al. 2002). Thermostable chitinase from *Bacillus* sp. BG-11 was resistant to the action of proteases and allosamidin (Bhushan 2000). N-terminal amino acid sequence analysis of *B. cereus* exochitinase showed 25 amino acids. Alanine was the first N-terminal amino acid residue indicating the cleavage of a signal peptide from chitinase precursor to form a mature extra cellular chitinase. The N-terminal sequence showed significant similarity with other bacterial chitinases (Wang et al. 2001).

7. CONCLUSIONS

Chitinase offer tremendous industrial potential as biocontrol agent as well as for the treatment of chitinous wastes from sea-food industry. Utilization of chitinous waste as substrate in SSF could be of great relevance for both economical and environmental aspects. Extensive literature is available on improving chitinase yield using immobilized as well as recombinant microorganisms. Mathematical modelling can be employed for optimization and control of large-scale fermentation systems.

8. PERSPECTIVES

Recently, there has been an increased attention on the production of microbial chitinases mainly due to its application in various fields. Even though there is considerable increase in chitinase production through improved screening methods and genetic modifications, studies on large-scale production of chitinases is very limited. Therefore, a wide scope exists for extensive research in this area. Detailed studies on the influence of engineering variables such as aeration, water activity, etc. on the production of chitinase would be useful for understanding the kinetics of production. A complete study on the nature of chitinolytic enzymes will make them more useful in a variety of processes in near future. Chitinases with specific characteristics would be of much interest for commercial applications. SSF should be looked extensively as tool for their production.

Table 2. Characterization of microbial chitinases

Microorganism	Type of chitinase	Molecular weight (kDa)	Optimum pH& pH Stability	Optimum & Stable temperature	Effect of metal ions	Reference
Microbispora sp.v2	exo	35	3.0 3.0-11.0	60	Hg^{2+} inhibited	Nawani et al. 2002
Serratia plymuthica	endo	60.5	5.4	55	Inhibited by Ca^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+},	Frankowski et al. 2001
	exo	95.6	6.6	43		
Colletotrichum gloeosporioides	Endo	43	7.0	50	Completely affected by K^{+}, Hg^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+}, Zn^{2+} , Mn^{2+} , Fe^{2+}	Souza et al. 2003
Bacillus cereus	exo	36	5.8, 2.5-8.0	35, 4-70	NS	Wang et al 2001
B. brevis	endo	85	8.0, 6-10	60	Ag^{2+}	Li et al 2002
Streptomyces RC 1071	endo	70	8.0, 4-9	40, 30-70	NS	Gomes et al 2001
Acremonium obclavatum	NS	45	3-4, 3-7	50	Inhibited by Hg^{2+} & Mn^{2+}	Gunaratna & Balasubramanian 1994
Ewingella americana	endo	33	6.5	50	Zn^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Co^{2+} had a drastic effect	Peter & John 1997

NS-Not Studied

Acknowledgements

PB acknowledges the grant of a Junior Research Fellowship by the Council of Scientific and Industrial Research, New Delhi.

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Invertases

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1. INTRODUCTION

1.1 History and general concepts

β-D-fructo furanoside fructohydrolase, commonly called invertase and also known as β-Ffase, β-fructofuranosidase, β-fructosidase, β-h-fructosidase-fructofuranosidase, β-fructosylinvertase, glucosucrase, invertin, maxinvert, saccharase and sucrase; was first discovered by Berthelot in 1860 (Neumann & Lampen 1967). The detachment reaction of the terminal non-reducing L-D-fructofuranoside residue is catalysed by invertase. Its preferred substrate is sucrose but invertase has the ability to catalyse the hydrolysis of rhamnose and stachyose (Fiedurek et al 2000). Actually, invertase belongs to the first protein that has been identified as biocatalysts and it fits into one of the most fundamental principles of enzymology, the Michaelis-Menten equation (Chen et al 1999). Then it was quickly framed into the Lineweaver-Burk lineal form. For this reason, invertase has been used to establish many of the models that are currently used in the kinetic of enzyme-catalysed reactions.

Although invertase can be widely found in nature, it is usually produced from baker's yeast. It can be obtained at different strength levels. Typically, the strength of an invertase preparation is expressed as Sumner Units/gram. That means that one pound of invertase contains just a fraction of the proper enzyme mixed with modified starch as a carrier.

Actually, invertases are the most used enzymes in the food industry, and are especially used for the preparation of jams, candies and preserves, and production of lactic acid from fermentation of cane sugar molasses (Acosta et al 2000). Invertase are also used to produce ethanol from sucrose as a carbon source; by using this method the presence of sorbitol during the fermenting process is avoided (Lee & Huang 2000).

1.2. Classification of invertases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, invertases are classified in the subgroup 2 of group 3 (glycosilases). Glycosylases are placed on the hydrolases subgroup as they hydrolyse the glycosidic linkage in sucrose to liberate D-glucose and fructose. Glycosylases are subdivided into those enzymes that hydrolyse O- (EC 3.2.1), S-glycosyl (EC 3.2.3) and N-glycosyl compounds (EC 3.2.2). However, invertases do not easily fit into the general nomenclature system and some of them can transfer glycosyl residues to oligosaccharides, polysaccharides and other alcoholic acceptors.

While the systematic name always includes 'hydrolase', the common name is constructed by adding the suffix –ase to the name of the substrate in which the enzyme exerts catalytic action. It is understood that the name of the substrate with this suffix, and no other indicator, means a hydrolytic enzyme. Furthermore, common names for invertase enzymes acting on D-sugars or their derivatives do not contain D, unless ambiguity would result from the common existence of the corresponding L-sugar.

2. MICROBIAL INVERTASES

Invertases are produced by bacteria, fungi, higher plants and some animal cells (de la Vega et al 1991, Mátrai et al 2000, LuxhØi et al 2002, Huang et al 2003). However, most of the research on invertase production has been done with *Saccharomyces cerevisiae* (Waheed & Shall 1970, Zech & Görish 1995, Vrábel et al 1997, Acosta et al 2000, Belcartz et al 2002, Codón et al 2003). *S. cerevisiae* is particularly an interesting microorganism and while most sucrose-consuming yeast species have the ability to either synthesise only one form of invertase in intra- or extracellular type (Belcartz et al 2002), *S. cerevisiae* synthesises both of them. One is a glycosylated periplasmic protein and the other is a cytosolic and nonglycosylated protein. It is known that both of these enzymes are synthesised on the matrix of the same structural gene (SUC2), which encodes two different mRNAs (1.8 and 1.9 kb). The glycosylated form has been used as a model for the study of synthesis and excretion of enzymes and other proteins with carbohydrate domain (Zech & Görisch 1995). Other yeast strains such as *Kluyveromyces fragilis* (Workman & Day, 1983), *Phaffia rhodozyma* (Persike et al 2002), *Rhodotorula glutinis* (Rubio et al 2002), *Pichia anomala* (Rodríguez et al 1995) and *Schwanniomyces occidentalis* (Rubio et al 2002) produce and excrete a single form of invertase. However, in *Candida utilis,* the enzyme was described as a glycoprotein composed by two identical subunits with a molecular mass of 150 kDa each. Both subunits contained approximately 60% of carbohydrates (Chávez et al 1997, Belcartz et al 2002). Chávez et al (1997) suggested that *C. utilis* has the ability to synthesise and excrete only one form of invertase. In contrast, Belcarz et al (2002) reported that in certain liquid media *C. utilis* simultaneously produced not one but two types of invertases. It was found that the extra-cellular invertase activity was higher (70 U/mL) when the yeast was cultured in YEP medium. However, high specificity was observed when *C. utilis* was grown in Ld culture media (291 U/mg protein).

Rubio et al (2002) reported that the biosynthesis of invertase by *R. glutinis* was strongly affected by the type of sugar used as carbon source (fructose, glucose, lactose, maltose, raffinose and sucrose). They also reported that the enzyme was only produced with sucrose and raffinose as carbon sources, indicating that the enzyme was substrate inducible. Costaglioli et al (1997) reported similar results for *S. occidentalis* and *A. niger.*

Chaudhuri & Maheshwari (1996) showed that invertase could be produced in culture broth containing sucrose and asparagines by *Thermomyces lanuginose.* Invertase has also been produced by *A. niger* in solid-state fermentation (SSF) and higher titres of invertase compared to those obtained in submerged fermentation (SmF) were produced (Viniegra-González et al 2003).

Several works have reported the use of bacteria for invertase production (Looten et al 1987, Ro & Kim 1991, Nadkarni et al 1993, Liebl et al 1998). Table 1 presents some of the microorganisms that have been used to produce invertases.

Table 1. Micro-organism reported as invertase producers

Yeast	Reference	Fungi	Reference
Candida utilis	Belcarz et al. 2002	*Aspergillus candidus*	Mátrai et al. 2000
Hansenula polymorpha	Acosta et al. 2000	*Aspergillus ficcum*	Ettalibi & Baratti 2001
Kluyveromyces fragilis	Workman & Day 1983	*Aspergillus flavus*	Mátrai et al. 2000
Phaffia rhodozyma	Persike et al. 2002	*Aspergillus fumigatus*	Fiedurek et al. 2000
Pichia anomala	Rodríguez et al. 1995	*Aspergillus japonicus*	Duan et al. 1993
Pichia pastoris	Acosta et al. 2000	*Aspergillus nidulans*	Chen et al. 1996
Rhodotorula glutinis	Rubio et al. 2002	*Aspergillus niger*	Mátrai et al. 2000, Romero-Gómez 2000; Montiel-González et al. 2002
Saccharomyces cerevisiae	Neumann & Lampen 1967	*Aspergillus nomius*	Mátrai et al. 2000
Schwanniomyces occidentalis	Cited by Mukherjee et al. 2002	*Aspergillus ochraceus*	Mátrai et al. 2000
Schizosaccharomyces pombe	Tanaka et al. 1998	*Aspergillus parasiticus*	Mátrai et al. 2000
		Aspergillus sclerotium	Mátrai et al. 2000
		Aspergillus sydowii	Mátrai et al. 2000
		Aspergillus terreus	Mátrai et al. 2000
		Aspergillus versicolor	Mátrai et al. 2000
		Aureobasidium sp.	Hayashi et al. 1992
		Aureobasidium pullulans	de Aguiar Oliveira & Park 1995
Fusarium oxysporum	Onodera & Maruyama 1979, Nishizawa et al. 1980	*Azotobacter vinelandii*	de la Vega et al. 1991
Fusarium moniliforme	Mátrai et al. 2000	*Azotobacter chroococcum*	de la Vega et al. 1991
Neurospora crassa	Meachum et al. 1971	*Lactobacillus reuteri*	Cuezzo de Gines et al. 2000
Neurospora sitophila	Dixon & Fogarty 1974	*Clostridium perfringens*	Ishimoto & Nakamura 1997
Penicillium aurantiogriseum	Mátrai et al. 2000	*Clostridium acetobutylicum*	Looten et al. 1987
Penicillium crustosum	Mátrai et al. 2000	*Corynebacterium murisepticum*	Nadkarni et al. 1993
Penicillium duponti	Maheshwari et al. 2000	*Pseudomonas fluorescens*	Bugbee 1984
Penicillium griseoroseum	Mátria et al. 2000	*Pseudomonas syryngae*	de la Vega et al. 1991
Penicillium janthinellum	Mátrai et al. 2000	*Thermotoga maritime*	Liebl et al. 1998
Phytophthora megasperma	West et al. 1980	*Zymomonas mobilis*	Lee & Huang 2000
Thermomyces lanuginosus	Chaudhuri & Mahesshwari 1996		
Termitomyces clypeatus	Mukherjee et al. 2002		

3. PRODUCTION OF INVERTASES

Most of the works on invertase production have been done in SmF using sucrose and molasses as main carbon source. Belcarz et al (2002) observed that 1% sucrose induced invertase synthesis by *C. utilis*. It has been reported that *P. anomala* and *S. cerevisiae*, at low glucose and fructose concentration (products of sucrose cleavage) induced the invertase-coding gene. *S. cerevisiae* has been cultured in different media to produce high quantity of invertase. Workman & Day (1983) used *Kluyveromyces fragilis* (ATCC 12424) to produce invertase in a 20 L aerobic reactor using a defined medium with inuline (10 g l^{-1}) as carbon source. Invertase production by *P. rhodozima* was detected when the yeast was cultured in a medium containing sucrose. No significant invertase activity was found in the cell-free culture from the lag to the stationary phase. Intracellular invertase production occurs between 24 and 26 h (Persike et al 2002).

A method to prepare immobilised cell-associated enzyme by entrapping mycelia has been reported by Chien et al (2001). The column reactor packed with the cell-immobilised gluten particles was tested for long-term operation and it was found to have a great potential to scale-up the process for industrial production.

Several attempts have been made to improve invertase production by fungi. A selection of *A. fumigatus* strains by using classical screening and mutagenesis techniques have been reported (Fiedurek et al 2000). Maximum extracellular and intracellular invertase activities (5.27 U/mL and 0.44 U/mL) were reached after 96-h incubation. The increment of invertase activity compared to the parental strain was about two folds. Mukherjee et al (2002) reported the invertase production by *Termomitomyces clypeatus* when the fungus was cultured for five days in synthetic medium with 1 % (w/v) of sucrose. They also reported the constitutive character of the invertase when the yeast was cultured in sucrose, glucose, cellobiose, fructose or mannose as alternative carbon sources leading to equivalent cellular levels of invertase obtained by extensive sonication of the respective cell mass.

Montiel-González et al (2002) compared haploid mutants of *A. niger*, previously characterised as enzyme-over-producing strain and diploid constructs of *A. niger* originated from such haploid mutants. Higher invertase production was obtained by SSF than the conventional SmF. Romero-Gómez et al. (2000) also reported higher invertase titres by *A. niger* on SSF than SmF.

Baig et al (2003) showed that the use of different sources of organic nitrogen affected invertase production. They observed that when peptone and yeast extract were used as nitrogen sources, maximal activity was obtained by different strains of *Saccharomyces* sp. De la Vega et al (1991) reported invertase production by *Azotobacter chroococcum* and *Azotobacter vinelandii* growing on nitrogen free Burk medium supplemented with sucrose (1 %) as the sole energy and carbon source.

3.1. Inhibition of invertase activity

Von Euler & Landergren (1922) were the first to report that a low concentration of iodine has inhibitory effect on yeast invertase (cited by Waheed & Shall 1970). They observed that about 55 % inhibition occurred when invertase was reacted with iodine solutions (Waheed & Shall 1970). It has also been reported that invertase activity is inhibited by sucrose concentrations higher than 5 %. However, studies carried out with co-immobilised enzymes on chitin showed that inhibition occurred at sucrose concentrations higher than 20 % (Ro & Kim 1991).

The invertase produced by *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) was inhibited (45 %) with Hg^{+2} (2 mM). Aniline caused a lower inhibitory effect (10 mM) on the invertase produced

by *Xanthophyllomyces* than the inhibitory effect observed for *Saccharomyces* or *Neurospora* invertases (Ki = 1.7 and 1.5 mM, respectively) (Persike et al 2002).

Zech & Görish (1995) showed that invertase activity was 60 % reduced by ethanol at 120 g L^{-1} when it was incubated for 20 min. After 5 h incubation, the activity decreased 15 % more, and after 48 h, the enzyme was completely inactive. Similar pattern was observed when the enzyme was incubated with sodium chloride (7 g L^{-1}); enzyme inactivation was parallel to the enzyme dissociation into subunits. The inactivation and dissociation effect may be reversible when ethanol and NaCl concentration are reduced, then the enzyme activity is restored and the subunits re-associated to dimmers and tetramers.

Gianfreda et al (1993) studied the interaction between invertase molecules, tannic acid, hydroxyl-aluminium (OH/Al) in presence or absence of montmorillonite to obtain information about the effect of each component on inhibition and recovery of the enzymatic activity in enzyme-complexes. This study was performed to understand the behaviour of the invertase activity in the ground. It was found that a significant proportion of soil-immobilised enzyme was associated with the humic fraction forming enzyme-polyphenolic copolymers during the formation of humic substances.

De la Vega et al (1991) showed that *Azotobacter crococcum* produced an invertase, which was sensitive to a variety of divalent metals. They reported that the presence of 50 mM Cu (II) or Cd (II) in the assay mixture resulted a total inhibition on invertase activity, while Ca (II), Ba (II) and Zn (II) caused 30 % inhibition and Ni (II) caused 65 % when tested at the same concentration (50 mM). However, aniline had a less inhibitory effect.

Nevertheless, in order to study the activity of invertase, it is sometimes desirable to inhibit the invertase activity at a particular time. According to Kouassi & Roos (2000), any enzyme may be deactivated by using acetonitrile but Kochhar et al (1999) reported that invertases obtained from plant sources can be inhibited by pyridoxine hydrochloride. Table 2 shows the principal inhibitor agents for invertase.

Table 2. Inhibitory agents of invertase activity

Inhibitors	Organism	Comment	Reference
2,5-anhydro-D-mannose	*Kluyveromyces fragilis*	Competitive	Workman & Day 1983
2,5-dideoxy-2,5-imino-D-glucitol	*Saccharomyces cerevisiae*	-	Legler et al. 1993
2,5-dideoxy-2,5-imino-D-mannitol	*Saccharomyces cerevisiae*	Very good inhibitor	Legler et al. 1993
2-amino-2-hydroxy-methyl propane-1,3-diol	*Yeast*	Extra-cellular enzyme	Goldstein & Lampen 1975
2-mercaptoethanol	*Lactobacillus reuteri*	-	Cuezzo de Gines et al. 2000
Ag+	*Aspergillus japonicus*	$AgNO_3$	Duan et al. 1993
Ag+	*Clostridium perfringens*	-	Ishimoto Nakamura 1997
Ag+	*Fusarium oxysporum*	-	Nishizawa et al. 1980
Ag+	*Kluyveromyces fragilis*	-	Workman & Day 1983
ammonium sulphate	*Yeast*	Intra-cellular enzyme	Goldstein & Lampen 1975

Inhibitors	Organism	Comment	Reference
Aniline	*Yeast*	Extra-cellular enzyme	Goldstein & Lampen 1975
Aniline	*Clostridium perfringens*	-	Ishimoto & Nakamura 1997
Aniline	*Azotobacter chroococcum*	-	de la Vega et al. 1991
Ba^{2+}	*Azotobacter chroococcum*	-	de la Vega et al. 1991
Ca^{2+}	*Lactobacillus reuteri*	-	Cuezzo de Gines et al. 2000
Ca^{2+}	*Azotobacter chroococcum*	-	de la Vega et al. 1991
Cd^{2+}	*Kluyveromyces fragilis*	-	Workman & Day 1983
Cd^{2+}	*Lactobacillus reuteri*	1 mM, 66% inhibition	Cuezzo de Gines et al. 2000
Cd^{2+}	*Azotobacter chroococcum*	-	de la Vega et al. 1991
Co^{2+}	*Aspergillus niger*	-	Rubio & Maldonado 1995
Cu^{2+}	*Aspergillus niger*	-	Rubio & Maldonado 1995
Cu^{2+}	*Clostridium perfringens*	-	Ishimoto & Nakamura 1997
Cu^{2+}	*Brevibacterium divaricatum*	$CuCl_2$	Yamamoto et al. 1986
Cu^{2+}	*Kluyveromyces fragilis*	-	Workman & Day 1983
Cu^{2+}	*Lactobacillus reuteri*	1 mM, 90% inhibition	Cuezzo de Gines et al. 2000
Cu^{2+}	*Azotobacter chroococcum*	-	de la Vega et al. 1991
Dithiothreitol	*Lactobacillus reuteri*	-	Cuezzo de Gines et al. 2000
$FeSO_4$	*Brevibacterium divaricatum*	-	Yamamoto et al. 1986
Hg^{2+}	*Aspergillus niger*	1 mM	Hirayama et al. 1989, Rubio & Maldonado 1995
Hg2+	*Aspergillus japonicus*	HgCl2	Duan et al. 1993
Hg^{2+}	*Clostridium perfringens*		Ishimoto & Nakamura 1997
Hg^{2+}	*Triticum aestivum*	$HgCl_2$; 0.002 mM, complete inhibition	Krishnan et al. 1985
Hg^{2+}	*Fusarium oxysporum*		Nishizawa et al. 1980
Hg^{2+}	*Brevibacterium divaricatum*	$HgCl_2$	Yamamoto et al. 1986
Hg^{2+}	*Kluyveromyces fragilis*		Workman & Day 1983
Hg^{2+}	*Lactobacillus reuteri*	1 mM, 67% inhibition	Cuezzo de Gines et al. 2000
I_2	*Saccharomyces cerevisiae*	-	Baseer & Shall 1971
I_2	*yeast*	Extra-cellular enzyme	Goldstein & Lampen 1975
K^+	*Aspergillus niger*	-	Rubio & Maldonado 1995
K_2PtCl_3	*Saccharomyces cerevisiae*	Non-competitive	Baseer & Shall 1971
Mg^{2+}	*Aspergillus niger*	-	Rubio & Maldonado 1995

(Some data are from www.brenda.uni-koeln.de)

3.2. Modelling

Herwing et al (2001) proposed a simple non-structural and non-segregated kinetic model. The model is quite simple but predicts the evolution of sucrose, fructose and glucose by using only four parameters when one of them is fitted at every run. The authors concluded that the invertase expression is controlled by the extra-cellular monosaccharide concentration. Viniegra-González et al (2003) proposed a model to predict the enzyme productivity. In this model, the fermentation system can be expressed in different ways. The model was used to compare the enzymatic titres obtained by SSF and SmF, remarking the advantages of SSF over SmF.

4. ASSAYS FOR INVERTASE DETERMINATION

Different methods have been proposed to assay the activity of invertase. However, the saccharolytic method is commonly used. The method is based on the dinitrosalicylic acid (DNS) test for reducing sugar determination. One unit of invertase is the amount of enzyme that hydrolyses sucrose at 1 mmol/min at 60 °C and pH value of 5.0 (Illanes & Gorgollon 1986, Acosta et al 2000, Ettalibi & Baratti 2001).

Several authors (LuxhØi et al 2002, Montiel-González et al 2002, Persike et al 2002) have used the DNS method to determine the invertase activity by using 250 mL of 0.1 M sucrose as substrate. The substrate is mixed with 200 mL of 0.1 M acetate buffer (pH 5) and 50 mL of the diluted enzymatic extract. After 30 minutes incubation at 30 °C, the reaction is stopped by adding of the chromogenic reagent sodium dinitrosalicylate (DNS). One invertase unit (IU) is defined as the amount of enzyme needed to release one μmol of reducing per minute under the assay conditions.

Chen et al (1999) reported a colorimetric method where invertase samples are dissolved in 1 mL of 0.36 M with phosphate buffer at pH 4.2, and then one millilitre of sucrose solution (40 mg/mL) is added. The reaction is stopped by addition of ice cold KOH and the solution is kept on ice in order to maintain the colour in samples (Chen et al 1999, personal communication). Thereafter, the solution is mixed with a Trinder reagent solution and absorbance is recorded at 550 nm. One enzyme unit is defined as the amount of enzyme needed to release one μmol of glucose per minute at 25 °C.

A similar method for sucrose hydrolysis was reported by Vrábel et al (1997). The method uses a McIlvain buffer (with citric acid and sodium phosphates and KCl at pH 4.6). The assay differed from that described by Trinder (1969) in the chromogen used (4-chloro-3-cresol) and the monitoring wavelength (490 nm). The method used tris solution to stop the enzymatic reaction.

Invertase activity may be also estimated by the measurement of glucose released by using the YSI27 glucose analyser (Yellow Springs Instruments, Yellow Springs, OH). One unit of activity is defined as the amount of enzyme required to release one nmol of glucose per microgram of protein per minute at 30 °C (Codón 2003).

5. PURIFICATION

According to Neumann & Lampenn (1967), Berthelot published the first work on invertase purification in 1860. Chen et al (1996) purified and characterised two invertases (S –form) from *A. nidulans* by SDS-PAGE (185 KDa and 178 KDa, respectively). They suggested that the native enzyme existed probably as a dimmer. Rubio et al (2000) reported a purification methodology where the yeast was isolated from a 36 h old-sucrose culture by centrifuging at 15,000 g for 20 min. Intracellular enzyme

was prepared by re-suspending the cell into 0.2 M acetic acid–sodium acetate buffer (pH 4.5) plus 1.4 mM of 2-mercaptoethanol and disrupting in a ballistic disintegrator. Disrupted cells were centrifuged at 15,000 g for 10 min and the supernatant solution was used as intracellular enzyme source (crude extract). The crude extract was first precipitated with ammonium sulphate (40 % saturation). The precipitate obtained was collected by centrifugation at 15,000 g for 20 min and dissolved in 0.2 M acetic acid–sodium acetate buffer with 1.4 mM of 2-mercaptoethanol. Ammonium sulphate was added to the supernatant solution to achieve 80% saturation. Precipitated proteins were collected by centrifugation at 15,000 g for 20 min and dissolved in 1 ml of the same buffer. Purification was made by gel filtration (Sephadex G-150 column) equilibrated and eluted with 0.2 acetic acid–sodium acetate buffer (pH 4.5) containing 1.4 mM of 2-mercaptoethanol. The active fractions were pooled and applied to a DEAE-Sephacell column and then eluted with a linear gradient from 0 to 1 M NaCl solution in the same buffer. The purified extract was dialysed against 0.2 M acetic acid–sodium acetate buffer plus 1.4 mM of 2-mercaptoethanol and stored at 4 °C. Similar purification methods have been reported by other authors (Zech & Görish 1995).

A purified invertase preparation from *A. chrococcum* was prepared by centrifugation of the culture; the cell-free supernatant was adsorbed into a DEAE-cellulose column. Homogeneous enzyme was eluted with a lineal gradient of KCl. This purified enzyme preparation was stable at 4 °C for at least three months and a room temperature for one week. (de la Vega et al 1991).

6. CHARACTERISATION

Invertase shows stability at pH values between 3 and 7 and optimum pH at 6.5 (Acosta et al 2000). The S- and F-forms of invertases produced by *C. utilis* showed high tolerance to temperature changes and were active from 30-90 °C. However, optimum activity was obtained at 70 °C for both of them. The enzymes were highly active between pH 3.6 and 5 with optimum pH at 4.4 (Belcarz et al 2002). pH and temperature values for the optimal invertase activity are listed in Table 3.

The isoelectric point for the invertase produced by *A. chrococcum* was determined to be 4.1 by both electrofocusing and chromatofocusing methods. Belcarz et al (2002) reported two extracellular invertases from *C. utilis*, the S- and F-form, which had the same isoelectric point (pH 3.35). Chaudhuri & Maheshwari (1996) observed that *T. lanuginosus* produced active and inactive invertase forms, depending on the oxidation state of the cysteine residue, essential for the catalytic activity of enzyme. Colonna et al (1975) reported an invertase as a glycoprotein with approximately 50 % mannan and 3 % glucosamine.

Kouassi & Ross (2000) studied the glass transition and water effects on sucrose inversion by invertase from yeast. The results showed that sucrose hydrolysis was perceptible at a_w value of 0.444. Above this value, sucrose inversion increased with water activity and reached a maximum at a_w value of 0.746. It was also observed that sucrose inversion did not occur in the glassy state but rather in the rubbery state, where crystallisation occurred.

The molecular weight of the native enzyme has commonly determined by gel filtration and SDS-PAGE. Different molecular weights have been reported for invertases. A molecular weight of 59 KDa was reported for invertases produced by *A. chrococcum* (de la Vega et al 1991). Acosta et al (2000) obtained a molecular size of 60 KDa (SDS-PAGE) for a recombinant invertase expressed in a methylotrophic yeast. The molecular mass of the F-form the invertase produced by *C. utilis* under native and denaturing condition (SDS-PAGE) was 62 KDa and 62.5 KDa, respectively.

Table 3. pH and temperature values for optimal invertase activity produced by different micro-organisms

	pH	T (°C)	Reference
Yeast			
Candida utilis	4.4 both forms	70 (both forms)	de la Vega et al. 1991, Belcarz et al. 2002
Kluyveromyces fragilis	4.5	n. r.	Workman & Day 1983
Rhodotorula glutinis	4.5	60	Rubio et al. 2002
Saccharomyces cerevisiae	3.5	n. r.	Duan et al. 1993
Schizosaccharomyces pombe	4.0	n. r.	Zarate & Belda 1996
Yarrowia lipolytica	4.5 - 6.0	30	Mansfeld et al. 1995
Fungi			
Aspergillus fumigatus	4.5	n. r.	de Rezende & Felix 1999
Aspergillus japonicus	3.5	n. r.	Duan et al. 1993
Aspergillus nidulans	3.75 - 6.0	n. r.	Chen et al. 1996
Aspergillus niger	4.0	60	Shiomi & Onodera 1988, Rubio & Maldonado 1995
Aureobasidium sp.	5.5	n. r.	Hayashi et al. 1992
Aureobasidium pullulans	5.5	n. r.	de Aguiar Oliveira & Park 1995
Fusarium oxysporum	4.5	n. r.	Nishizawa et al. 1980
Phytophthora megasperma	4.2	n. r.	West et al. 1980
Bacteria			
Azotobacter chroococcum	6.5	30	de la Vega et al. 1991
Lactobacillus reuteri	n. r.	37	Cuezzo de Gines et al. 2000
Clostridium perfringens	6.0	n. r.	Ishimoto & Nakamura 1997
Clostridium acetobutylicum	5.0	n. r.	Looten et al. 1987
Corynebacterium murisepticum	7.0	n. r.	Nadkarni et al. 1993
Pseudomonas fluorescens	6.5	n. r.	Bugbee 1984
Thermotoga maritime	n. r.	90	Chavez et al. 1997

Table 4. Kinetic parameters for invertase produced by different microorganisms

	Km	V_{max}	Reference
Yeast			
Candida utilis	2 mM (S-form) 1.54 mM(F-form)	2.8 mM/min 1.5 mM/min	Belcarz et al. 2002 Chavez et al. 1997
Kluyveromyces fragilis	21 mM	n. r.	Workman & Day 1983
Rhodotorula glutinis	0.227 M	0.096 µmol/min	Rubio et al. 2002
Saccharomyces cerevisiae	7.2 mM	n. r.	Arruda & Vitolo 1999
Schwanniomyces occidentalis	0.020 M	n. r.	Rubio & Maldonado 1995
Fungi			
Aspergillus fumigatus	7.6 mM	n. r.	de Rezende & Felix 1999
Aspergillus nidulans	0.037 M (S-form) 0.046 M (F-form)	n. r.	Chen et al. 1996
Aspergillus niger	0.06 mM	n. r.	Rubio & Maldonado 1995
Aureobasidium sp.	0.75 mM	n. r.	Hayashi et al. 1992
Fusarium oxysporum	2.17 mM	n. r.	Nishizawa et al. 1980
Bacteria			
Azotobacter chroococcum	5 mM	n. r.	de la Vega et al. 1991
Lactobacillus reuteri	6.62 mM	n. r.	Cuezzo de Gines et al. 2000
Corynebacterium murisepticum	57 mM	n. r.	Nadkarni et al. 1993
Pseudomonas fluorescens	90 mM	n. r.	Bugbee 1984
Thermotoga maritime	19 mM	n. r.	Liebl et al. 1998

Bayramoğlu et al (2003) reported Km values of 14 and 39 mM, and Vmax values of 410 and 254 U mg^{-1} for free and immobilised invertases, respectively. Table 4 shows the some of the reported kinetic parameters for invertases produced by different microorganisms.

7. CONCLUSIONS

There is constant growth in the R&D on invertase. Among the several aspects being explored in this regard, two important ones include metabolic regulation involved in invertase production and the development of recombinant microorganisms. Recent studies have contributed to understand some aspects involved in the biosynthesis of the enzyme. Moreover, it has been established that each isoenzyme presents its own physicochemical characteristics. Without a doubt, most of the studies have been done with invertases produced by *S. cerevisiae.* However, other microorganisms could produce invertases offering an excellent research field. The analytical tools used so far have provided valuable information on the invertase characterisation and the mechanisms of the enzyme regulation by microorganisms reported as invertase producers are well known. Aspects of the inhibitory substances have been also investigated. Furthermore, the increasing market for fructose syrups has opened a very interesting field to explore.

8. PERSPECTIVES

The future perspective for the invertase production is promising. Recent advances in molecular biology and bioengineering could help to improve invertase production and application in a variety of processes related to the food and pharmaceutical industries. Fermentation process yielding high titres of invertase should be developed in order to reduce expenses related to the protein recovery. Since catabolic repression is reduced in solid-state fermentation, this type of culture can be used to obtain higher extracellular invertase yields. Studies related to the structure-function relationship of invertase should be carried out in order to improve the stability and activity of the enzyme. New approaches for invertase immobilisation with effective adsorption of the protein on the support and reversible immobilisation would reduce losses of protein allowing the reuse of the supports.

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Mannanase

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1. INTRODUCTION

1.1 Mannans

Mannan exists in nature in two forms; as acetylated galactoglucomannan, a principal component of hemicellulose found in softwoods (accounting for up to 25% of the dry weight), which has a heterogeneous backbone of β-1,4-linked mannose and glucose residues in the ratio 3:1, and galactomannan, which is found in seeds of leguminous plants, nuts and beans of carob trees (function as storage carbohydrates) and is composed of a homogeneous backbone of β-1,4-linked mannose residues. Mannose residues often possess α-1,6-galactose as side groups and may be acetylated at the O-2 and O-3 positions (Ethier et al. 1998).

1.2 Mannanases

Mannan-degrading enzymes are found in multiple enzyme forms (www.brenda.com, 2003). This multiplicity might result from the requirements to bind and to degrade substrates of varying complexity. Mannanases are enzymes that degrade 1,4-β and 1,6-α glycosidic bonds of mannans. Mannanase enzymes involved in the degradation of mannans are: Mannan 1,4-β-mannobiosidase (EC 3.2.1.100), mannan endo-1,6-α-mannosidase (3.2.1.101), mannosyl-oligosaccharide 1,2-α-mannosidase (EC 3.2.1.113), α-mannosidase (3.2.1.24), β-mannosidase (3.2.1.25), and mannan endo-1,4-β-D-mannosidase (EC 3.2.1.78). Enzymatic depolymerization of the mannan backbone is affected by endo-β-1,4-mannanases, which cleaves the chain randomly, producing mannobiose and longer manno-oligosaccharides from which mannose is released by exo-acting β-mannosidase. α-galactosidase and β-glucosidase are required for the complete breakdown of galactoglucomannan (Halstead et al. 1999).

Endo-β-1,4-mannanases (β-mannanases) have been grouped into two of the 85 sequence-based glycoside hydrolase families (GHs), 5 and 26 (Mc Cleary 1988). The GH5 family has the $(\alpha/\beta)_8$-barrel fold (Sabini et al. 2000), while family 26 β-mannanases stabilize a $B_{2,5}$ transition-state conformation for the glycosylation step of glycoside hydrolysis (Davies et al. 2003). GH26 family is exclusively from prokaryotic origin, while GH5 β-mannanases could have been derived from both bacteria and fungi (Hogg et al. 2003). These authors found that three β-mannanases produced by *Cellvibrio japonicus* (Man5A, Man5B and Man5C) contained both non-catalytic carbohydrate-binding modules (CBM), and GH5 catalytic domains, which targeted mannans integral to the cell wall. Another β-mannanase (Man26B) contained a GH26

catalytic domain, which targeted the storage polysaccharide galactomannan. Man26B, a modular enzyme composed of an N-terminal signal peptide and a family 26 β-mannanase domain (Kurokawa et al. 2001).

β-mannanases are available commercially, e.g. endo-β-mannanase from Megazyme (*Aspergillus* niger) and Hemicell Feed Enzyme from Hubbard Feeds Inc. (*Bacillus lentus*); or as one component of an enzyme powder such as in Mannaway from Novozymes and Procter & Gamble (recombinant *Bacillus* strain).

This chapter focuses on mannan endo-1,4-β-D-mannosidase (EC 3.2.1.78), which is also known as β-mannanase and endo-1,4-mannanase. β-mannanase catalyses the random hydrolysis of the 1,4-β-D-mannosidic linkages in mannans, galactomannans, glucomannans and galactoglucomannans, producing manno-oligosaccharides. The extent of the hydrolysis depends on the degree of substitution and the distribution of the substituents.

1.3 Applications

β-mannanases have numerous applications in food industry, which include fruit and vegetable maceration, brewery and wine, juice clarification, oil extraction from legume seeds, and viscosity reduction of coffee extract in instant coffee process, in textile and laundry, animal feed, pulp and paper industry (bio-bleaching in soft wood Kraft pulp), etc.

2. SOURCES OF β-MANNANASES

Since mannans are found in softwood hemicellulose and in seeds as storage carbohydrates, microorganisms growing in these environments are able to produce mannan-degrading enzymes. β-mannanase are produced by a number of fungi, yeasts, bacteria, marine algae, germinating seeds of terrestrial plants (Feng et al. 2003; Nonogaki et al. 2000; Viikari et al. 1993), and by invertebrates (Yamaura & Matsumoto 1993). In fruits, β-mannanase enzyme is cell wall associated; its activity increases during ripening, and is concentrated in the skin and outer pericarp of the fruit. In contrast to the situation in fruits, the seeds produce active enzymes, especially following germination (Banik et al. 2001). Table 1 lists some of the microbial sources. Novozyme, Procter & Gamble, and Hubbard Feeds use the genus *Bacillus* for β-mannanase production, becoming until now the most successful bacterial genus for industrial purposes. Among fungal cultures, only *Aspergillus niger* has been used in β-mannanase industrial production by Megazyme.

3. PRODUCTION

Production of β-mannanases has been mainly focused on microbial sources due to their industrial potential. Several bacterial and fungal cultures have been used for this purpose in submerged (SmF) and solid-state fermentation (SSF). β-mannanase biosynthesis by *B. subtilis* 168 employing a shake culture was extremely influenced by cell growth, using glucose as catabolite repressor, and galactomannan (4 g/L) as inducer (el-Helow & Khattab 1996). Inoculum size was very significant to achieve optimized conditions, which produced an enzyme activity of 33 U/mL in 4 h. This activity is among the highest reported for β-mannanase production. Blocking of the sporulation process did not significantly affect enzyme production, but allowed an extended enzyme production phase, especially in a continuous culture. The β-mannanase activity produced by *Flavobacterium* sp. was the highest in a culture medium containing: 1.0% guar gum, 0.3% $(NH_4)_2SO_4$, and 0.06% yeast extract and five-day cultivation at 4°C (Zakaria et al. 1998). The optimal temperature of this β-mannanase was 35°C, but the activity decreased

Table 1. Sources of β-mannanases

Microorganism	Reference
Vibrio sp. MA-138	Tamaru et al. 1995
Bacillus subtilis KU-1	Zakaria et al. 1998
B. stearothermophilus	Ethier et al. 1998
Cellulomonas fimi	Stoll et al. 1999
Caldocellulosiruptor saccharolyticus	Morris et al. 1995
Caldibacillus cellulovorans	Sunna et al. 2000
Sclerotium (Athelia) rolfsii	Grobwindhager et al. 1999
Schizophyllum commune	Gübitz et al. 2000
Sclerotium rolfsii	Gübitz et al. 2000
Thermomyces lanuginosus	Puchart et al. 2000
Trichoderma reesei	Sabini et al. 2000
Aspergillus niger	Regalado et al. 2000
A. oryzae	Hashem et al. 2001
Mytilus edulis	Xu et al. 2002b
Ripening tomato fruits	Sean-Carrington et al. 2002
Germinating coffee grains	Marraccini et al. 2001

to 25% of the optimal at 10°C. Addition of 0.5% lactose or 0.5% locust bean gum (LBG) to a LB medium increased significantly the β-mannanase productivity from *B. subtilis* JS-1 (Yim et al. 2003). However, induction of the enzyme depended on the carbon source used, which suggested different induction mechanisms.

A batch fermenter was used to test the effect of different environmental conditions on the production of β-mannanase by *B. licheniformis*. Temperature was the most significant factor, since it affected the other parameters, such as pH, dissolved oxygen, and reducing sugars. The best enzyme activity was obtained by using 0.7 vvm of aeration rate, agitation of 600 rpm, and a temperature of 30°C. The activity obtained in shake flask (after 48 h) was very close to that obtained using the fermenter (212.0 U/mL), after 36 h of cultivation (Feng et al. 2003). It was concluded that the mRNA coding for this enzyme was unstable, and within a temperature range, a decrease in temperature enhanced the mRNA stability prolonging the duration of enzyme production. Medium optimization for β-mannanase production by *Bacillus* sp. WS-42 in submerged fermentation was conducted by using various carbon sources (Kim et al. 1997). The optimized medium had the following composition: Locust bean gum of 10.0 g/L, soytone of 5.0 g/L, KH_2PO_4 of 2.0 g/L, NaCl of 10.0 g/L, $MgSO_4 \cdot 7H_2O$ of 0.2 g/L, Na_2CO_3 of 2.0 g/L. A maximum β-mannanase activity of 20.8 units/mL was obtained after 14 h fermentation, corresponding to a productivity of 1.48 units/mL-h.

A strain of *Streptomyces craterifer* was immobilized in luffa pulp (LP) as a porous support. Maximum production of β-mannanase (34.7 U/mL) by immobilized cell cultures was obtained at a concentration

of 7.5 g/L LBG, pH 8 and 30°C, with a productivity of 34.7 U/mL (Hassan 2000). Enzyme production was increased by 1.2-fold when a fixed-bed bioreactor for fermentation was used. The effect of aeration rate was tested and repeated batch fermentation was carried out in the column reactor.

High yields of the enzyme were obtained by inducing its production with galactomannan guar gum and beef extract/peptone as carbon and nitrogen sources, respectively (Kataoka & Tokiwa 1998). *Sporolactobacillus* sp. M201 produced extracellular β-mannanase in a medium containing 2% LBG, 0.5% peptone, 0.2% KH_2PO_4, 80 mg/L $MgSO_4$, and 8 mg/L $ZnSO_4$ (pH 6.0). The optimal culture temperature for production of β-mannanase was 37°C. Under optimal culture conditions, β-mannanase reached the highest level of 10.6 units/mL after 30 h of fermentation (Park et al.1998). Alcaraz-Sandoval et al. (1997) obtained good β-mannanase activity from SSF of copra meal using *A. ochraceus* and *Penicilliium fellutanum.* Regalado et al. (2000) obtained several β-mannanases from SSF of copra paste and spent soluble coffee wastes, using *A. niger* and *A. oryzae*. Higher β-mannanase production was achieved from SSF of copra paste using *A. oryzae.* This enzyme could be important for viscosity reduction of concentrated coffee extract in instant coffee manufacture.

β-mannanase production maximization was conducted by response surface methodology in SSF of coffee wastes using *A. oryzae* (Regalado et al. 1999). The optimized media included a nutritive supplement included in the solution to get 55% moisture content, of 1x minerals concentration, 4 g/L ammonium sulphate, 11.5 g/L yeast extract, and four days fermentation time. The maximum activity achieved, which was also predicted by the fitted second order model, went up to 108 mg mannose/(min L).

4. ASSAYS

β-mannanase activity has normally been evaluated by using the amount of reducing sugars released from a suitable substrate, under conditions near optimal enzyme activity. The substrate has usually been locust bean galactomannan (LBG) from *Ceratonia siliqua* seeds (Rätto et al. 1993, Regalado et al. 1999, 2000, Xu et al. 2002a). Another substrate that has been used is ivory nut (*P. macrocarpa*) mannan, which is an unbranched β-1,4-linked mannan homopolymer (Tenkanen et al. 1995, Hägglund et al. 2003), sold by Megazyme (Bray, Ireland). The reducing sugars released from the LBG substrate by the β-mannanase activity can be determined using a variety of methods. Typically, this enzyme assay is carried out by mixing appropriate dilutions of a β-mannanase solution with 50 mM sodium citrate buffer, pH 5.5, containing 0.5% LBG. The mixture is then incubated at 40°C for 20 min and the reaction is stopped by adding the DNS reagent. This is followed by placing the sample for five min in a boiling water bath and quickly cooled to room temperature. The degree of LBG hydrolysis is determined spectrophotometrically by measuring the absorbance at 540 nm. A mannose standard curve is usually done to determine the reducing sugars released. The activity units may be expressed as the nmoles of mannose released per second from the LBG substrate under the experimental conditions.

A modified form of gel-diffusion assays (Wood et al. 1988, Downie et al. 1994) has been used to monitor β-mannanase activity (Still et al. 1997). In this method, the enzyme diffuses from wells through a 0.8% agarose gel containing the substrate (LGB; 0.05% w/v in citrate buffer, pH 5.0) in a Petri dish. The substrate is hydrolyzed by the enzyme, reducing the binding of the dye Congo Red (3,3'-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[4-amino, disodium salt), and leaving a clearing zone which is considered to be proportional to the enzyme concentration. The clearing zone diameter was greater using Congo Red than using Remazol Brilliant Blue colored Carob substrate at identical concentrations, enzyme activities

and assay conditions (Downie et al. 1994). Congo red is highly specific to polysaccharides containing a backbone of (1 → 4) or (1 → 3) β-linked D-glucans and galactoglucomannans (Wood 1980). Each well receive the enzyme solution, and is allowed to diffuse for 24 h at 25°C. Pure endo β-mannanase standards at different concentrations placed in another gel-diffusion plate are used to plot a standard curve. A Congo Red solution (0.5% w/v) is used to stain the plates and after 20 min, they are washed and a citrate-phosphate buffer, pH 7.0 is added to reveal (after a short incubation time at room temperature) red hydrolyzed zones and a dark background. The activity is calculated from a plot of the diameter of the hydrolyzed area versus the log of the β-mannanase enzyme activity (Still et al. 1997, Still & Bradford 1997). Thus, this method gives a transient visibility of the hydrolyzed zones; however, it results in high contrasts, allowing easy activity diameter measurements (Nonogaki et al. 2000). The β-mannanase detection sensitivity was similar to that of the viscometric assay (0.14 pkat) (Downie et al. 1994).

5. PURIFICATION

Most purification schemes have made use of traditional biochemical methods to purify β-mannanases. This includes precipitation, ion exchange, size exclusion, chromatofocusing and hydrophobic interaction chromatographies. Other works separate the proteins by their isoelectric point, and most recently recombinant fused β-mannanases have been purified using immobilized metal affinity chromatography (IMAC).

A thermostable β-mannanase from *B. stearothermophilus* was purified using a combination of ammonium sulphate fractionation, gel filtration, hydrophobic interaction and ion-exchange chromatographies, followed by chromatofocusing. The enzyme was pure and was a dimer of 162 kDa with identical subunits (Talbot & Sygusch 1990). The β-mannanase from *T. reesei* was purified by ammonium sulphate precipitation, followed by cation (CM-Sepharose) and anion exchange (DEAE-Sepharose) chromatographies, hydrophobic interaction chromatography (Phenyl-Sepharose) and gel filtration (Sephacryl S-100 HR). The β-mannanase showed a single band after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Rätto et al. 1993). A β-mannanase from SSF of copra using *A. oryzae* was highly purified by a combination of acetone precipitation, cation exchange and gel filtration chromatographies to obtain a purification factor of 90.7, with an activity yield of 10.2% (Regalado et al. 2000). However, after SDS-PAGE and Coomassie staining, the gel showed three bands, of which only one had activity as determined by using the Congo Red staining, having a molecular weight of 110 kDa.

To study the properties of the carbohydrate binding module (CBM) of the β-mannanase from *T. reesei* (Man5A), a mutant lacking the part encoding the CBM was expressed in *T. reesei* under the regulation of *Aspergillus nidulans gpd*A promoter. The culture filtrates were first concentrated 10 times by ultrafiltration, followed by ion exchange chromatography (Sepharose HP). Man5A was further purified using a Mono-P chromatofocusing column. The purified enzymes appeared as single bands on SDS-PAGE (Hägglund et al. 2003). It was concluded that CBM binds to cellulose but not to mannan. A gene coding for β-mannanase in the hepatopancreas of the blue mussel (*Myrtilus edudis*) was identified, cloned and expressed in *Pichia pastoris*, which produced the recombinant protein at a level of 900 mg/L (Xu et al. 2002a). However, the expression level was strongly affected by the induction temperature. The β-mannanase was expressed as a His-tag fusion protein, and therefore the first purification step was IMAC on Ni^{2+} saturated Chelating Sepharose Fast Flow, followed by elution with imidazole gradient. Further fractionation

was conducted by using ion exchange chromatography (SP Sepharose HP column). The recombinant β-mannanase was purified to homogeneity, and showed very similar properties to those showed by the native enzyme, and belonged to the family 5 of glycoside hydrolases. Xu et al. (2002b) purified two isoforms of β-mannanases from the digestive tract of blue mussels. The purification steps involved a Streamline chelating saturated with Zn^{2+}, considering that the proteins had many histidine residues. After eluting with imidazol, the fractions showing β-mannanase activity were purified by size exclusion chromatography (SEC) (Superdex 75). After this step, the active fraction achieved 22-fold purification. The pooled active fraction was subjected to cation exchange chromatography (Mono S column), where the proteins eluted with the salt gradient at about 200-250 mM NaCl. To remove trace contaminants, SEC was conducted on the denatured proteins (added with 6 M Gu-HCl). The fractions showing activity were re-natured by removing the Gu-HCl and were finally re-run on the cation exchange column (Mono S) at pH 5.5. β-mannanase activity was separated into two peaks, ManA and ManB with purification factors 3.5 and 2.3, respectively. Overall, a 1300-fold purification was achieved for ManA with an activity yield of 20%. For ManB the overall purification factor was 900-fold with 16% activity yield. SDS-PAGE and isoelectric focusing showed a single band for each of the two β-mannanases. They showed the same isoelectric point (7.8), and slightly different molecular weight as evaluated by MALDI-TOF mass spectrometry (39216 versus 39265 Da).

6. CHARACTERIZATION

6.1 Physicochemical and kinetic characterisations

Despite the industrial potential of β-mannanases and the increasing number of research reports and patents, there is not enough information about their physicochemical and kinetic characterization. Tables 2 and 3 show some of these properties. Fungal β-mannanases generally have acidic isoelectric points and molecular masses of 40-110 kDa. Optimum pH of all the enzymes reported until now are in the acidic range from 2.9 to 6.5 pH units. This fact could be related to the natural habitat of the organism studied. Because of the variety of enzyme sources, the optimal temperatures reported for β-mannanases secreted by organisms ranges from 40 °C for *A. niger* (Regalado et al. 2000) to 90 °C for *Thermatoga neapolitana* (McCutchen et al. 1996, Duffaud et al. 1997). Some thermostable β-mannanases from thermophilic bacteria have also been characterised (McCutchen et al. 1996, Duffaud et al. 1997) and sequenced, but no archaeal β-mannanase has been described so far (Sunna et al. 2000).

Industrial application aspects of enzyme catalytic properties are high reaction rate, extension rate and stability. Another criterion to be taken into account to select an enzyme is the specificity. Quantitatively, specificity is measured by V_{max}/K_M or k_{cat}/K_M, called the specificity coefficient. The higher this value, the enzyme shows more specificity for a substrate. To enhance enzyme production and performance, recombinant enzymes production has been studied (Cann et al. 1999, Sunna et al. 2000, Setati et al. 2001). However, heterologous expression is not always successful. Setati et al. (2001) have observed loss of catalytic performance of recombinant enzymes. These authors expressing the *Aspergillus aculeatus* endo-β-1,4-mannanase encoding gene (*man1*) in *Saccharomyces cerevisiae* found that the recombinant β-mannanase activity decreased its specificity coefficient by four times related to the native strain.

6.2 β-mannanase enzyme activity inhibition

Information on β-mannanase enzyme activity inhibition too are scarce. Generally, metal ions have been shown to be relevant in enzyme activity. It has been reported (www.brenda.com) that some metal

Table 2. Physicochemical properties of endo-β-mannanases

Organism	MW (kDa)	T optima (°C)	T stability (°C)	pH optima	pH stability	pI	Reference
Vibrio sp MA-138	49	40		6.5		3.8	Tamaru et al. 1995
C. saccharolyticum		80		6.0			Morris et al. 1995
C. cellulovorans	30.7	85		6.0			Sunna et al. 2000
(*) *E. coli*	116	65		5.8			Cann et al. 1999
(*) *S. cerevisieae*	50		< 50		4.0-6.0		Setati et al. 2001
S. rolfsii	61.2	74	< 50	2.9	3.0-6.0	3.5	Gübitz et al. 2000
S. rolfsii	46.5		50	3.0-3.5	4.5	2.75	Sachslehner and Haltrich, 1999
A. niger	45	60	< 70	3.0	3.0-8.0	3.5	Megazyme
A. oryzae	110	40		6.0		3.5-4.5	Regalado et al. 2000
M. edulis	39	50-55	< 40	5.2	4.0-9.0	7.8	Xu et al. 2002b

(*) Recombinant organism

Table 3. Kinetic properties of endo-β-mannanases

Organism	K_M	V_{max}	Specific activity	Reference
Vibrio sp MA-138	10 mg mL^{-1}	450 U mg^{-1}		Tamaru et al. 1995
(*) *E.coli*	2.4 mg mL^{-1}	384 U mg^{-1}		Ethier et al. 1998
(*) *S. cerevisieae*	0.3 mg mL^{-1}	82μM/min mg		Setati et al. 2001
A. niger	102 μM	37300 U mg^{-1}	1760 U mg^{-1}	Regalado et al. 2000

(*) Recombinant organism

cations, such as Ag^{+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+} inhibited β-mannanase activity. On the other hand, Ca^{2+} stimulated enzyme activity from *Aeromonas hydrophila* (Araki 1983). However, substrate inhibition has only been recently reported for β-mannosidase (Zechel et al. 2003).

6.3 β-mannanase structure

The amino acid sequence of several β-mannanases, deduced from the genes sequences encoding them, place them in families to 5 and 26 of the glycosyl hydrolases (Stoll et al. 1999).

6.3.1 Bacterial β-mannanase structure

The β-mannanase genes encode 362 amino acid residues. The 26 amino acid stretch (from ^{1}Met to ^{26}Ala) in the N-terminus of the predicted amino acid sequence has properties similar to typical signal peptides in *Bacillus* sp, consisting of positively charged amino acids followed by a hydrophobic amino acid stretch. A homology search of the deduced primary structure β-mannanase from *Bacillus* sp 5H

was carried out with sequences in the SWISSPROT and PIR databases by BLASTp program. β-mannanase showed homology with that from *Bacillus* sp belonging to glycosyl hydrolase family 26 (Khanongnuch et al. 1999).

The complete sequence of a β-mannanase gene from an anaerobic extreme thermophile, *Caldocellum saccharolyticum* has been determined and expressed in *E. coli*. It showed that the expressed protein consisted of two catalytic domains showing β-mannanase and endoglucanase activity. The recombinant β-mannanase showed optimal enzyme activity at 85°C, pH 6.0 and was extremely thermostable at 70 °C. It has been shown that the mannanases can significantly increase the brightness of the bleached *Pinus radiate* kraft pulp in peroxide delignification sequences (Sunna et al. 2000).

The crystal structure of *Pseudomonas cellulose* β-mannanase 26A was solved by Hogg et al. (2001). The enzyme comprises $(\alpha/\beta)_8$-barrel architecture with two catalytic glutamates at the ends of β-strands 4 and 7 in precisely the same location as the corresponding glutamates in other 4/7-superfamily glycoside hydrolase enzymes. Trp-360 played a critical role in binding substrate at the -1 subsite, whereas Tyr-285 was important to the function of the nucleotide catalyst. It was also suggested that Trp-217 and Trp-162 are important for the activity of β-mannanase 26A against monnooligosaccharides but are less important for activity against polysaccharides. Structure elucidation of a β-mannanase from the thermophilic actinomycete *Thermonospora fusca* was obtained from the electron-density map to the DNA sequence (Hilge et al. 2001).

6.3.1 Fungal β-mannanase structure

Interestingly, in family 5, fungal β-mannanases from *T. reesei* and *Agaricus bisporus* as well as two bacterial β-mannanases from *Caldocellum saccharolyticum* possess in addition to the catalytic core, a cellulose-binding domain (CBD) linked to the catalytic core by proline-threonine and serine-rich linkers (Ethier et al. 1998). Most *Trichoderma* cellulases and some hemicellulases are two-domain protein composed of a catalytic domain connected via a linker peptide to a small cellulose-binding domain (CBD). The fungal CBDs share high sequence identity whereas the catalytic domains usually belong to different sequence families having different folds. Endoenzymes have active sites situated in clefts on enzyme surfaces allowing the cleavage to happen in the middle of the chain. Endoglucanases, xylanases and β-mannanase belong to this type of enzymes (Harjunpää et al. 1999).

Sabini et al. (2000) determined the crystal structure of the catalytic core domain of *T. reesei* β-mannanase from glycoside hydrolase family 5. The authors compared their result with that of the homologous β-mannanase from the bacterium *Thermonospora fusca*. *T. reesei* β-mannanase consists of two functional domains: a catalytic core joined to a putative substrate-binding domain by a linker unit. This enzyme carries its polysaccharide-binding domain at the C-terminus, in contrast to the fungal cellulases belonging to the same hydrophobic cluster analysis family, which have N-terminal cellulase-binding domains.

Sectioning β-mannanase gene from *C. thermocellum* coupled with functional analysis of the truncated gene products and primary sequence comparisons revealed that the enzyme contained a family 26 CD, a domain that bound mannan weakly, which was conserved in β-mannanases from anaerobic fungi and the ubiquitous dockerin that was characteristics of all *C. thermocellum* cellulosomal subunits (Halstead et al. 1999).

7. CONCLUSIONS

Industrial production of β-mannanases is favoured by microorganisms. This is due to its low cost, high production rate, and controlled conditions. Molecular biology and protein engineering are playing an important role to understand and to improve enzyme catalytic properties. Sequencing and cloning of β-mannanase genes for homologous and heterologous expression in bacterial and fungal strains is common tool to increase enzyme yields. Nowadays, the use of β-mannanases has become a fact mainly in the detergent industry and in animal feed. Furthermore, β-mannanases have a great potential in pulp and paper industry, food processing and in the near future as a diet supplement for human beings with digestive problems.

8. PERSPECTIVES

Addition of mannanase to an enzyme cocktail to remove carbohydrates residues increased the sales by 10% in 1999. Hence, Novozymes predicted that mannanases could one day be as important to Procter & Gamble as amylases, cellulases, and lipases-all basic cleaning enzymes-are today. Performance enhancement of β-mannanase biocatalytic activity could be reached by looking for new enzyme microbial sources such as extremophiles (hyperthermophiles and alkalophiles). Like other extreme enzymes, thermotolerant β-mannanase production can be achieved using recombinant techniques to express gene encoding the hyper-thermal enzyme in a host organism growing in a conventional cultivation system.

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Bioreactor Analysis and Design

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1. INTRODUCTION

The bioreactor is the centre - the "heart" - of all biological processes. It is the thing that makes or breaks the process economically (Levenspiel 1999), even though in any process the bioreactor should not be regarded as an isolated unit but as part of an integrated process with both upstream and downstream processing units. Its main function is to provide a perfectly controlled environment so that one can obtain the optimal growth or the optimal formation or transformation of a product. This includes:

- sterile conditions;
- control of the shear stresses created by agitation. High constraints can be lethal to micro-organisms, but low constraints can lead to the formation of pellets or development on the walls of the reactor, in particular in the case of cultures of filamentous micro-organisms;
- sufficient aeration, if the micro-organisms or the biotransformation require it;
- dissipation of the heat released by the culture.

Residence times in bioreactors are generally significant and range from a few hours to a few days, even a few weeks. The residence time in the industrial process of biotransformation of acrylonitrile to acrylamide, an important bulk chemical, by immobilised cells of *Rhizopus rhodocrous* was about five hours. The cell production process lasted 72 hours. Similarly immobilised cells of *E. coli* transformed fumaric acid to aspartic acid, an intermediate of aspartame, in 0.07 to 0.75 hour, depending on the process (Liese et al. 2000). The time for one batch in the production process of heptanone-2 from octanoic acid, a flavour component, produced by the spores of *P. roquefortii* has been about 300 hours (Larroche & Gros 1997).

Most biological reactors are multiphase systems. Biological reactions are always carried out in one solvent, water, and substrates and products concentrations are relatively low. This is also true for the enzymatic reactions carried out in two-phase (aqueous/organic) reactors, even if the presence of water can be almost negligible. The biocatalyst may be present as a solid phase, for example as immobilised enzymes or cells or as an individual cell, the substrate may also be solid. Typically when necessary, gas is sparged into reactors to supply oxygen or a gaseous substrate and remove carbon dioxide. Thus the number of phases involved can be from one (homogeneous reactors) to four (heterogeneous systems).

Bioreactors can be operated in discontinuous mode, with frequently a sequential or continuous feed of substrate (fed-batch operation) or in continuous mode. The hydrodynamic behaviour of each phase needs to be considered. In the idealised case, batch reactors have perfectly-mixed phases. Ideal continuous reactors are of two types: perfectly mixed and plug flow or tubular reactors. The choice of the operating mode depends mainly on the reaction characteristics. Table 1 summarises the evaluation of reactor operation by the chemical reaction engineering approach based on kinetic characteristics (Levenspiel 1999). The term perfectly-mixed very often applies to the liquid phase only. In an aerobic culture or biotransformation, it can be advantageous to contact the perfectly-mixed liquid phase with a gas phase that goes through the reactor in plug flow since it will give the highest rate of mass transfer of a gaseous substrate to the liquid; this contributes to an optimal utilisation of the gas phase.

Table 1. Best (**) reactor types and operation on the basis of reaction kinetic characteristics

Reactor Operation / Reaction characteristics	Batch	Fed-batch	Continuous	
	Stirred-tank	Stirred-tank	Stirred-tank	Tubular plug flow
Michaelis or Monod constant high	**	**		**
High conversion				**
Substrate inhibition		**	**	
Product inhibition	**			**

Culture and biotransformation processes can be divided generally into submerged cultures or biotransformations, and solid-state fermentations. The major difference between these two processes is the amount of free liquid in the reactor. In submerged cultures, water represents up to 95-98 % of the reacting volume, when absence or near absence of visible liquid water characterises solid-state fermentations. Although solid-state fermentation was documented as long as 3000 years BP in China, its use is restricted to enzyme production in cases where enzymes obtained have improved thermostability compared to enzymes produced in submerged cultures.

The main reactor types used in submerged cultures and biotransformations are the mechanically stirred-tank reactors, aerated or not, and the fixed or fluidised beds reactors (Fig 1). At a first approximate, stirred-tanks and fluidised beds behave as perfectly mixed when fixed bed reactors are plug flow reactors. Notice that many fixed-bed processes are operated with an external recycle of fluids; when the recycle ratio is high, the fixed-bed is nothing more than a continuous stirred-tank reactor.

In solid-state fermentation processes, the principal reactor types are the tray reactors, fixed-bed, and drum reactors (Fig 2).

The majority of industrial products are obtained in stirred-tank reactors operated in batch, fed-batch, or continuous mode and in fixed-bed reactors (Table 2).

Stirred-tanks are used also for the production of microorganisms or enzymes. To the biologists, the stirred tank reactor is what the "Grignard" is to the chemists: a general-purpose reactor with low

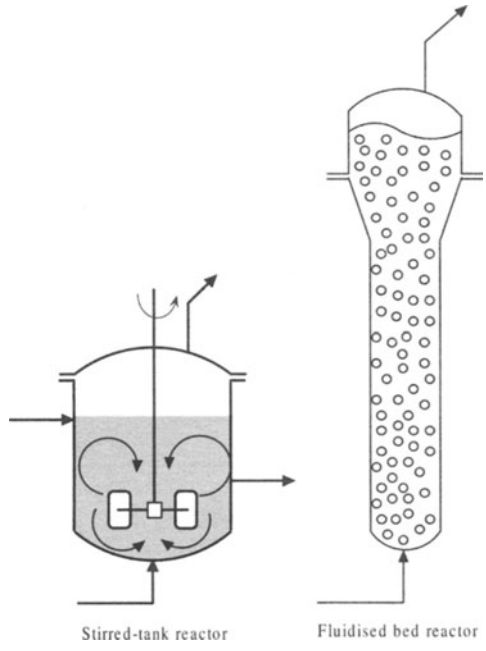

Figure 1. Various types of liquid phase reactors

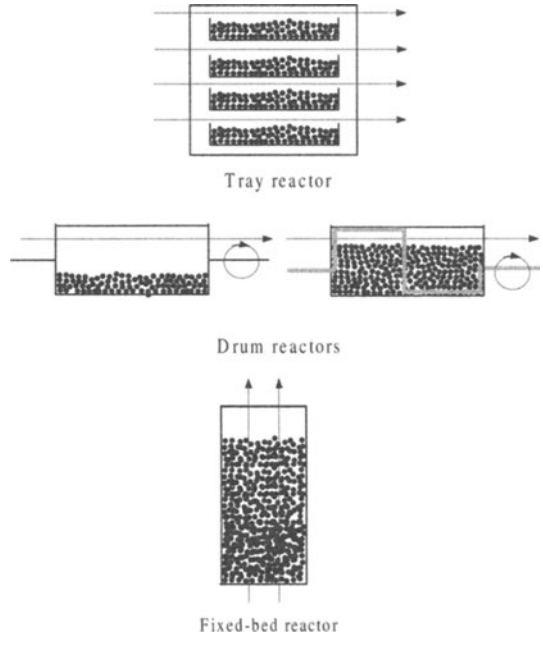

Figure 2. Main reactor types in solid-state fermentation or bioconversion processes

Table 2. Type of reactors and operation mode in some industrial biotransformations by enzymes

Enzyme	Substrate and product	Biocatalyst	Type of reactor and operation	Company
Nitrile hydratase	Acrylonitrile to acrylamide	Immobilised cells of *R. rhodocrous* J1	Stirred-tank reactor Fed-batch	Nitto Chemical Industry Co
Aspartate β-decarboxylase	Aspartic acid to L-alinine	Immobilised cells of *P. dacunhae*	Fixed-bed reactor Continuous	Tanabe Seyaku Co
Tyrosine phenol lyase	L-DOPA	Cells of *E. herbicola*	Stirred-tank reactor Batch	Ajinomoto Co
Alcohol and aldehyde dehydrogenase	Ethanol to vinegar	Cells of *Acetobacter* sp.	Acetator (aerated stirred-tank reactor) Repeated batch or continuous	Frings Ltd
Aspartase	Fumaric acid to aspartic acid	Immobilised cells of *E. coli*	Fixed-bed reactor Continuous	Tanabe Seyaku Co
Aspartase	Fumaric acid to aspartic acid	Cells of *B. flavum*	Stirred-tank reactor Batch	Mitsubishi Petrochemical Co
Aspartase	Fumaric acid to aspartic acid	Immobilised enzymes from *E. coli*	Fixed-bed reactor Continuous	BioCatalytics
Xylose isomerase	High fructose corn syrup	Immobilised whole cells or isolated enzymes of *B. coagulans*	Fixed-bed reactor Continuous	Gist-Brocades, Novo Nordisk, etc.
Penicillin amidase	Penicillin G to 6-APA	Immobilised enzymes of *E. coli*	Stirred-tank reactor Batch	Unifar
Alcohol dehydrogenase	Anticholesterol drug intermediate	Solubilised enzymes of *A. calcoaceticus*	Stirred-tank reactor Batch	Bristol-Meyer-Squibb
Multiple	Octanoic acid to heptanone-2	Immobilised spores of *P. roquefortii*	Stirred-tank reactor Biphasic water/solvent	Degussa

operation and capital costs. It is the "workhorse" in the fermentation and enzyme industry. Thereafter, we will consider mainly stirred-tank reactors, which remain the reference reactors.

The sizes vary from 1 L approximately up to 500 m^3. At the laboratory scale, the tanks are made of glass, up to 20 L, or, for larger volumes, of stainless steel. The height/diameter ratio can vary between 1:1 (laboratory scale) and 6:1 (industrial tanks). Baffles avoid the formation of a vortex and can be used to maintain the tanks at constant temperature if heat removal is necessary. The reactors, in traditional configuration, are equipped with six blade turbines, and they are characterised by:

- efficient mixing, a rather low mixing time;
- effective agitation, so that the concentrations of substrates, products, micro-organisms or enzymes are uniform, just as the temperature;
- strong shear stresses;
- important aeration capacity.

Even though the principal selection criterion of a reactor is, in addition to costs, the kinetic characteristics of the culture or of the biotransformation, operating conditions influence the rate and the yield, in particular (Riba 1998):

- power consumption;
- mixing time and suspension of cells or immobilising material;
- shear stress;
- mass transfer between phases;
- mass transfer in phases.

Design of a reactor is not a routine matter and it is not possible to give precise design advice for designing and operating bioreactors. We will consider in this text the interactions between cells or enzymes, and the above parameters defining their macroenvironment. We believe that a proper analysis and understanding of the topics presented are of great help for properly designing new processes or improving existing ones.

2. OPERATING PARAMETERS

2.1. Power consumption

Power consumption represents an important operation cost. It influences drastically performance in aerobic conditions or for a high viscosity medium. The ideal agitation device is that for which the power consumption is minimal for a given mixing time and a given aeration capacity. Power consumption P_d, in the absence of aeration, for a fluid of density ρ and of viscosity μ, an agitation mobile characteristic length d_a (in general the impeller diameter) and a stirring rate N is correlated by

$$P_d = N_P \ \rho N^3 d_a^5 \tag{1}$$

N_p is the power number. If the stirrer rate is sufficient so that flow is turbulent ($Re = \rho N d_a^2/\mu$ greater than 10^4) the power number is constant. It depends only on the agitator type, and of the shape of the tank and the baffles. In the case of fluids of high consistency, the mode of agitation can become laminar; there is no

risk of formation of vortex and baffles are not used. In this case

$$N_p\, Re = C^{ste} \tag{2}$$

In industrial tanks, but also laboratory-scale tanks with multiple impellers, the situation is more complex (Nielsen et al. 2003), but one can obtain a good approximation of the power dissipated by multiplying the consumption by an agitator with the number of impellers.

When a gas is injected, as in aerobic conditions, the bubbles are attracted in the zones of the apparatus at low pressure and gas accumulates by forming cavities behind the blades of the impeller. The power consumption is decreased, all other things being equal, compared to the power dissipated without gas injection.

One then defines the ratio P_{dg}/P_d which is a function of the aeration number $N_a = Q_g/Nd_a^3$, where Q_g is the gas volume flow rate. It is of course of interest that P_{dg}/P_d does not decrease too much when the number of aeration increases, which is not the case with the Rushton-type turbines, but what can be obtained with more modern impellers (Nielsen et al. 2003).

2.2. Mixing time

Mixing time is the time necessary to make the liquid phase homogeneous in concentration following a disturbance, for example the introduction of a reagent. This time depends on the choice of a criterion of homogeneity, in general 95% or 99%. It is a very important parameter because it makes it possible to know whether the reactor, whatever it is, can be regarded as homogeneous in every point, one says whereas it is "perfectly mixed". One admits that a reactor can be regarded as perfectly mixed when the mixing time is approximately 10 times lower than the time characteristic (the time-constant) of the biological reaction (Roels 1983). Thus for a growing microorganism, the time-constant is $\tau = 1/\mu_{max}$, μ_{max} being the maximum specific growth rate. By way of example, for *E. coli* under optimum conditions for growth, if it is admitted that the generation time is 20 minutes, μ_{max} is worth 2.08 h^{-1} and τ is approximately 30 minutes. A mixing time of a few minutes is thus tolerable. It is always the case at laboratory scale for an agitated tank and one can consider that the liquid phase is perfectly mixed. It is generally not true at industrial scale.

The situation can be more complex if the activity of a microorganism is not limited to its growth and if its "metabolic flexibility" is important. The time-constant related to the modifications of the metabolism can then be much smaller. This partly explains why some metabolite by-products are found in the liquid medium, the presence of which seems incompatible with the conditions of culture. Recent techniques make it possible to reach these time-constants. Thus in a 22 m^3 reactor, Enfors et al. (2001) noted that the growth of *E. coli* was carried out with simultaneous accumulation of formate, although the aeration capacity was sufficient; this was due to the existence of zones in which there was oxygen limitation. The enzymatic systems implied in the fermentation of *E. coli*, although inhibited and in certain cases repressed by oxygen, are present at a sufficient concentration in the cell in aerobic conditions to produce measurable quantities of acids (formate, acetate, lactate), during the few seconds when the cells are in anaerobic conditions. Moreover as soon as the oxygen concentration is sufficient in the reactor, the produced lactate and acetate are re-assimilated and only formate accumulates.

For stirred-tanks, an estimate of the mixing time t_m is proposed by Nienow (1997), as a function of the power dissipated per unit of fluid mass P_{dm}:

$$t_m = 5.9 \left(\frac{d_T^2}{P_{dm}} \right)^{1/3} \left(\frac{d_T}{d_a} \right)^{1/3} \tag{3}$$

This corresponds for a standard vessel for which the height to diameter d_T ratio is one , and for which $d_T/d_a = 3$, to

$$N\, t_m = 28 \tag{4}$$

Scale-up of a tank is based on geometrical similarity, at least for volumes up to 1 m^3. The type of impeller and its position in the tank are unchanged. If an equivalent mixing action is desired, it is necessary, for a not very viscous Newtonian medium, to maintain the power dissipated in the medium per unit of mass. One must then satisfy

$$N_1^3 \; d_{1a}^2 = N_2^3 \; d_{2a}^2 \tag{5}$$

where subscripts 1 and 2 refer to plant scale and laboratory scale. The mixing time strongly increases with the increase in size. Let us recall that these relations give only orders of magnitude, but they show that t_m is a very important parameter to consider at industrial scale.

2.3. Shear stress

In stirred-tanks, there are great variations of the shear rates according to whether one is near or far away from the impeller. A rough estimate of the mean velocity gradient or shear rate is:

$$\dot{\gamma}_m = a \; N \tag{6}$$

a is an empirical constant, N the stirring rate. a varies between 4 and 13 depending on the type of impeller and the value most classically adopted for the Rushton turbines is a = 11. The maximum shear rate is also approximately proportional to the impeller tip speed. As the stirring rate is typically lower in a large scale reactor than at small scale, this means that the maximum shear rate is lower at large scale. The tip speed, however, is normally higher and the maximum shear rate is higher.

3. EQUILIBRIUM BETWEEN PHASES

3.1. The phase-equilibrium concept (Vidal 1997, Prausnitz et al. 1999)

The concept of equilibrium between phases is fundamental for the comprehension and the quantification of the distribution of components i of a mixture between several phases. Several phases in contact are in equilibrium when any infinitesimal transformation being able to imagine is reversible. The necessary and sufficient condition so that p phases are in equilibrium is that:

- the temperature and the pressure are the same for all phases I, II, ..., p;
- each component i has the same chemical potential μ_i in all the phases.

This leads to the equation:

$$\mu_i^I = \mu_i^{II} = ... = \mu_i^p \tag{7}$$

For a pure substance, the chemical potential is the molar free enthalpy G. In the case of mixtures one can write:

$$G = \sum n_i \mu_i \tag{8}$$

Taking into account the Gibbs-Duhem equation $(\sum_i \mu_i \ dn_i = 0)$, eq. [8] can be rewritten:

$$\mu_i = \left(\frac{\partial G}{\partial n_i} \right)_{T,P,n_{j(j \neq i)}} \tag{9}$$

One can thus define the thermodynamic equilibrium starting either from the chemical potentials or from the free enthalpy function.

The problem is, however, not solved. It is necessary to know how μ_i is connected to T, P and to the concentrations within the phase. To establish these relations, it is convenient to introduce auxiliary relations such as fugacity or activity. These functions are closer to our senses than the abstract concept of chemical potential; thus if I is a gas and II a liquid, equation [7] can be written as follows:

$$\varphi_i y_i P = \gamma_i x_i f_i^\circ \tag{10}$$

y_i is the molar fraction of i and φ_i the fugacity coefficient in the gas phase, P the total pressure; in the liquid phase γ_i indicates the activity coefficient, x_i the molar fraction of i and f_i° the fugacity of i in a reference state called standard state. $\gamma_i x_i$ is the activity of i in the liquid. There is no question of clarifying eq. [10] here; it has however the decisive advantage to reveal directly the measurable quantities y_i, x_i and P. All its utility comes from the concept of ideality. If one defines ideal mixtures as being those for which $\varphi_i = 1$ (ideal gas) and $\gamma_i = 1$ when f_i° is equal to the vapour pressure of pure compound i in liquid form P_i° and at low pressure, one obtains a very simple relation:

$$y_i = x_i \frac{P_i^\circ}{P} \tag{11}$$

For mixtures not obeying this law, known as nonideal mixtures, one must establish the relationships

$$\varphi_i = f(T, P, y_1, y_2 \ldots) \tag{12}$$

$$\gamma_i = f(T, P, x_1, x_2 \ldots.) \tag{13}$$

In the case of cultures of micro-organisms and for the majority of the biological processes, one can estimate that the vapour phase has a perfect gas behaviour. It thus comes

$$y_i P = P_i = \gamma_i (T, P, x_i) \ x_i \ P_i^\circ(T) \tag{14}$$

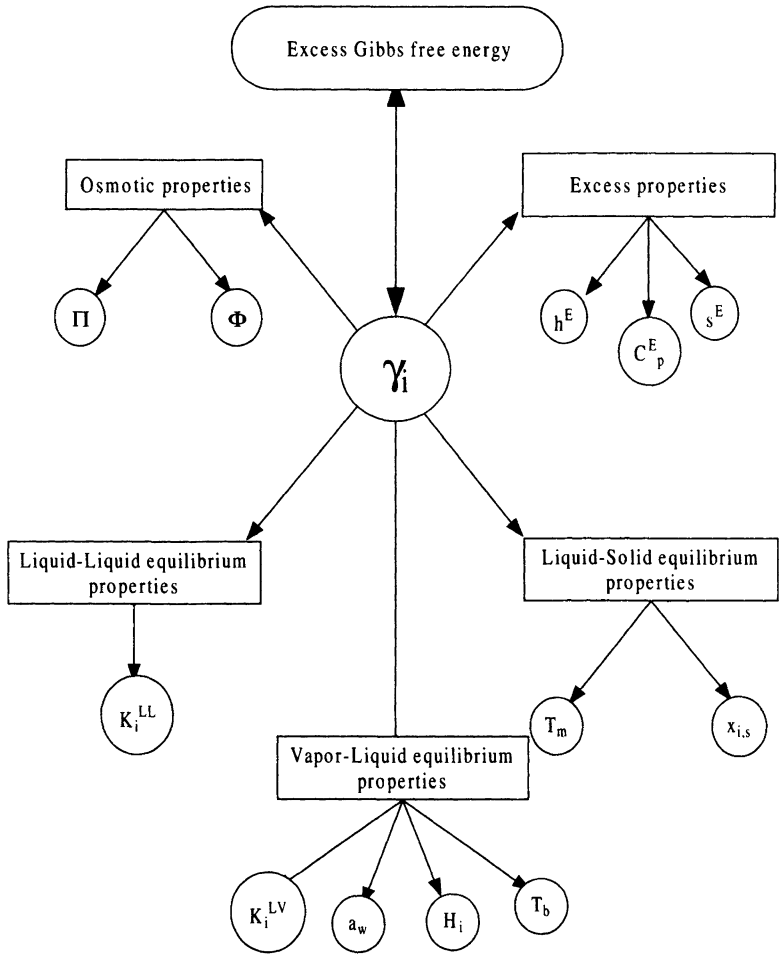

Figure 3. Equilibrium properties related to the activity coefficient in liquid phase

This equation shows the central role of the activity coefficient for the determination of liquid-vapour equilibrium. In Figure 3 are summarised the main equilibrium properties related to the activity coefficient of species i in solution: excess properties, liquid-vapour equilibrium (LVE), liquid-liquid equilibrium (LLE) or liquid-solid properties (LSE), and osmotic properties. h^E, C_p^E and s^E are excess enthalpies, excess specific heats and excess entropies. T_m and x_{is} are the melting point temperature (change from the liquid state to a solid state) and the solubility of i in the mixture. K_i^{LV}, a_w, H_i, T_b, are respectively the partition coefficient of i between the gas phase and the liquid phase, the activity of water, Henry's constant giving solubility of i, and the boiling point temperature of the mixture. K_i^{LL} indicates the partition coefficient of i between the two liquid phases. Π and Φ are the osmotic pressure and the osmotic coefficient.

3.2. Models of activity coefficients

There are many activity coefficient models for nonelectrolyte liquid mixtures. Among those, let us quote the models of Margules, Van Laar, Scatchard and Hildebrand, Wilson, NRTL (Non Random Two Liquids) and UNIQUAC (UNIversal QUAsi Chemical) models. Several comparative reviews of these various models have been published (Sandler 1994, Chen & Mathias 2002).

The UNIQUAC model (Abrams & Prausnitz 1975) was developed with the aim of having a general equation making it possible to take into account the differences of size and the shape of the molecules in solution (entropic or combinatorial contribution) and the energy interactions of physical nature (enthalpic or residual contribution), i.e. of short range, between the molecules. The UNIQUAC model involves two kinds of parameters: the first are representative of molecular size r_i and of external surface area q_i of each component and they have been obtained starting from the volume and surface area of molecules suggested by Bondi. The seconds (a_{ji} and a_{ij}) are binary interaction parameters (for the molecules taken 2 by 2) and they must be evaluated from binary experimental data.

This type of model has limited applications. It applies to polar and nonpolar compounds but not to electrolyte solutions. It is not completely predictive because the binary parameters must be obtained from experimental data. There are thus databases, which provide the interaction parameters a_{ji} for a great number of binary systems. The most known is the Dortmund Data Bank (www.ddbst.de). But its merit is to allow a prediction of the equilibrium properties of more complex systems, i.e. multicomponent systems.

However, the number of compounds in a mixture in liquid phase is so high that one cannot hope to get all the parameter values needed in this type of model. The idea has been then to develop group contribution methods; it is related to the fact that if it exists in nature several thousands of chemical or biological molecules, the number of groups, which constitute them is very much smaller. A mixture is no longer considered as a set of molecules, but as a set of functional groups obtained by decomposition of the molecules. They are the interactions between groups, which are considered for the representation of the non-ideality of the liquid solutions, giving the concept of "group-solution".

UNIFAC (UNIQUAC Functional Group Activity Coefficient) is the group-contribution method derived from UNIQUAC (Fredenslund et al. 1975). As in UNIQUAC, the activity coefficient is treated as the product of a combinatorial part and a residual part:

$$\gamma_i = \gamma_i^C \gamma_i^R \tag{15}$$

or

$$\ln\gamma_i = \ln_i^C + \ln\gamma_i^R \tag{16}$$

The combinatorial part takes into account the differences of size and form of the components of the mixture. The residual part accounts for the energy interactions between groups; each group is characterised by its molar fraction in the mixture and its activity coefficient.

The authors of UNIFAC ensure a regular update of the tables of parameters R_k (size), Q_k (external surface area) of the k groups and tables of interaction parameters (a_{mn} and a_{nm}) between groups. Many alternatives of the original model have been published, of which the most used are those of Larsen et al. (1987) and of Gmehling and co-workers (Gmehling & Schiller 1993, Yan et al. 1999, Gmehling 2003). However, this type of model cannot cope with the representation of the properties

of all aqueous solutions. There exist indeed many compounds which have the characteristic to dissociate completely or partially to give charged species. The presence of electric charges reveals new interactions, known as electrostatic, which appear even if the distance between the molecules is significant; they are thus called long range interactions. Many models being based on the theory of Debye and Hückel, stated in 1923, were conceived to take account of it. The work of Pitzer (1973, 1980, 1991) makes authority at the present time. In addition, in aqueous solutions, there are many chemical effects such as chemical association between ions and water and they cannot be neglected. The local structure of an electrolyte solution is consequently very different from that which was envisaged at the beginning by the theory of local compositions. Ions are surrounded by water molecules to form aggregates or "clusters". This phenomenon is called solvation.

To correlate the activities of ions in solution, Achard et al. (1994) have used the model of Pitzer to take account of the long range interactions and an UNIFAC with solvation model was added to take account of the short range interactions between solvated ions and molecules. This model, known as ULPDHS (UNIFAC Larsen Pitzer Debye Hückel with solvation), utilises two parameters per ion, the hydration number and the energy of interaction between the functional ion and water or groups. Each ion sets up a group. ULPDHS makes it possible to represent in a very satisfactory way the nonideality of ionic solutions up to 6 to 8 moles.kg^{-1}. It should be noticed that the most recent data on ions have been published by Marcus (1997).

The activity coefficient is then the sum of three contributions:

$$\ln\gamma_i = \ln\gamma_i^C + \ln\gamma_i^R + \ln\gamma_i^{PDH} \tag{17}$$

It is important to realise that the values of the mole fractions and thus γ_i^C et γ_i^R will be modified by the presence of the solvated ions.

For relatively diluted solutions (<0.5 mole.kg^{-1}), the long range interactions are largely dominating. They are sufficient to characterise the nonideality of the solutions. One can also use the model suggested by the National Office of Standards (Goldberg 1981) which is in fact a simplified alternative of the Pitzer model.

3.3. Liquid-Vapour Equilibrium

One often defines LVE starting from liquid-vapour equilibrium coefficient K_i^{LV} corresponding to the ratio of the vapour mole fraction y_i to the liquid fraction x_i of component i, i.e. according to [14]:

$$K_i^{LV} = \frac{y_i}{x_i} = \frac{\gamma_i P_i^0}{P} \tag{18}$$

The larger K_i^{LV} the more volatile is the compound. For a solid component in native state such as glucose, the equilibrium constant tends towards zero because the vapour pressure is often negligible. For a component such as oxygen (supercritical compound) it becomes very large. The calculation of K_i^{LV}can be carried out at fixed T and P, provided P_i^0 is known for each pure substance and that one has an activity coefficient model to estimate γ_i. The calculation of the compositions (molar fractions) in the two phases is carried out by solving the MES equations (Material balance, Equilibrium relationships and Sum of the molar fractions in the two phases).

In the manufacture of vinegar, one can thus calculate the activity coefficients of the volatile organic compounds (VOC) provided the composition of the cultivation medium is known. Table 3 gives the values computed by the ULPDHS model at infinite dilution in water and in a "model vinegar" made of acetic acid (120 $g.L^{-1}$), ethanol (16 $g.L^{-1}$) glucose (1 $g.L^{-1}$) and Frings salts (0.7 $g.L^{-1}$).

Table 3. Activity coefficient values at infinite dilution in water and in a model vinegar. Ethyl acetate concentration is between 0.05 and 0.3 $g.L^{-1}$ and acetaldehyde between 0 and 0.2 $g.L^{-1}$

Component	γ_i^{∞} (in water)	γ_i (model vinegar)	K_i^{LV} (model vinegar)
Ethanol	5.4	4.1	0.38
Acetic acid	3.8	3.0	0.075
Ethyl acetate	71	71	10.3
Acetaldehyde	2.7	2.7	3.28
Water	1		

It should be stressed that the activity coefficients of the VOCs vary with the composition of the medium and rather strongly differ from the values at infinite dilution. In this process the mass transfer coefficients are high (> 500 hr^{-1} for oxygen) and one can admit that the phases are in equilibrium. One can thus directly use the preceding results, knowing the aeration flow rate, and calculate the exhaust of VOCs for various concentrations in the culture medium, via the partition coefficients. Results show that these losses are primarily due to ethanol stripping and an effective way of decreasing them is the use of a fed-batch technique for ethanol supply, allowing ethanol concentration to remain at low value in the culture medium (Pochat-Bohatier 1999).

3.3.1. Water activity

The activity of water, denoted a_w, is also a LVE property, defined by the ratio

$$a_w = \frac{P_w}{P_w^0} \tag{19}$$

i.e., while referring to equation [14]:

$$P_w = y_w P = \gamma_w x_w P_w^0 \tag{20}$$

P_w is the vapour pressure in the gas phase at equilibrium with the liquid, P_w° is the vapor pressure of water. Calculation of the activity coefficient thus gives a direct access to water activity.

The vapour pressure of pure substances can be estimated through many methods if they are not available in databases (Reinhard & Drefahl 1999). Let us highlight the software developed by ACD (Advanced Chemistry Development Inc, Toronto), which gives, even with complex molecules (terpenes, terpenoïds, etc.) results within ± 10%.

3.3.2. Solubility of gases

The compounds called commonly gases are in a supercritical state under the conditions of temperature and pressure of the system. At thermodynamic equilibrium, there is a given amount of gas dissolved in the liquid phase. In the conditions of temperature and pressure classically met in biological processes, the solubility of gases is low and one is near to the infinite dilution conditions. Henry's law makes it possible to calculate the solubility of a compound i in a solvent:

$$P_i = H_i x_i \tag{21}$$

H_i is the Henry's coefficient; for any given solute and solvent, it depends only on temperature and to a lesser degree on total pressure. A comparison with eq. [14] shows that

$$H_i = \gamma_i^{\infty} f_i^{oL} = \gamma_i^{\infty} P_i^{o}(T) \tag{22}$$

When calculating the solubility of gases, one can have two types of situation: evaluation of solubility in pure water (or a pure solvent) at a given temperature, or estimate of the changes of solubility in a complex solution. In the last case, the resulting value is less than that in pure water, and solubility decrease is called "salting out". For the first situation, the most precise method is to use selected empirical correlations (Wilhelm et al. 1977, Fogg & Gerrard 1991).

In the second case, one must be able to account for systems known as multi-solvent systems (for example water-ethanol, water-methanol) and for the presence of salts.

The first attempt of description of the "salting out" effect was by Setchenov who wrote in 1889 for a solute i in aqueous solution:

$$\log_{10} \frac{H_i}{H_{iw}} = K_i C_i \tag{23}$$

H_i is the Henry's coefficient in the mixture, H_{iw} that in pure water, C_i the concentration of i in the solution. This work was continued for biological mixtures by Schumpe (1993) and its team (Quicker et al. 1981, Weissenberger & Schumpe 1996, Rischbieter et al. 1996) who introduced Setchenov pseudo-constants for each species in solution and used these constants in an additive law:

$$\ln \frac{H_i}{H_{iw}} = \sum_i K_i C_i \tag{24}$$

Catté et al. (1993) developed a group-contribution method, which allows to obtain predictions of gas solubility in pure solvents and mixtures of solvents at low temperature and pressure. Seven gases (O_2, N_2, H_2, CO, CO_2, CH_4, C_2H_4) were studied on a large variety of solvents (alkanes, alcohols, ketones, esters, ethers, aromatic and cyclic compounds, water, etc.). This model was extended, in the particular case of O_2 and CO_2, to mixtures containing ions in solution (Gros et al. 1999). The structure of the model is as follows:

$$\ln \frac{H_i}{H_{iw}} = \sum_j \phi_j \ln \frac{h_{ij}}{H_{iw}} \tag{25}$$

ϕ_i indicates the volume fraction of group i and h_i is the Henry's pseudo-constant for the solubility of the gas considered in a solution containing only groups of the i type. The summation of this equation extends to all the species in solution, water, organic solvents and dissolved substances (cations, anions, sugars, etc.). The dissolved species are considered solvated as in the ULPDHS model.

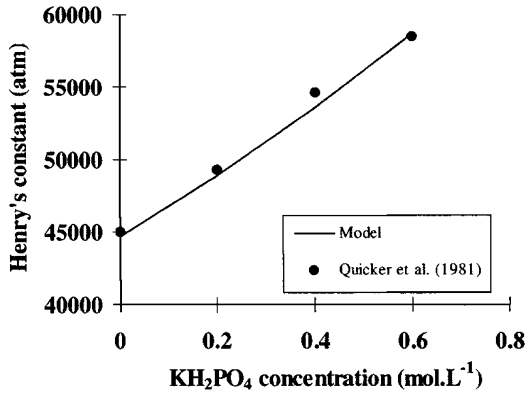

Figure 4. Henry's coefficient values as a function of KH_2PO_4 in a water, lactose 0.1M and KH_2PO_4 mixture

Reliable experimental data on oxygen or carbon dioxide solubility in complex aqueous media are not plentiful. Figure 4 shows changes in the oxygen solubility in water-lactose 0.1M-KH_2PO_4 mixtures at 25°C against salt concentration. The Henry's coefficient is strongly modified by the presence of salts,

Table 4. Oxygen and carbon dioxide solubility in two biotransformation media at 25°C. Values in water are given for comparison

Culture medium	Composition ($g.L^{-1}$)		H_{O2}(atm)	H_{CO2} (atm)
Basic medium for *C. glutamicum* growth	Glucose	10.0	47820	1747
	$(NH_4)_2 SO_4$	7.0		
	$Na_2 HPO_4$	6.0		
	$KH_2 PO_4$	3.0		
	NaCl	0.5		
	NH_4Cl	1.0		
Growth medium for *A. niger*	Sucrose	30.0	46695	1682*
	Yeast extract*	1.0		
	$NaNO_3$	2.0		
	KCl	0.5		
	K_2HPO_4	1.0		
	$MgSO_4 7H_2O$	0.5		
	$CaCl_2$	5.5		
Water			43690	1635

* Yeast extract is not used for calculation

and the values of the Henry's coefficients estimated by the method of Catté (Gros et al. 1999) are satisfactory.

Table 4 reports examples of solubility of oxygen and carbon dioxide in two culture media. *Corynebacterium glutamicum* is a bacterium industrially used for the biotransformation of fumaric acid to malic acid, used in food, cosmetics and pharmaceutical industries. The medium suggested here is a basic medium for growth, which must be supplemented with vitamins and salts sterilised separately.

The medium for *Aspergillus niger,* a prolific producer of many types of enzymes (over 40 different commercial enzymes) is also a growth medium. The results show that in certain cases, and this will be more true as the media will be richer in metabolites, to suppose that the solubility of oxygen or carbon dioxide is the same one as in water, can lead to errors up to 20%.

3.4. Liquid-liquid equilibrium

It can exist several liquid phases in a system when the solvents are not completely miscible. LLE properties are very useful in solvent extraction and in biotransformations or enzymatic syntheses in two-solvent systems. One speaks about LLE in two cases:

- if the two solvents are not completely miscible; it is said that there is partial miscibility of two solvents;
- if there is distribution of a compound in the two non-miscible solvents.

At equilibrium, equality of chemical potentials in the two phases for a component i leads to:

$$\gamma_i^{L1} X_i^{L1} = \gamma_i^{L2} X_i^{L2} \tag{26}$$

As for vapour-liquid systems, one can define a liquid-liquid equilibrium coefficient K_i^{LL} :

$$K_i^{LL} = \frac{x_i^{L2}}{x_i^{L1}} = \frac{\gamma_i^{L1}}{\gamma_i^{L2}} \tag{27}$$

Knowledge of K_i^{LL} and solving for the system made of the MES equations (component Material balances, Equilibrium relationships, and Sum equations i.e. mole-fraction constraint) make it possible to calculate the composition of each phase.

3.4.1. Solubility of hydrophobic organic compounds

Let us imagine a biphasic system in which a liquid organic compound is in equilibrium with an aqueous phase. The aqueous phase is then saturated with this compound. The equilibrium conditions for this compound is:

$$\gamma_{is}^{w} x_{is} = 1 \tag{28}$$

$$x_{is} = \frac{1}{\gamma_{is}^{w}} \tag{29}$$

x_{is} is the molar fraction of i and γ_{is}^{w} its activity coefficient in water at saturation. If i is a sparingly water-soluble compound:

$$x'_{is} = \frac{1}{\gamma_{is}^{w}} \cong \frac{1}{\gamma_{i\infty}^{w}} \tag{30}$$

The solubility limit is then rather low so that one can introduce molar solubility C_{is} (in mole.L^{-1}) as:

$$C_{is} = \frac{55.6}{\gamma_{i\infty}^{w}} \tag{31}$$

The accuracy on the estimate of the solubility of i relies then on the estimate of γ_i in water at infinite dilution. Group-contribution methods fail as soon as molecules become too complex and have a structure too different from those included in the UNIFAC data base.

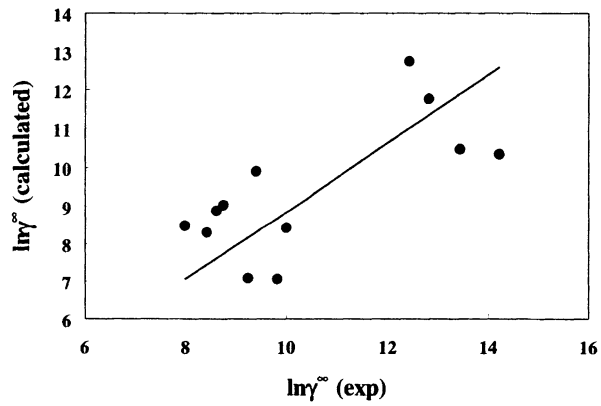

Figure 5. Activity coefficients values compared to values computed using the UNIFAC-Larsen (UL) model. The slope of the straight line is 0.88 ± 0.08 (95% confidence interval). Components are limonene, α-pinene, β-pinene, myrcene, borneol, α-pinene oxide, limonene oxide, carveol, linalool and α-terpineol

Monoterpenes such as α-pinene, β-pinene, limonene and β-myrcene are used as precursors in biotransformations for the production of terpenoïds such as borneol, carvone and α-terpineol. Development of these processes requires to know the solubility of these poorly water-soluble compounds (or in the same way, their activity coefficient at infinite dilution together with the vapour pressure of pure substances) to estimate the losses in substrates and products during biotransformations. Figure 5 shows that activity coefficients of terpenes and terpenoïds in water at infinite dilution and at 25°C are badly predicted by the UNIFAC-Larsen model (UL). The average deviation is about 220%. It is thus necessary to be very careful when using this type of model.

3.4.2. Water-octanol partition coefficient K_i^{ow}

The partition coefficient K_i^{ow} corresponds to the distribution of a compound i present in two phases, an aqueous phase saturated with 1-octanol and a phase with 1-octanol saturated with water, at equilibrium.

$$K_i^{ow} = \frac{C_i^o}{C_i^w} \tag{32}$$

It is relatively easy to perform experiments and there are several data bases giving K^{ow} or $\log_{10} K^{ow}$, such as Starlist (Leo 1993) or LOGKOW (Sangster 1993). It is very often used in environmental studies to estimate the distribution of polluting organic compounds between soil and water, water and dissolved organic matter, to predict toxicity parameters in aqueous phase, or the biodeterioration of components. K^{ow} is also noted P or $P_{ow,}$ and $\log_{10} K^{ow}$ is used as a relative measurement of the hydrophobicity of components.

The $\log_{10} K^{ow}$ or $\log_{10} P$ concept has been used to predict solvent toxicity (Tramper 1996). Even though $\log_{10} K^{ow}$ is a rather crude parameter, there is presently no better or easier alternative. Solvents with a $\log_{10} K^{ow}$ lower than 2 can be expected to be toxic to all viable cells and most enzymes, whereas those ranging between 2 and 4 are unpredictable; solvents with $\log_{10} K^{ow}$ above 4, i.e. the most hydrophobic ones, are generally not toxic.

When the experimental value of K^{ow} is not available, one can use estimation methods. Reinhard & Drefahl (1999) index 22 methods, including 10 group-contribution methods. An example is the approach of Lin & Sandler (1999) who developed a prediction model called $GCSK_{ow}$. K_{ow} is calculated starting from three parameters, molecular volume, surface area and charging free energy. The molecules are broken up into functional groups according to UNIFAC. However the most reliable estimates are up to now obtained with KowWin (http://esc.syrres.com/interkow/kowdemo.htm) or CLOGP (http://www.daylight.com/daycgi/clogp) both being available for free.

3.4.3. Estimate of hydrophobic organic compounds solubility using K^{ow}

$$K_i^{ow} = \frac{C_i^o}{C_i^w} = \frac{C_{o\,total}\, x_i^o}{C_{w\,total}\, x_i^w} \tag{33}$$

On the basis that at equilibrium, the 1-octanol phase contains 72.5% (mole basis) of octanol and that the aqueous phase contains 99.99% (mole basis) of water, one can write that the molar total solvent concentration in the octanol phase is $C_{o\,total} = 8.23$ mole.L^{-1} and that $C_{w\,total} = 55.68$ mole.L^{-1} in the aqueous phase. It comes then:

$$K_i^{ow} = 0.148 \frac{x_i^o}{x_i^w} \tag{34}$$

At equilibrium, $a_i^o = a_L^w$ which implies

$$\gamma_{i\infty}^w = \frac{K_i^{ow} \gamma_{i\infty}^o}{0.148} \tag{35}$$

If the compound is not very soluble in water, eq. [30] applies, and

$$x_{is}^{w} \cong \frac{0.148}{K_i^{ow} \gamma_{i\infty}^{o}} \tag{36}$$

The solubility of i in water can thus be estimated for these compounds starting from the value of K_i^{ow} and the calculation $\gamma_{i\infty}^{o}$ of, which is more accurate than if it is estimated by a group-contribution method such as UNIFAC. If the concentration $C_{is} = x_{is} / V_w$ is reintroduced eq. [36] becomes:

$$\log_{10} C_{is} = -\log_{10} K_i^{ow} - \log_{10} \gamma_{i\infty}^{o} + 3.916 \tag{37}$$

Eq. [37], where C_{is} is expressed in mmoles. L^{-1}, has been tested on a data base of 164 components (Fig 6) including components with odorous properties (alkyne and aromatic compounds, esters, acyclic and cyclic alkanes, cyclic and acyclic monoalcenes, polyenes, primary, secondary and tertiary alcohols, diols, ketones, aldehydes, epoxides). The polyaromatic ones (stilbene, anthracene, benzopyrene, biphenyl) are not well estimated by this correlation.

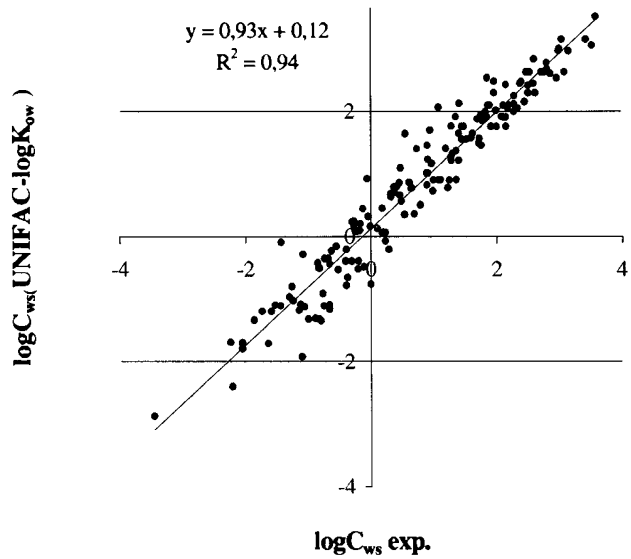

Figure 6. Performance of eq. [37] for estimating aroma compounds solubility in water. Experimental versus calculated values ($mg.L^{-1}$)

3.5. Chemical equilibrium. Calculation of pH

The culture media are always complex and many species dissociate completely or partially in aqueous medium. Partially dissociated species belong to many families: carboxylic acids, amino acids, organic bases (nitrogen bases), inorganic weak acids (H_3PO_4, H_2CO_3), inorganic weak bases (NH_3) and salts of acids or weak bases. Moreover several acids and weak bases present several dissociation reactions in water (H_3PO_4, H_2CO_3), which create a significant number of ions.

Thus, if one carries out a biotransformation in the simplest possible medium, glucose in a phosphate buffer for example, one can find in the medium the following compounds: glucose, H^+, OH^-, K^+, H_2O, H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}. Taking into account chemical dissociation equilibrium is essential insofar as these species modify the physicochemical characteristics of the solution, in particular the ionic force, and thus the non-ideality of the medium.

Achard et al. (1992, 1994) have developed a procedure, which makes it possible to determine on one hand the number and the nature of the species present in the solution and on the other hand to use information obtained to calculate the molar fraction and activity coefficient of each compound. The necessary and generally available data are the stoechiometries of the reactions of dissociation and the dissociation constants. One must then solve a system of equations comprising the material balances on the acids, the bases and on water, the equilibrium equations and the electroneutrality condition. Activity coefficients are calculated from the ULPDHS model. In practice the activity coefficients and the mole fractions cannot be calculated separately since the expressions of the dissociation constants involve these two data. The activities of all compounds present in the solution are then directly accessible, just as pH defined as:

$$pH = - \log_{10} (a_{H+}) \tag{38}$$

Table 5 shows that in the case of a reference solution made of a phosphate buffer, the calculated pH is very close to the experimental value, as given in handbooks. It also demonstrates that the commonly allowed assumption in a calculation of pH, ideality of the solution, cannot be accepted.

Table 5. pH of two phosphate buffers at 25°C. Comparison between data (Weast 1993) and values obtained by calculation assuming the nonideal and ideal mixture behaviour

Na_2HPO_4 (mole.L^{-1})	KH_2PO_4 (mole.L^{1})	pH (experimental)	pH (calculated with ULPDHS)	pH (ideal solution assumption)
0.025	0.025	6.865	6.873	7.210
0.0304	0.0087	7.413	7.417	7.750

For the culture media described previously, the calculated pH has been compared with the experimental ones (Table 6) measured before sterilisation. The absolute deviations between the measured and calculated values have been found always lower than 0.1 pH unit.

Table 6. pH of liquid fermentation media at 25°C. Experimental values compared to values calculated from the ULPDHS model or assuming ideality of solutions

Culture medium	pH (experimental)	pH (ULPDHS model)	pH (ideal solution assumption)
Basic medium for *C. glutamicum* growth	6.9	6.96	7.43
A. niger medium	5.65	5.69	5.69

The calculated pH, if the solutions are supposed to be ideal, can be rather far away from the experimental data. It is sometimes experimentally observed that the pH of growth or biotransformation media can be modified by sterilisation. To take this phenomena into account, it would be necessary to couple the method of calculation with vapour-liquid balances and equilibrium equations and possibly solid-liquid balances. This point is not yet entirely solved.

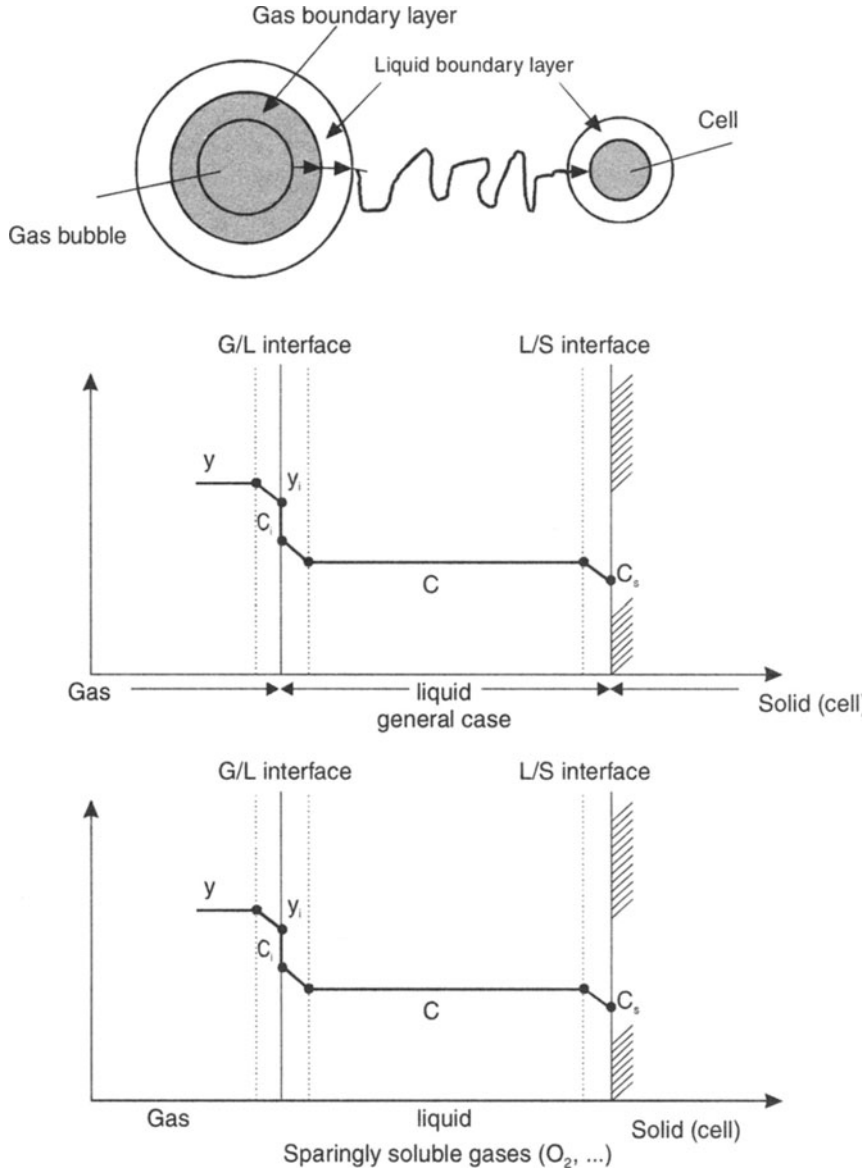

Figure 7. Steps in mass transfer from a gas phase to cells and corresponding concentration profiles

4. CONTACT BETWEEN PHASES. MASS TRANSFER

In many cases, the transport of substrates to the cells and that of metabolites from the surface of the cells to the culture medium are carried out at rates characterised by time-constants of the same order of magnitude than those of the biological reactions. Transport, or transfer, of matter must thus be included in an analysis of the behaviour of a bioreactor as well as the cellular kinetic rates (Scragg 1991, Nielsen & Villadsen 1994).

4.1. Gas-liquid dispersion

As it was stated in previous sections, many gases are poorly soluble in culture media. It is the case for oxygen and CO_2. For example, the oxygen reserves contained in the culture medium satisfy the oxygen demand of the microorganism only for one short length of time, which can become lower than a few seconds. It is thus necessary to bring these substrates continuously from the gas phase to the liquid phase according to the mechanism described in Figure 7:

1. transport by convection in the gas bubble.
2. diffusion through the gas boundary layer in the vicinity of the gas-liquid interface.
3. transport across the gas-liquid interface.
4. diffusion in the liquid boundary layer in the vicinity of the interface gas-liquid.
5. transport by convection in the liquid phase.
6. diffusion in the liquid layer in the vicinity of the microorganism.

Normally, the following assumptions are carried out: 1) transport by convection is sufficiently fast so that dissolved gas or gas concentrations are homogeneous, 2) the crossing of the gas-liquid interface is instantaneous; there is no resistance to transfer. There is thus thermodynamic equilibrium at the interface. This assumption should be discussed.

In most of the processes, steps 1, 3, 5 and 6 are in pseudo-steady-state and the mass transfer is governed by diffusion through the gas-liquid layers (steps 2 and 4). An additional step can appear if one deals with aggregates of cells (pellets), but we will not examine this case. Transfer by diffusion through the boundary layer is given by a relation of the type:

[Transfer rate] = [Transfer coefficient] [Exchange area] [Driving Force]

In the general case of gas-liquid transfer, one can write for each step:

$$Q = k_g A (y - y_i) = k_l A (C_i - C) \tag{39}$$

with

$$y_i = H C_i \tag{40}$$

H is a Henry's coefficient.

As y_i and C_i are difficult to obtain by experiment, one prefers to introduce an overall exchange coefficient

and an overall driving force so that the transferred rate is put in the form

$$Q = K_L A (C^* - C) \tag{41}$$

with

$$y = H C^* \tag{42}$$

K_L is the overall mass transfer coefficient based on the liquid phase. C* thus corresponds to equilibrium with the gas phase of composition y.

The link between these coefficients is simple to obtain. Indeed one can write

$$Q = k_g A (y - y_i) = k_l A (C_i - C) = K_L A (C^* - C) \tag{43}$$

and

$$\frac{1}{K_L} = \frac{1}{k_l} + \frac{1}{Hk_g} \tag{44}$$

In the case of oxygen or a sparingly soluble gas, H is large and thus $(1/Hk_g)$ is negligible compared to $1/k_l$. Therefore $K_L \approx k_l$. Resistance to mass transfer is located in the liquid phase. Thus one can write $K_L A \approx k_l A$. This is why, in the literature, one finds the two notations K_L or k_l with sometimes a risk of ambiguity.

In a bioreactor one is interested in the transfer per unit of volume of reactor, called $K_L a$ or volumetric mass transfer coefficient. a is the interfacial surface per unit of volume of liquid. In a perfectly mixed tank, C has an identical value in any point and C* depends on the conditions in the gas phase at the outlet of the reactor. Several authors (Nielsen & Villadsen 1994) consider that a better estimate of the driving force is given by the logarithmic mean concentration difference between the entry and the exit of gas.

4.1.1. Models of k_l

What has been just described previously corresponds implicitly to the two-film theory formulated by Whitman in 1923, which supposes that transfer is carried out by molecular diffusion through two stagnant films. From the Fick's law, it comes then:

$$k_l = \frac{D_i}{l} \tag{45}$$

D_i is the coefficient of diffusion of i in film and l the thickness of liquid film. l depends on the operating conditions and the physical properties of the medium. According to the boundary-layer theory, l is a function of viscosity η, density of the medium ρ_l, and diffusivity of i so that

$$l \div \left(\frac{\eta}{\rho_l D_i} \right)^{-\frac{1}{3}} \tag{46}$$

This leads to:

$$k_i \div D_i^{\frac{2}{3}} \tag{47}$$

This relation supposes that the liquid film thickness is identical in all the points of the reactor, which is seldom the case. Another well-known category of models in mass transfer is that of the surface-

renewal theory of Denbigh; it considers that aggregates of fluid are renewed near the gas-liquid interface at a frequency depending on the flow conditions. This leads to:

$$k_i \div D_i^{\frac{1}{2}} \tag{48}$$

In fact none of these models of exchange coefficient are of much use for the calculation of k_i values in real reactor conditions and we have to obtain them by experiments. However, these models can be used as a guide to estimate the influence of the physical properties of the medium. They also make it possible to consider relative values of $k_i a$ for compounds for which in experiments the value of $k_i a$ is not measurable as easily as for oxygen.

4.1.2. Interfacial gas-liquid area and gas bubble diameter

In the definition of $k_i a$ or $K_L a$, the interfacial surface area is in general based on the liquid volume in the reactor V_l:

$$a = \frac{A}{V_l} \tag{49}$$

A is the total interfacial area in the gas-liquid dispersion. This definition is consistent with the material balances on the reactor and in particular the gas-phase balances. However, in a few correlations published for $k_i a$ values, the authors use a specific area based on the total volume of the gas-liquid dispersion:

$$a_d = \frac{A}{V_d} = \frac{A}{V_l + V_g} \tag{50}$$

a_d and a are connected via gas hold-up ε :

$$\varepsilon = \frac{V_g}{V_d} \tag{51}$$

hence

$$a_d = (1 - \varepsilon)\, a \tag{52}$$

a is a function of the size of the gas bubble dispersion:

$$a = \frac{6\varepsilon}{(1-\varepsilon)\, d_b} \tag{53}$$

There are correlations making it possible to estimate ε and d_b as a function of the viscosity of the medium, its surface tension, its density, the characteristics of the gas injector, surface gas velocity and power dissipated by mixing (Nielsen & Villadsen 1994). If we remember the complex composition of culture media where the presence of inorganic ions, proteins etc., strongly affects the gas-liquid interface and thus coalescence, they can be used only to detect tendencies.

4.1.3. Empirical correlations for $K_L a$

Even if the remarks expressed above apply to $K_L a$, many correlations of experimental results were published (Perry & Green 1997). Most of these correlations are written in the form:

$$K_L a_d = k\, u_s^a \left(\frac{P_d}{V_l}\right)^b \tag{54}$$

Table 7. Coefficient values for estimating volumetric gas-liquid transfer coefficient by Eq. [54]. P_d in W, u_s in $m.s^{-1}$, V_l in m^3, K_La_d in s^{-1}

Culture medium	k	a	b	Stirrer type	Reference
Coalescing	0.025	0.5	0.4	6-bladed Rushton turbine	Moo-Young and Blanch, 1981
	0.0049	0.4	0.59	6-bladed Rushton turbine	Linek et al. 1987
	0.01	0.4	0.475	Various types	Moo-Young and Blanch, 1981
	0.026	0.5	0.4	Various types	Van't Riet, 1979
Non-coalescing	0.0018	0.3	0.7	6-bladed Rushton turbine	Moo-Young and Blanch, 1981
	0.00135	0.4	0.95	6-bladed Rushton turbine	Linek et al., 1987
	0.02	0.4	0.475	Various types	Moo-Young and Blanch, 1981
	0.002	0.2	0.7	Various types	Van't Riet, 1979

Coefficients k, a and b depend on the reactor design (Table 7). u_s is the surface velocity of gas and P_d the power consumption. It is observed that the volumetric mass transfer coefficient for a non-coalescing medium is higher by about a factor of two than that measured for a coalescing medium under the same operating conditions. Table 7 shows this distinction; fermentation media are in general non coalescent, but biotransformation media, even very simple, can be coalescent.

Culture or biotransformation media are generally Newtonian, but a high cell concentration and some metabolites produced (exopolysaccharides, etc.) can induce a non-Newtonian behaviour. In these cases, it becomes difficult to calculate the apparent viscosity of the medium. In stirred-tank reactors, the rheological properties are taken into account by means of an average viscosity η_m representative of the liquid behaviour in the reactor. One can treat simply the only case of non-Newtonian fluids obeying the law power model:

$$\tau = K \dot{\gamma}^{n} \tag{55}$$

If it is considered that the mean gradient velocity in the reactor is proportional to the stirring speed N, the average viscosity is given according to eq. [6] by

$$\eta_m = K (a N)^{n-1} \tag{56}$$

Correlations are then put in the form

$$K_L a_d = k u_s^a \left(\frac{P_d}{V_l} \right)^b \eta_m^c \tag{57}$$

P_d is calculated starting from an apparent Reynolds number defined by $Re = \rho N d_a^2 / \eta_m$. It is the principle of the Metzner and Otto method (1957) which remains the reference method to treat fluids

with complex rheology. There are equivalent correlations for air-lift reactors and bubble columns or fluidised beds. The accuracy which one can expect, even in the case of Newtonian fluids is about 20 % to 30 %. If one needs accurate values, the gas-balance method, operated during the course of a culture, is the only one that give a $K_L a_d$ or $K_L a$ value averaged on the whole reactor.

4.2. Liquid-liquid dispersions

In liquid-liquid reacting systems, one of the important parameters is the surface area per unit volume a in dispersion, which can be related to the Sauter mean drop diameter d_{32}. In some processes, the drop size distribution and especially the minimum drop size or the maximum stable drop diameter are also important factors in analysing the process results. Whether or not a drop can be broken depends on the relative magnitude of the external deforming forces:

- viscous stress due to velocity gradients in the surrounding continuous phase;
- turbulent pressure fluctuations.

The restoring forces are as follows:

- stress due to interfacial tension σd;
- internal viscous stress.

Hinze (1955, 1987) suggested two dimensionless groups to account for the forces balance, a generalised Weber group We, and a viscosity group Vi:

$$We = \frac{\tau d}{\sigma} \tag{58}$$

$$Vi = \frac{\mu_d}{\sqrt{\rho_d \sigma_d}} \tag{59}$$

For dilute dispersions with a non-viscous dispersed phase where the viscous energy within a drop is negligible compared to the surface energy, the maximum stable drop diameter d_{max} is given by

$$\frac{d_{max}}{D} = c\, We_{st}^{-0.6} \tag{60}$$

where $We_{st} = \frac{\rho_c N^2 D^3}{\sigma}$ is the Weber number in the stirred tank; ρ_c is the density of the continuous phase, N the impeller speed rate and D the impeller diameter. c lies between 0.05 and 0.06 for 6 bladed Rushton turbines depending on published correlations (Zhou & Kresta 1998). When the volume fraction ϕ of the dispersed phase becomes important, eq. [60] is multiplied by the correcting factor $(1. + 4\,\phi)$.

It was Sprow (1967) who first assumed that the Sauter mean diameter is proportional to the maximum stable drop diameter, i.e.

$$d_{32} = c\, d_{max} \tag{61}$$

and then verified the relationship with his experimental data. c lies between 0.42 and 0.69 and decreases with an increase in N, but is independent on the geometry of tanks and impellers (Zhou & Kresta 1998). When N is high (greater than 20 s^{-1}), c approaches a constant value and c = 0.5 can be considered as a design value.

It is worth noting that in some particular cases, an emulsion is obtained due to the medium characteristics. Fontanille (2002) has used a water-hexadecane two-phase system, which when stirred gives an oil-in-water microemulsion, to obtain the enzymatic transformation of α-pinene oxide to isonovalal. Due to the high exchange area created, the biotransformation was not limited by mass transfer between phases and the reaction rate obtained was higher than 25 $g.L^{-1}.h^{-1}$.

4.3. Case study: production of hydroxylated compounds of β-ionone

During these last decades, a great number of compounds comprising a trimethylcyclohexane skeleton were isolated from tobacco. These compounds have a typical tobacco flavour, detectable even at very

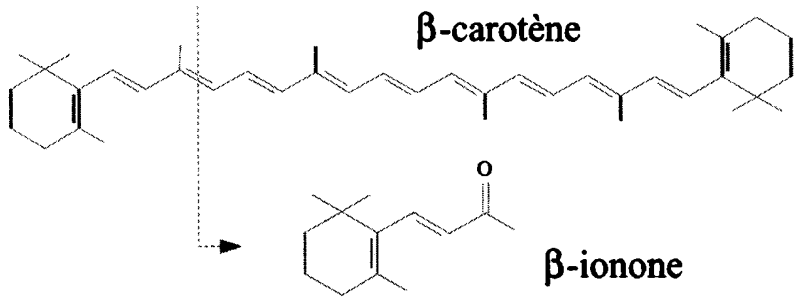

Figure 8. An example of production of ionone from a carotenoid

low concentrations. They probably originate from the degradation of carotenoids by complex enzymatic pathways. Many researchers then thought to obtain flavour of tobacco by the microbial transformation of carotenoids, but especially of ionones which are regarded as resulting from the degradation of carotenoids (Figure 8) and would be essential intermediates.

Ionones are very widespread in nature. By way of example, an essential oil with strong odor of tobacco was produced by *Lasiodiplodia theobromae*. In the same way the Givaudan Company used non-proliferating cells of *L. theobromae* ATCC 28570 for the conversion of β-ionone, and it asserted in a patent the transformation of 10 $g.L^{-1}$ of β-ionone with a 90% yield, the main products being β-cyclohomogeraniol. *Aspergillus niger*, known as being able to hydroxyle terpenes and terpenoïds was also tested as well as more than 160 moulds. The experiments showed that, together with *Botryosphoeria* and *Lasiodiplodia*, this mould was the only one capable of hydroxylation of β-ionone in position 2 and 4 of the cycle.

The main products are 4 hydroxy-β-ionone, 2 hydroxy-β-ionone, 2-oxo-β-ionone and 4-oxo-β-ionone in the order of decreasing productivity. These aromatic compounds are difficult to obtain by chemical

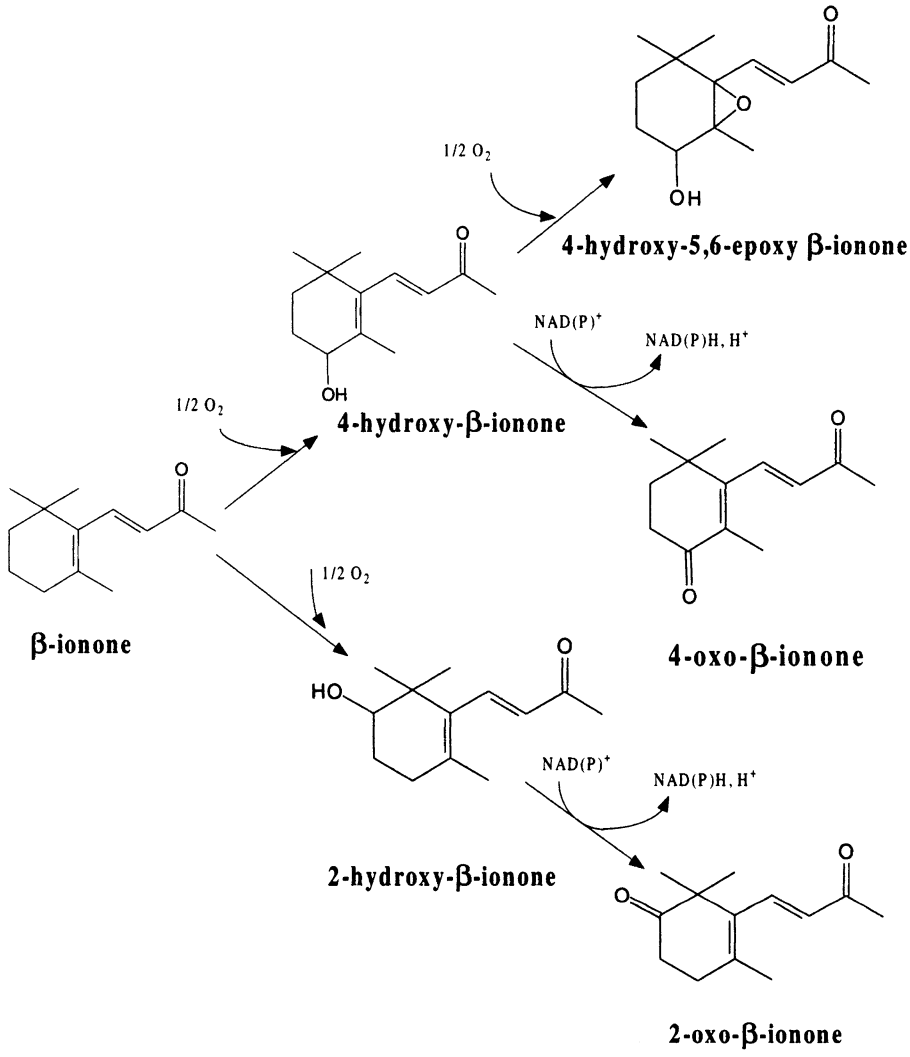

Figure 9. Products obtained from β-ionone by hydroxylation

way; the hydrophobic fraction of the products resulting from the biotransformation, even at very low concentration (1 to 5 ppm), makes it possible to correct the defects of mild tobaccos of poor quality, making them qualified as "sweet and mild." The most powerful strain is *A. niger JTS 191* (Mikami et al. 1981) but it is a proprietary strain. Larroche et al. (1995) selected the strain *A. niger* IFO 8541 which produces the same metabolites according to the pathways in Figure 9.

Even if these compounds are remarkably effective at low concentration, an industrial development could be considered only if the contents of this fraction in the final medium were strongly increased. However, β-ionone has a strong anti-bacterial and anti-fungal behaviour. This mainly explains why over more than 1000 strains tested (160 mushrooms, 830 bacteria, 30 yeasts), very few have been

found able to perform the targeted biotransformation. Reasons are multiple. The substrate concentration must remain lower than 1.5 $g.L^{-1}$ to avoid any inhibition. The growth of *A. niger* is carried out in the form of pellets and the mycelium attached itself on the walls of the reactor, baffles, impeller and measurement probes.

The reactions of biotransformation correspond overall to an oxidation, i.e. there is oxygen uptake, brought by the gas phase, and risks of stripping of the products. These characteristics make it possible to suggest conditions of culture. In a first outline of process, the resistance of *A. niger* to β-ionone was increased by entrapping the mycelium in hydrophobic polyurethane cubes and by adding an organic solvent, isooctane (Sode et al. 1989). Another approach consisted in immobilising *A. niger* in beads. In this last case, a complete process was proposed. The spores of *A. niger* are obtained by fermentation on solid substrate in a fixed bed reactor and they are extracted by distilled water. To ensure the growth in the form of pellets, the medium is inoculated by spores immobilised in calcium alginate beads. The beads are generated directly in the culture medium enriched by 0.05 $mole.L^{-1}$ (~6.9 $g.L^{-1}$) of calcium chloride. After a 24 to 140 hours growth, the biotransformation is carried out in a repeated fed-batch mode with an aeration flow rate of 0.3 vvm. One obtains approximately 4 $g.L^{-1}$ of hydroxylated compounds in 400 hours, which makes the process profitable. A thorough analysis, however, highlights points to be ascertain for the improvement of the process:

- lifetime of the catalyst;
- increase in biotransformation yield.

The lifetime of the catalyst can be increased by the addition, at the same time as β-ionone, of an easily consumed carbonaceous substrate (such as glucose). Determination of the apparent yield defined as the ratio "product recovered in the liquid-medium substrate consumed" shows that this yield is close to 35%. Two questions arise then:

- which are the reasons of such a low yield value?
- which value takes the true biotransformation yield (the "biological yield")?

Two different phenomena are responsible of the yield decrease. Initially β-ionone is degraded by self-oxidation in aqueous medium, according to a 1st order kinetics, to give undesirable products, but also 4-oxo-β-ionone. Then, part of the precursor is stripped by the gas flow. The reduction in the aeration flow rate does not make it possible to improve the apparent conversion yield because the reduction in the losses by stripping is counterbalanced by the increase in self-oxidation degradation. The system is quadriphasic, or triphasic if one excludes the solid phase containing the microorganism. Indeed the very low solubility of β-ionone in water (~0.88 $mmole.L^{-1}$) made that, even if a fed-batch culture is performed, three phases are present: the aqueous phase, an organic phase consisting in pure β-ionone and the gas phase. The modelling of the exchanges within these phases (Fig 10) in the presence of self-oxidation and the calculation of corresponding exchanged flow rates (Fig 11) result in thinking that the major part of the flow rates exchanged between the organic phase and the bioconversion medium (aqueous medium) is carried out via the gas phase.

This corresponds to the diagram in Figure 12. It is only when the organic phase is exhausted that the flow of transfer is reversed and carried out, for β-ionone, from the aqueous phase to the gas.

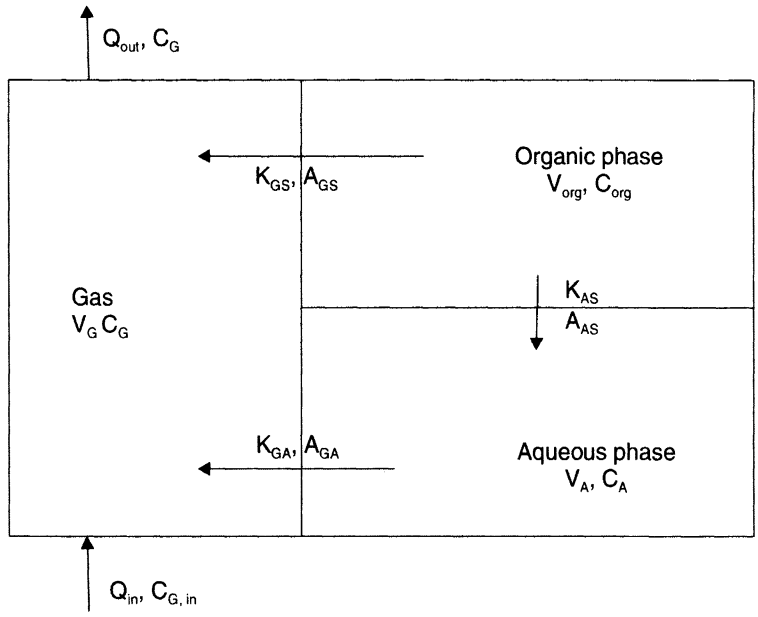

Figure 10. General scheme of mass transfer between phases in a triphasic biotransformation process. The Q are volumetric flow rates, V volumes, C concentrations, A exchange surface areas, and K overall mass transfer coefficients

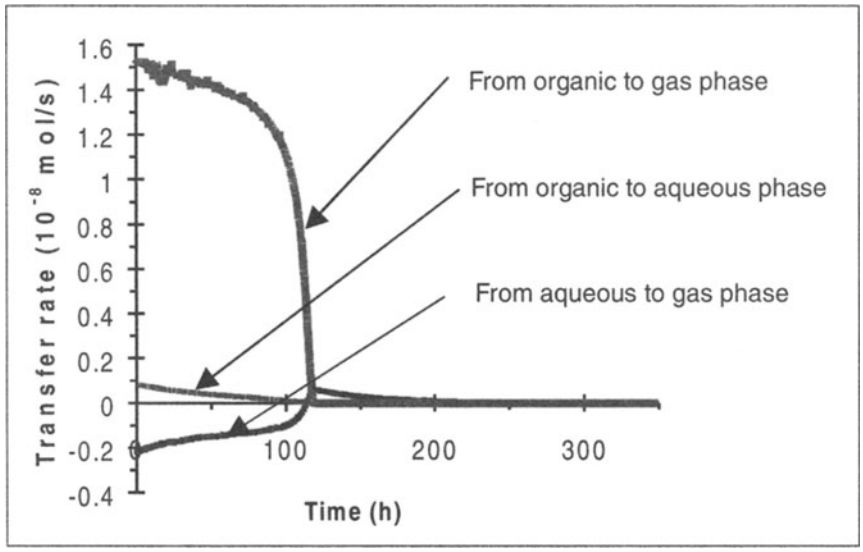

Figure 11. Fluxes between phases in the β-ionone hydroxylation process

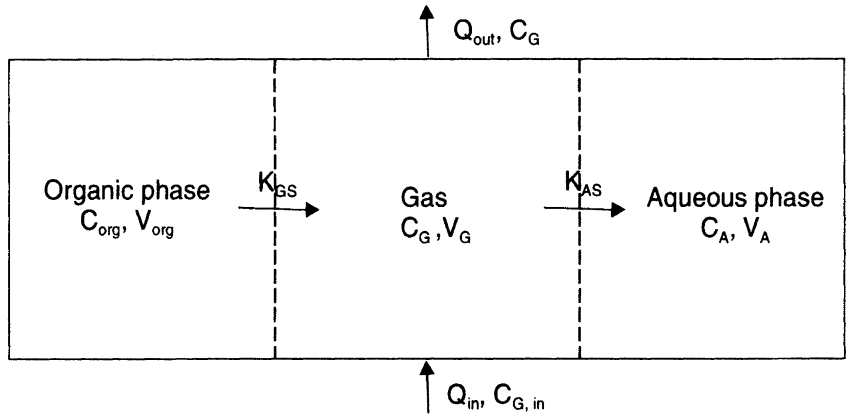

Figure 12. Specific scheme of mass transfer between phases in the β-ionone hydroxylation process. The Q are volumetric flow rates, V volumes, C concentrations, A exchange surface areas, and K overall mass transfer

This partly explains the strong losses of substrate.

Introduction of this information into a complete mathematical model (rate of exchange between phases, self-oxidation rate, biological reaction rate) shows that the biotransformation yield is very close to 100%. Further improvement of the process implies then the reduction in self-oxidation and losses by stripping (Grivel et al. 1999). Let us notice that the biomass concentration at the time of the feeding of the precursor is a critical factor. The synthesis of mycelium is stopped when β-ionone is added to the medium and the second period (active biotransformation) proceeds with constant biomass concentration. The fungus reacts to the presence of the precursor, a toxic molecule, there is a period of adaptation, then the biotransformation itself takes place only after this period. Without one being able to provide explanation, the higher the biomass concentration, the shorter the adaptation period; the latter vanishes when the biomass concentration is 6.5 to 7 $g.L^{-1}$, and the "true" yield of biotransformation becomes close to 100%.

5. INTRAPARTICLE DIFFUSION

When enzymes or micro-organisms are immobilized, i.e. entrapped in a pellet, substrates have to be transported not only to the pellet through the boundary layer surrounding it; they also have to be transported into the pellet and products have to be released out of the pellet. These transports occur by diffusion. Transport diffusion resistance may cause the interior of the pellet to be starved for reactants and substrate exhaustion (precursor, carbon source, oxygen, etc.) may occur in the centre.

It is important to analyse and compare the rate of reaction and the rate of the diffusion process. To measure how much the reaction rate is lowered because of diffusion resistance, a quantity called the effectiveness factor is defined as the ratio between the observed volumetric reaction rate and that obtainable in the absence of any mass transport resistance.

$$\eta_{eff} = \frac{\bar{r}_{S\,\text{with diffusion}}}{r_{S\,\text{without diffusion resistance}}} \tag{62}$$

It can be shown that for first order reactions, the effectiveness factor in a sphere particle is given by

$$\eta_{eff} = \frac{1}{\phi}\left[\frac{1}{\tanh 3\phi} - \frac{1}{3\phi}\right] \tag{63}$$

where ϕ is the so-called Thiele modulus, a dimensionless quantity defined by $\phi = \frac{R}{3}\sqrt{\frac{k_s}{D_S^{eff}}}$. R is the pellet radius, k_s the kinetic constant for the first order reaction and D_S^{eff} the so-called effective or apparent diffusivity. It should be stressed that D_S^{eff} is not the molecular diffusivity for two reasons : first, diffusion only takes place in the void volume ε_p of the pellet and second the length from the surface to the centre of the pellet is increased due to the tortuosity τ_p of the pellet so that

$$D_S^{eff} = \varepsilon_p \frac{D_S}{\tau_p} \tag{64}$$

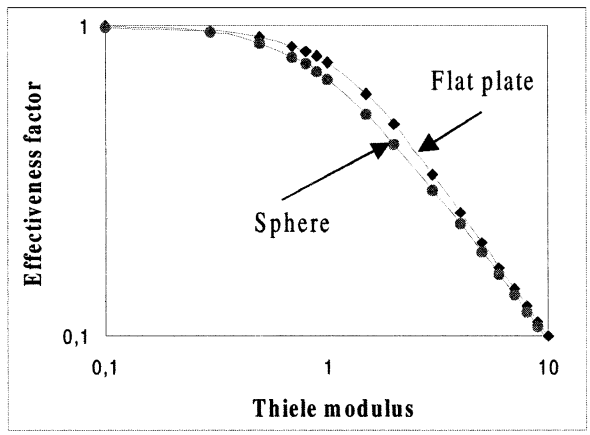

Figure 13. The effectiveness factor versus Thiele modulus.

The effective diffusivity in a pellet is therefore lower than the molecular diffusivity by at least a factor of 2, often of 10.

η_{eff} is shown in Figure 13 and with this figure we can tell whether diffusion modifies the apparent rate of reaction.

Two limit cases are observed. For small values of the Thiele modulus (or $\phi \leq 0.4$), $\eta_{eff} = 1$ and the substrate concentration does not appreciably drop within the pellet. There is no resistance to diffusion, i.e. it is a kinetic or diffusion-free regime. For large values ($\phi \geq 4$), $\eta_{eff} \cong 1/\phi$, hence diffusion strongly influences the rate of reaction. This regime is called diffusion regime. A large value of the pellet diameter, fast reaction, low diffusion, all three factors tend to increase the resistance to diffusion.

Analysis of the diffusion process helps in finding whether diffusion resistance is or not intruding to lower the rate of reaction. Suppose we have an observed experimental rate of reaction for a given biotransformation by immobilised cells and that negligible external film diffusion can be assumed. It is possible to define another module ϕ_w, which only includes observable and measurable quantities. This is the Weisz modulus (Levenspiel 1999).

$$\phi_w = \phi^2 \eta_{eff} = \frac{R^2 k_s}{9 D_S^{eff}} \eta_{eff} = \frac{R^2 k_s}{9 D_S^{eff}} \frac{\bar{r}_{S\ observed}}{k_S S_S} \tag{65}$$

$$\phi_w = \frac{R^2}{D_S^{eff}} \frac{\bar{r}_{S\ observed}}{S_S} \tag{66}$$

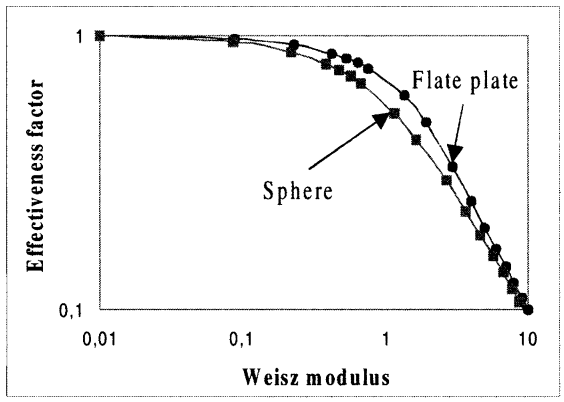

Figure 14. The effectiveness factor versus Weisz modulus.

It can be shown that the diffusion-free regime occurs when $\phi_w \leq 0.15$ and that the strong diffusion regime occurs when $\phi_w \geq 4$ (Fig. 14).

This simple criterion give access to the desirable processing range. Fine pellets are generally free of diffusion resistance but are difficult to use in packed beds due to the pressure drop they create; large particles create small pressure drop but are liable to be in the diffusion regime. For most effective operations, the best is to use the largest particle size which is still free of diffusional resistance, i.e. $\phi_w \cong 0.15$

The production of 2-heptanone from octanoic acid by spores of *P. roquefortii* provides the last example; this reaction is performed by spores immobilised in Ca-alginate beads, 3 mm diameter, in a continuous stirred-tank reactor. In steady-state, for an acid concentration of 0.34 mmol.L^{-1} in reactor exit, the reaction rate is 0.166 mmol.L^{-1}.hr^{-1}. The Michaelis constant is 1.69 mmol.L^{-1} and the diffusion coefficient of octanoic acid in the beads is 7.7×10^{-10} m^2.s^{-1}. In this case, the Weisz modulus can be evaluated assuming that the reaction is first order and it equals $\phi_w = 0.044$. The reaction rate measured is thus not limited by diffusion. It is easy to calculate that limitation will occur for 5.5 mm alginate beads diameter, value for which $\phi_w = 0.15$.

Note that Aris (1975) demonstrated that the relationship $\eta_{eff} = \tanh \phi/\phi$ obtained for cylindrical pores, gives a satisfactory approximation for the effectiveness factor for any kinetics and any reasonable pellet shape if the characteristic length R_p is taken as the ratio between pellet volume and pellet external surface area V/A and provided that a generalised Thiele modulus is used. For a Michaelis-Menten kinetic rate, this module is (Nielsen et al. 2003):

$$\phi = \frac{k R_p}{3\left(1+\frac{K_s}{S_s}\right)\left[2D_s^{eff} S_s k\left(1-\frac{K_s}{S_s}\ln\left(1+\frac{S_s}{K_s}\right)\right)\right]^{1/2}} \tag{67}$$

k is the rate constant, S_s the substrate concentration and K_s the Michaelis-Menten constant.

6. CONCLUSIONS

We hope to have demonstrated that the physical-chemical interactions between enzymes or cells and their macroenvironment have to be clarified to get a better understanding of their behavior in a bioreactor. It helps determining the limiting steps in the biotransformation or cultivation processes. This analysis is the only way to avoid pitfalls at laboratory scale or when designing and scaling-up a new process.

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Isolation and Purification of Enzymes

Rintu Banerjee

1. INTRODUCTION

Most of the biological reactions are enzyme mediated. There are several standard protocols, techniques and methods available to purify and characterize such biocatalysts present either in plant, animal or microbial systems. Most of the regulatory enzymes catalyzing several such reactions are intracellular in nature. But in the lower organisms such as microbes, the enzyme is also extracellular in nature and in these cases the level of contaminants is not that high as compared to the intracellular enzyme, the product (enzyme) is generally in diluted form. Thus, in developing a downstream process for isolating and purifying an enzyme, one of the main objectives remains to bring down the cost. This requires strategic selection of the purification processes. Though there are plenty of protocols and methods available for this, the selection of process largely depends upon the nature of the targeted enzyme. If the enzyme is intracellular, contamination level will be high and if it is extracellular, dilution problem predominates (Atkinson & Mavituna 1983, Bailey & Ollis 1996, Bejurstrom 1985, Dunhill 1983, Michaels 1984, Belter et al 1988). There is no standard protocol available, which could be universally accepted either for intracellular or extracellular enzyme purification. Belter et al (1988) suggested that one should take a look on the following points before determining the purification process for any enzyme:

i. The nature of the product, i.e. whether the product is intracellular or extacellular?

ii. Nature of the major contaminants.

iii. During the purification where the desired product lies?

iv. The end use of the final processed product.

v. Any alternative techniques available to make the process economically viable.

Based on the above points, the purification strategy can be divided into four different steps as below:

i. **Separation of insolubles:** Depending up on the nature of the enzyme, i.e. extracellular or intracellular, the separation of the insolubles should consider the filtrate (supernatant), or cells (biomass), respectively as the target. For this filtration and centrifugation are suitable processes.

ii. **Extraction and isolation of the products:** Not only the enzyme or protein based molecules, but also if the product is any alkaloid/phenolic compound, separation or removal of contaminants are mainly done through extraction technique.

iii. **Removal of contaminants through advanced chromatographic technique:** For most of the enzymes, chromatographic purification is playing a vital role. In this process, a number of properties should be taken into consideration before going for purification:

a. Size and shape – Gel Filtration Chromatography (GFC)

b. Net charge – Ion Exchange Chromatography (IEC)

c. Hydrophobicity – Hydrophobic Interaction Chromatography (HIC)

d. Metal Binding – Metal Chelate Affinity Chromatography (IMAC)

e. Thiol Group – Covalent Chromatography (CG)

f. Isoelectric Point – Chromato Focussing (CF)

g. Biospecific Ligand Binding Properties – Affinity Chromatography (AC)

iv. **Crystallization of the purified product:** The end use of the product dictates the final form of the purified product where crystallization of the desired product is taken into consideration.

2. SEPARATION OF INSOLUBLES

2.1. Filtration

2.1.1. General theory

The nature of insoluble during fermentation process is mainly compressible in nature, which is totally different from that of inorganic materials, where the insolubles are incompressible in nature as they are not as fluffy in nature as the cell biomass (compressible insoluble). Darcy's law hold good to explain the simple process of filtration through a process bed of solids to a pressure drop which is causing the flow of liquid through the membrane (Belter et al 1988).

$$V\alpha\frac{\Delta P}{\mu} \tag{1}$$

Where V is the velocity of the liquid (fermented beer), ΔP is the pressure drop across the thickness of the membrane l and Δ is the viscosity of the liquid broth.

$$\text{Or, } V = \frac{k\Delta P}{\mu} \tag{2}$$

Where k is the proportionality constant, which is called Darcy's law of permeability. Darcy's law holds good when

$$dV\rho/\mu(1\text{-}\varepsilon) < 5 \tag{3}$$

Where d is the particle size or pore diameter in the filter cake, ρ is the liquid density and ε is the void fraction in the cake. During filtration, resistance can be offered either by the medium itself (i.e. the fermented broth) or due to the deposition of insoluble on the filter membrane. Sometimes the fermented

broth is highly viscous and is difficult to filter because of high non-Newtonian viscosity, or because of the fluffy insoluble present in the broth in the form of cell biomass. In such conditions, the broth requires pretreatment.

2.1.2. Pretreatment processes

There are several techniques of pretreatment of the fermented broth such as heating, coagulation and flocculation addition of filter aids etc (Belter et al 1988). Though heating is one of the least expensive techniques, but as the biological are mostly heat sensitive, depending upon the nature of the product, heating process is selected. With the rise in temperature of the broth, viscosity is decreasing which makes it much easier to filter. Another pretreatment method is the addition of electrolytes to promote coagulation and flocculation. With the addition of electrolytes in the solution, electrostatic repulsion exists between the colloidal particles. When this repulsion forces are reduced, attractive Van der Waals forces predominate and thus result in the coagulation of the particle, which makes the liquid easy to filter.

With the addition of acid or bases in the fermented broth, coagulation takes place, which makes the broth easy to filter. Sometimes polyelectrolytes (such as polyacrylamide, polyethyleneimines, polyamines etc) pretreatment also reduce the electrostatic repulsion and adsorption on adjacent particles – resulting in aggregation of the molecule.

Pretreatment of the fermented broth before filtration is a common practice to enhance the efficiency of the filtrate collection. But depending upon the nature of the desired product, the additives are to be selected so that there won't be any denaturation of the product. Addition of filter aids as an adsorbent is another very common and popular practice for high-density filter broth. Diatomaceous earth (standard-cell, hyflo-supercel), perlites (Terracel, Celatom etc) are used for different applications such as beer, oils, wine, vinegar, alcohol, citric acid, gelatin, fruit juice, antibiotics fermentation etc. One of the disadvantages of the addition of filter aids in the broth is that though it increases the amount of filtrate collection but decreases the filtrate clarity. Sometimes, some antibiotics have been noticed to bind irreversibly resulting in the loss of final product. On the whole the filtration process can be divided into Microfiltration, Ultrafiltration and Reverse Osmosis.

2.1.3. Microfiltration

During the filtration process the insolubles get deposited on the filter membrane resulting in the decrease/fall in the filtrate collection. Prolonged filtration with a minimum deposition of insolubles is called microfiltration. This is achieved by using a large cross-flow tangential to the surface of the filter membrane/cloth. As there is a tangential flow along with the vertical flow, the rate of deposition of the insolubles is less, which in other way improves the efficiency of the filtration process.

2.1.4. Conventional filtration units

The configuration of filtration unit varies from conventional funnel or buchner's funnel with filter paper, for the laboratory scale operation to plate and frame type or rotary vacuum filtration type for large-scale filtration process. Several filtration units, as given below, are available for moderate quantity to large-scale operation of the processes. **Plate and frame type** filtration unit is one of the most popular filtration units where insolubles are retained on the filter membrane and there are some flushed plates that are used by open frames where the cakes are formed. **The horizontal plates** are for small scale operation. Filtration occurs

from the top of each plate. Vertical filter requires small floor area but the surface area is more as compared to the other two types. This type of filtration unit is very difficult to clean. **Candie type** filtration unit has the highest filtration area per volume. Filter cake is formed on the outer surface of the filtration tube rapped with the filtration unit. These units can be cleaned through back flushing.

2.1.5. Rotary vacuum filters

Rotary vacuum filters are widely used for large-scale operation of fermented broth. The perforated rotating drum is wrapped with the filter cloth, which is connected with the vacuum pump. The drum remains submerged partially in the trough containing fermented beer and rotates with a very slow speed. As there is a partial vacuum inside the drum, the liquid is sucked inside the drum and the insolubles are deposited on the outer surface of the membrane filter. Since the drum rotates at a very slow speed, the deposited insolubles get sufficient time to dry. There is water header also to wash the insolubles, followed by drying. Due to the vacuum inside, liquid present along with the biomass get absorbed. There is a blade (called Doctor's blade) inside the drum, which is attached in such a way that the deposited insolubles are removed as soon as they come to the other end of the trough. Once again the filter cloth is submerged inside the liquid containing drum (Belter et al 1988). Thus, there is a continuous discharge of the insolubles and the efficiency of the filter membrane/cloth remains same as the pores blockages are removed. Sometimes pre-coated membranes are also used for this purpose.

2.2. Centrifugation

2.2.1. General background

To separate the insolubles, an alternative to the filtration process is centrifugation. It is a process of solid-liquid separation in which sedimentation relies on gravity settling where solids are deposited at the bottom. Centrifugation involves the application of centrifugal forces mechanically to clarify the liquid supernatant by precipitating the solid. It is one of the vital steps in the separation of insolubles from the fermented broth in the fermentation process. The concentration and sizes vary widely and it may be as high as 60% per volume to as low as 0.1%. If the insolubles are large and rigid, they can be separated easily by ordinary filtration process. When the insolubles are not easily filtered, another alternative to separate these insolubles is centrifugation (Binder et al 1982). Centrifugation utilizes the density difference between the solids and the surrounding fluid. When the suspension is allowed to stand, the denser particles slowly settle under the influence of gravity and the process is called sedimentation. The terminal velocity for a solid particle settling under the influence of gravity is given by Stoke's law as

$$V_g = ((\rho_p - \rho_t)/18\mu)\,\alpha_{p^2}\,g \tag{4}$$

Where ρ_p is the density of the particle, ρ_t is the density of the surrounding medium, μ is the viscosity of the suspension, α_p is the diameter of the particle and g is the gravitational acceleration. This relation is valid for spherical particles with a Reynolds number ($\alpha_{p\,p}$Vg ρ_p/μ) < 1 for a low particle concentration where particle hindrance is absent.

$$V_c = ((\rho_p - \rho_t)/18\mu)d_p^{\,2}\omega^2 r \tag{5}$$

Where ω is the angular velocity and r is the radial position of the particle. This equation is valid for R_e < 0.4. By dividing equation (5) by equation (4), one can get

$$Z = \omega^2 r/g \tag{6}$$

Where Z is the relative centrifugal force, or g number. The range of Z for industrial centrifuge is of the order of 300 to 16,000 (Hsu 1981, Sokolov 1971, Moo-Young 1985).

2.2.2. Configuration of centrifuges

As discussed earlier, w^2r can be made as large as possible for higher bowl speed or large radial distances from the center of the axis. With this basic principle, a number of geometries are available for different types of centrifuges such as (Moo-Young 1985).

i) Tubular Bowl – It is simplest configuration, which provides a very high centrifugal force. Suspension is fed from the bottom and the clear liquid is collected from the top. It is good in dewatering, easy to clean and dismantling the bowl. However, it has limited solid capacity, causes foaming unless special skimming or centripetal pump is used and solid recovery is difficult.

ii) Chambered Bowl – It has small L/D ratio and clarification efficiency remains constant until sludge space is full. It has large solid holding and good dewatering capacity, along with bowl cooling. However, it cannot discharge solids and the recovery of solid is difficult. Moreover, compared to tubular type, cleaning is also difficult.

iii) Decanter Type – It is a special type for high solid content in which solid discharge is possible and liquid discharge under pressure eliminates foaming. In this case also, bowl can be cooled. However, it has poor dewatering configuration and that's why it is difficult to clean.

iv) Disc Type – It splits the liquid into thin layers, which makes particles to settle easily. It discharges solids continuously with high solids concentration. Low centrifugal force and turbulence created by scroll are drawbacks in this system.

3. EXTRACTION AND PURIFICATION OF SOLUBLES

3.1. Ultrafiltration

3.1.1. Definition and principle

Ultrafiltration is a separation process where the selectivity is based on particle size. The molecular mass ranges between 500 – 5,00,000 g/mole, which reflects the pore diameter of 1 to 20 nm (Moo-Young 1985, Belter et al 1988). The separation of macro solutes by ultrafiltration has the following advantages:

i. Low energy requirement

ii. The process can be operated at adjustable temperature

iii. Along with filtration, sterilization is also possible.

iv. The diluted liquid can be considerably concentrated and thus there is a lower consumption of chemicals for precipitation.

v. It can be operated in batch and continuous mode.

vi. No phase transition

vii. Uncritical scaling up

The main application of ultrafiltration in enzyme manufacturing is in their concentration, if extracellular in nature. The efficiency of ultrafiltration membrane can be defined through its excellent performance. The basic principle of ultrafiltration is the semi permeable membrane. A good membrane should have the following characteristics: high hydraulic permeability to solvent, sharp molecular weight cut off, high fouling resistance, good mechanical durability, and it should be sterilizable, easily cleaned and prolonged shelf life.

Ultrafiltration always involves a large flow across the membrane, which is very essential. This type of flow is known as cross flow. When the macromolecular solution is ultrafiltered, cross flow reduces the macro-molecular deposition on the membrane surface. When such accumulation is there near the membrane surface, it is called concentration polarization, which reduces the rate of filtrate collection (Moo-Young 1985). The concept of semi permeable membrane is very old. Ultrafiltration is critically dependent on the membrane. The membranes are very thin having a

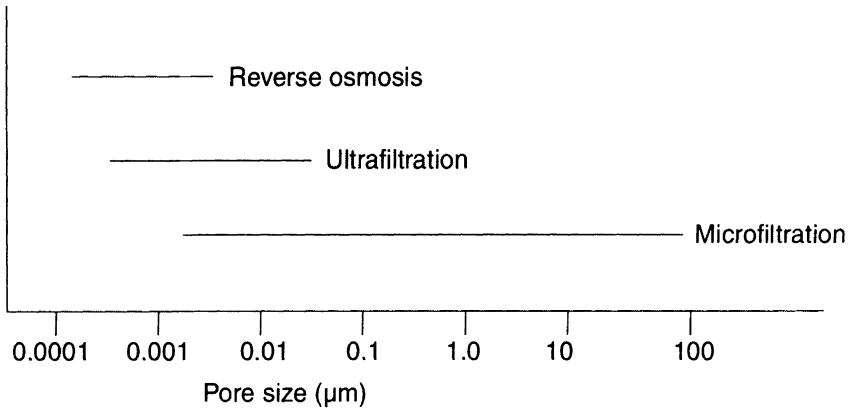

Fig. 1 Pore size distribution in various filtration systems

porosity of 0.01 to 1 μm (Fig. 1). Commercially available membranes are mainly made up of cellulose, polyelectrolyte, substituted olefins, aromatic polymers, cellulose esters, cellulose acetate, copolymer, polyacrylonitrile copolymer etc.

The most serious problem with ultrafiltration is the membrane fouling, which results in a progressive reduction in the filtrate ratio, which is due to the flux loss (because of the concentration polarization). Membrane fouling may be due to either adsorption of the solute to the membrane surface or due to the formation of gelatinous layer because of prolong running of the system, which blocks some of the pores of the membrane resulting in the fouling of the membrane.

3.1.2. Applications of ultrafiltration

General application of ultrafiltration can be broadly divided into three groups, *viz.* concentration, diafiltration and purification. A series of ultrafiltration units can be arranged for handling large quantities of liquid processing.

3.2. Liquid-liquid extraction

3.2.1. Organic solvent extraction

It is the separation of some of the constituents, which are poorly soluble in water but soluble in some other insoluble liquid. During the extraction, selection of suitable organic phase is very critical as water immiscible compounds immediately come to that phase. Final recovery is often achieved through evaporation. The advantage of organic extraction is that the separation can be done quickly on a large-scale. In the simplest system of two solutes distributed between two solvents, there is a wide choice of liquid to be used as a solvent of extraction (Binder et al 1982). Several points such as selectivity, distribution coefficient, insolubility of solvent in water, recoverability, density, interfacial tension, chemical reactivity, viscosity, non toxicity, etc have to be given due consideration before selecting a solvent. The extraction of the desired product is carried out mainly through counter current extraction. Continuous extraction of the mixer from an emulsion can also be made possible for large quantity product extraction.

3.2.2. Aqueous two-phase extraction

One of the alternative methods of extraction as well as concentration of enzymes is the aqueous two-phase extraction. The basic principle of separation of the molecules is based on the partition coefficient between two incompatible polymers such as PEG and dextran. When such polymers are dissolved in water, one polymer predominates in each phase. Biphasic conditions are also being possible if any one polymer such as dextran is substituted with water. Initially, there is no bilayer formation but after a certain concentration of polymers, bilayer formation takes place. Such mixture is called PEG-salt mixture. There are a number of polymers recommended for aqueous two-phase separation (Janson & Ryden 1998). Table 1 shows some examples.

In practice, aqueous two-phase extraction consists of three basic options such as the mixing of the phase component, separation of two phases and removal of enzymes/product along with the polymer removal. Uniform distribution of the component in the two polymers can be done within a few minutes of starting of the experiment. This type of quick equilibrium condition may be due to the low interfacial tension of the two phases, which also minimizes the energy input required to achieve rapid dispersal during mixing. In the subsequent stage, the recovery of the desired enzyme is carried out. The advantages of aqueous two-phase separation system include high biocompatibility and low interfacial tension resulting in minimum denaturation of the product, good resolution and yield, high capacity and easy scaling-up.

Examples of separation of any enzyme through aqueous two-phase separation are plenty. Firstly, PEG is a porous material, which absorbs the protein within it resulting in concentration. Moreover, it is easy to separate salt from the mixture either by ultrafiltration, diafiltration or through gel filtration chromatography. To make the process further cost effective, substitution of dextran

Table 1. Polymers for two-phase separation

Polymer I	Polymer II
Polyproplene glycol	Dextran Hydroxy propyl dextran Methoxy polyethylene glycol Polyethylene glycol Polyvinyl alcohol Polyvinyl pyrrolidone
Polyethylene glycol	Polyvinyl alcohol Polyvinyl pyrolidone Dextran Ficoll
Polyvinyl alcohol	Methyl cellulose Hydroxy propyl dextran Dextran
Polyvinyl pyrrolidone	Methyl cellulose Hydroxy propyl dextran Dextran
Methyl Cellulose	Hydroxy propyl dextran Dextran
Ethyl hydroxy ethyl cellulose	Dextran
Hydroxy propyl dextran	Dextran
Ficoll	Dextran

with salt is appreciated if the desired enzyme is not having any denaturation effect in the presence of salt. Aqueous two phase separation is not only concentrating the diluted products but partial purification can also be achieved through this process for which this process is considered to become a major unit operation method in biotechnology based industries.

4. RECOVERY AND PURIFICATION OF INTRACELLULAR PRODUCTS

4.1. Cell disruption

Till now we discussed about the extracellular enzymes, but if the enzyme is intracellular then cell disruption is an important step for the recovery of the enzyme (Belter et al 1988, Asenjo 1981). To achieve this, one can select physical, chemical or biological process. If the desired enzyme is shear sensitive, one should select any alternative pathway. Dyno mills or ball mills are the techniques where cells can be disrupted mechanically (i.e. with the increase in the agitation of the process). Sometimes frequent freezing and thawing of the cell result in the disruption. Non-mechanical methods include

treatment with alkali, acid, detergent, osmotic shock and enzymatic hydrolysis of the cell wall.

When mechanical disruption such as crushing in ball mills, ultra sound, dyno mill, hugues press, French press etc. is compared, most of the operations are harsher and cost of the process is moderate but if the enzyme/protein is shear sensitive, one should not select such process for operation.

4.2. Enzyme purification through chromatographic techniques

4.2.1. Gel filtration

4.2.1.1. General considerations

The chromatographic methods allowing biological molecules to be separated on the basis on their size are called Molecular Sieve Chromatography, Size Exclusion Chromatography, Gel Permeation Chromatography or Gel Filtration Chromatography. Various purposes such as desalting, purification of mixtures, determination of the distribution of molecular mass in gel matrices etc. can be solved through this kind of method (Scopes 1982). Similar to any other chromatographic techniques, selection of support matrices, pH, solvent, additives or pretreatment to any special type of protein play important role (Porath & Foldin 1959). The resolution of the gel may be crucial where properties of the bead size, slope of the selectivity curve and separation volume is playing an important role in case of normal as well as difficult problems.

Through this technique, the approximate molecular weight of the sample can be known as the separation is basically based on the size of the biomolecules.

$$k_d = (v_r - v_o)/v_i = (v_r - v_o)/(v_t - v_o) \quad (7)$$

Where v_t is the total liquid volume of the bed, k_d the distribution coefficient, v_o, v_i and v_r are the void, pore and elution volumes, respectively. The nature of the graph is sigmoidal. The pore volume of the bead can be calculated by substracting the void volume v_o from the total liquid volume v_t.

The well packed columns have a low, non separating void volume with a homogenous bed which prevents channeling of the mobile phase. If the packing of the materials is too high, this results in the reduction of the column life time. The choice of buffer, pH, ionic strength and additives should be properly selected so as to get a proper separation profile, as the basic principle is to separate the biological molecules in the matrices without any interactions with gel bed and protein molecule. Nonionic interactions can be avoided by increasing the pH and decreasing the ionic strength or with the addition of small quantities of detergents, ethyleneglycol or some organic modifier such as 1% propanol or acetonitrile along with the buffer. Based on the nature of the biological sample, selection of buffer pH and ionic strength is to be done (Janson 1995).

4.2.1.2. Working principle

As has been discussed above, based on the properties of the enzymes selection of pH, buffer and ionic strength is done. The operational flow rate of the mobile phase is kept in a slow phase for a better resolution i.e. 8-12 ml/hours. The gel matrices are loaded to the column in such a way that there is no bubble entrapped inside the column. The sample simply layered on the bed surface with the aid of either any pipette or Pasteur pipette, whereas more convenient way to apply sample is by injection technique.

The liquid buffer is to be degassed before use. The mobile phase then drags the molecule out of the column continuously with the help of the buffer. The length of the column is also another important parameter in gel filtration chromatography as the resolution of protein is proportional to the square root of the column length. Thus longer the column better is the resolution. The separation will be based on the molecular weight of the sample (enzyme). Using the standard protein sample and plotting the standard graph of K_d vs. log of molecular weight, calculation of the approximate molecular size of the unknown sample weight, unknown samples size can be determined. Some time instead of getting a sharp peak zone broadening occur resulting non-equilibrium between the mobile phase outside the particles and inside the pores that may be due to the slow diffusion of the solute through the pores.

4.2.2. Ion-exchange chromatography

Ion-exchange chromatography is one of the most popular techniques for purifying the mixture of protein molecules. Because of the high resolving power, high protein binding capacity, versatility, straight forward separation principle and ease of performance, this chromatography process in getting importance. The basic interaction and separation of enzymes in IEC method is based on the charge. The interaction between the molecules and the ion exchanger depends on the net charge and the ionic strength of the medium. There are many factors, which influence the IEC process such as net charge and ionic strength of the protein molecule, pH, nature of particular ions in the solvent, additives etc. The force of interaction between two charges Z_A & Z_B separated by a distance r is given by the following equation (Coulomb's law) (Janson & Ryden 1998), where F is the interaction of the two charged molecules and D is the dielectric constant of the medium.

$$F=Z_AZ_B/Dr^2 \qquad (8)$$

Just like other chromatographic procedures, IEC is also divided into two parts, ***stationary phase*** and **mobile phase**. The stationary phase is that part where the protein molecules are adsorbed with the matrices as there are either cationic groups or anionic groups attached (Peterson & Sober 1956, Peterson 1978). If the charge groups are of Dimethyl Amino Ethyl (DEAE) having a structure of $-OCH_2N^+H(C_2H_5)_2$, Trimethyl Amino Ethyl (TMAE) having a structure of - $OCH_2CH_2N^+(CH_3)_3$ or Quaternary Amino Ethyl (QAE) having a structure of - $OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$ where the functional groups are either ether linkage or amido linkage, they are called anion exchangers whereas the matrix having methacrylate (CH_2= $CH(CH_3)COOH$, carbomethyl CM-O- CH_2COOH, orthophosphate (P) $-O-PO_3H$ or sulphonate(S) $-OCH_2SO_3H$ are the cationic exchangers where the linkages are ether linkages. The charge groups are introduced to the matrices through chemical reaction where the binding of protein takes place. Introduction of DEAE is carried out to the cellulosic matrices by reaction with 2-Chlorotriethylamine whereas carboxymethyl cellulose is getting introduced by reaction with chloroacetic acid. The amount of protein adsorbed to the matrices is called the capacity or porosity of that particular adsorbent.

There are different types of ion exchangers such as resin ion exchangers, cellulose, dextran and agarose based ion exchangers, perfusion chromatography ion exchanger, continuous bed ion exchanger and nonionic interactions on ion exchangers. In case of resin based chromatography system, as the pore size of the resin is too small, the chromatography process is very much suitable for low molecular size peptide and amino acids (Jungbauer et al 1994, Gada & Cramer 1994). Generally the nonionic interactions of proteins with ion exchangers and hydrophilic matrices are normally low. However, under extreme conditions the proteins bind more strongly to the ion exchangers.

Normally the mobile phase for IEC system means either buffer or any solvent. pH of the buffer along with its concentration is playing an important role. Volatile buffers such as formic acid, pyridine/formic acid, trimethylamine/formic acid or HCl ammonia/acetic acid, ammonia/formic acid, ethanolamine/ HCl, trimethylamine/carbonate etc. are playing an important role. The main reason for the use of volatile buffer is to avoid the further dilution of the sample during isolation and extraction. The effect of anions on the retention of different protein molecules such as lysozome, chymotrypsinogen A, α-chymotrypsin and cytochrome c on cationic ion exchanger is in the order of

MOPS < acetate < chloride < sulphate < phosphate ions.

The chaotrophic effect of anions increases in the order of

$SO_4^{2-} \sim PO_4^{3-} < H\,PO_4^{2-} < CH_3COO^- < HCO_3^- < Cl^- < Br^- < ClO^- < I^- < SCN^-$

whereas the effect of cation increases in the following order

$NH_4^+ < K^+ < Na^+ < Li^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$

The sample after getting adsorbed to the matrix is eluted with the buffer. The protein molecules of same charge are carried out of the column and the process is called washing. After assuming that no unabsorbed proteins are there in the column, gradient is started to elute the binded proteins from the column. The elution can be with the salt gradient, pH gradient or by some affinity elution technique. Depending upon the elution technique and the nature of the biomolecules, selection of elution technique is done. Sometimes the elution is isocratic in nature where the sample and its properties are well known and the same kind of sample is run repeatedly. In isocratic elution, the components move simultaneously. Separation power increases with the square root of the column length. Stepwise elution or continuous elution is a serial application of several isocratic elutions. In these processes either with the increase in salt concentration intermittently or by the increase in the salt concentration continuously the sample is eluted. In Gradient elution, the concentration of the salt and pH of the eluting buffer is changed continuously. After each use, the gel matrices are regenerated. Regeneration procedures vary according to the stability of the matrix and the functional group. The manufacturer instruction is given on the level of the bottle nowadays. Before further use, the matrices are first washed with either 0.5 M NaOH or with 0.5 M HCl to remove the excess charge present. Before preservation sometimes sodium azide is added to the column.

4.2.3. Hydrophobic interaction chromatography (HIC)

4.2.3.1. General considerations

In Hydrophobic Interaction Chromatography (HIC), the biological molecules are self-associated due to hydrophobic interactions. The hydrophobic interaction is of prior importance that is one of the major driving forces for the folding of the globular proteins. Water is a poor solvent for non-polar solute and thus dissolving any non-polar substance in water is thermodynamically unfavorable (Hanstein 1979, Hanstein et al 1974). When any such compound is coming in contact with water, it forms a cavity in which the solute fits causing many hydrogen bond in water molecules to break and forming new hydrogen bond surrounding the cavity leading to a negative change in enthalpy that is ΔH. The interaction between any hydrophobic protein molecules in aqueous solution can be well explained in the same way as discussed earlier. Hydrophobic interaction is a big interaction and to strengthen this interaction there

are various factors, which play an important role. The effect of some additives such as salt promotes hydrophobic interaction, which are in the order of (Hofmeister 1887):

Anions:

$$SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$$

Cations:

$$Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$$

With the increase in salt concentration, the interaction becomes stronger as the added salt increases the surface tension of that particular interaction (Yon 1977, Tanford 1973).

Many types of matrixes are possible for HIC, but the most popular is the octyl or phenyl-sepharose containing matrix. The density of the ligand is also another important parameter that determines the capacity of the gel bed. Adsorption of the protein to the HIC gel is favored with high salt concentration. The different salts give rise to different strength of interaction of the molecules. The adsorption of the protein molecules in the HIC gel is increased in the order of

$$Na_2SO_4 > NaCl > (NH_4)_2SO_4 > NH_4Cl > NaBr > NaSCN$$

Temperature is also playing an important role as a decrease in temperature decreases the interaction and thus while working with cold labile protein the selection of the column should be properly done.

4.2.3.2. Working principle

The working principle of HIC is similar to the other types of the chromatographic processes where equilibration of the matrix is done with the same buffer having same molarity in which the protein is dissolved. After loading the sample, the unbind protein is washed out from the column and elution of the bound protein is done either step wise or with a gradient by applying three different techniques which are by changing the salt concentration, by changing the polarity of the solvent, by the addition of some detergents. It has already been mentioned that increasing salt concentration increases the strength of binding in HIC, and thus to elute the binded proteins decreasing the salt concentration drags the protein out of the column which is just reverse of Ion Exchange Chromatography (IEC). In IEC, the elution is done by increasing the salt concentration. The addition of ethylene glycol or isopropanol to the column decreases the polarity, resulting in the elution of the protein molecule. Sometimes detergent addition is also helps in the displacement of protein.

After the adsorbents are used, the matrix can be reused for several times after washing it with 6M Urea or Guanidine Hydrochloride to remove any left over protein present in the column matrix. After being washed, the column matrix can be stored at 4^0C in presence of 20% ethanol.

4.2.4. Immobilized metal chelate affinity chromatography (IMAC)

Metal chelate affinity chromatography is also known as Immobilized Metal chelate Affinity Chromatography (IMAC). Most proteins can form complexes with metal ions (Anderson & Porath 1986, Boden et al 1995). Many of these are multi dented complex (chelates) and allow the purification of proteins. Though it is also a type of ligand exchange chromatography, but there are some special features for IMAC as below:

i. Exposure of some amino acids such as histidine, cystine, tryptophan etc. on the surface of the targeted protein molecule.

ii. The steric arrangement of the protein molecule plays an important role that indicates that the protein having same molecular size, charge and amino acid composition but having differences in secondary and tertiary structure can be separated.

iii. Just like other chromatographic process, pH is also playing an important role in separation of protein molecule.

iv. The separation in IMAC chromatography is based on the metal ion interaction. So, metallo protein can't be separated through IMAC Chromatography.

There are different types of metal ions, e.g. transition metal ions such as Ca^{2+}, Fe^{2+}, Fe^{3+}, CO^{2+}, Cu^{2+}, $Ni^{2+}$and Zn^{2+}, which participate in the separation of protein molecules. There are various parameters that influence the adsorption and desorption of protein molecules. The parameters are chelaters and its structure, interaction of metal ion with the chelators, structure of the protein molecule, pH and ionic strength of buffer, types of buffer, effect of certain additives such as detergent, surfactants etc (Fanou-Ayi & Vijayalakshmi 1983, Berna et al 1997). Copper chelates with sepharose (Janson & Ryden 1998) in which one coordination site is occupied by water molecule that is substituted by a sample molecule during the course of chromatographic process. NNN tricarboxy methyl ethylene diamine coupled with sepharose is a five dented chelating group and is occupying 5 of the 6 co-ordination sites of Ni ions. Ni-TED sepharose is a weak adsorbent when IDA is used as a chelating group. Phospho serine is a tridented chelating group and can be compared with IDA, but it has a different selectivity of protein.

Similarly, the strength of protein, which is adsorbed on the IMAC surface, also plays an important role. Histidine and cysteine cause strong interaction with the metal in IMAC. As histidine has an indole group, tryptophan also contains indole structure, the binding is contributed through that site (Janson & Ryden 1998). The strength of the binding of the metal ions decreases in the order of

$$Cu^{2+} > Ni^{7+} > CO^{2+} > Zn^{2+}$$

In the absence of tryptophan and cysteine on the protein surface and by increasing the number of histidines, the binding strength enhances. In IMAC, histidine has been given a greater importance and it is suggested that if the protein molecule doesn't have any histidine or tryptophan on the outer surface, one should not recommend for IMAC. If the protein contains one histidine or more than one (i.e. a cluster of histidine) or several tryptophan and no histidine on the outer surface of the protein, one should suggest for Cu^{2+} or Cu^{2+}/Ni^{2+} or $Cu^{2+}/Ni^{2+}/Zn^{2+}/CO^{2+}$ or Cu^{2+} column chromatography. Just like other chromatographic technique, in IMAC buffer, ionic strength and different types of buffer are playing a major role to determine the characteristics of protein. High salt concentration suppresses the protein-protein interaction in IMAC. Detergents also enhance the binding of protein molecules.

As in other chromatographic methods, in IMAC the desorption of protein takes place with the changes in pH either in a gradient, stepwise reduction in pH or with competitive elution technique i.e. NH_4Cl, glycine, hystamine, histidine or immidazole can be used to elute the adsorbed protein. The reusability and stability of the matrix in IMAC is very high. The metal ion can be stripped off and reloaded before use.

Besides the coordination bonding between the protein and the metal ions there are some other interaction also existing such as electrostatic and charge induced interaction, coordination or electron donor acceptor interaction, covalent bond formation (Vijayalakshmi 2002, Vijayalakshmi 1989).

4.2.5. Covalent chromatography

Covalent chromatography is another technique of purifying any thiol-containing protein. As the name indicates, there is a covalent bond formed between the solute and the stationary phase. The binding of the protein molecules with the matrix is through the covalent bonding. If any thiol group (cysteine, methionine etc.) is not present on the outer surface of the protein molecule and is buried in a hydrophobic pocket, it can be demasked with either 8M Urea or 6M Guanidine containing EDTA solution without disturbing the three dimensional structure of the protein molecule (Janson & Ryden 1998). The optimization conditions for each protein molecule vary from species to species and also the sources (Belew et al 1987). Thiol can also be created in that particular protein with the reduction of the disulfide bonds. The most popular reducing agents are di-thio erythritol (DTE) or di-thio threitol (DTT) or β–mercaptoethanol. In some cases, the disulfides can be reduced without destroying the gross conformation of the protein molecule.

The thiol group can also be introduced to the protein molecule so that it can be processed under covalent chromatographic technique (Porath & Belew 1987). The most commonly used chemicals for this purpose is the N – succinimidyl-3-(2-pyridyldithio) propionate (SPDP). This reagent has a very mild action on the disulfide linkages and it functions with the equimolar amount of DTT resulting in the pyridyl disulfide substituted protein (Oscarsson & Porath 1990). When any such chromatography is in action, binding of the protein to the matrix is through covalent bonding. The excess protein is washed out from the gel bed and the desired protein is eluted with the reduction technique that is either 10 - 25 mM DTT or 25 – 50 mM β–mercaptoethanol. Sometimes the thiol group can also come out from the column, which acts as a ligand along with the eluted protein. Thus to quantify the amount of contaminants present in the protein in the form of thiol ligand, the optical density of the elutant is taken at 280 and 340 nm. One can get the actual optical density of the protein molecule by the difference of the two values. To separate the thiol group from the protein mixture, gel permeation chromatography is carried out. The preservation of the gel is always done by keeping the gel matrix at 4^0C. The use of sodium azide as a bacteriostatic agent is avoided, as it is a good nucleophile and reacts with the pyridyl sulfide group to form thio-pyridone and labile-sulfanyl azide.

4.2.6. Affinity chromatography

The different techniques whether it is HIC, Covalent, IMAC or histidine, are falling under the broad heading of Affinity Chromatography where the basic principle of separation of any molecule is based on the affinity ligands (Lowe & Dean 1974, Schott 1984, Scouten 1981). Whatever may be the chromatographic technique, a good ligand should have the following characteristics (Janson & Ryden 1998):

i. The ligands must be able to form reversible complexes with the targeted protein.

ii. It should have a good specificity.

iii. The association of the complex molecules should not be too strong or too weak which can

affect the chromatographic process. It should have chemical properties to immobilize with the matrix easily.

The ligands can be divided into two groups, the mono specific ligand, and group specific ligand. Generally mono specific ligands bind more strongly and require a harsher eluent to elute the desired protein whereas in case of group specific ligands, there is a specificity for a larger group of molecules which sometimes biomimic the biological molecules. The most widely used triazine dye is cibacron blue F3GA, which mimics NAD and thus any oxido-reductive group of enzyme can be purified through this technique, particularly those enzyme which are $NAD^+/NADP^+$ dependent. Multiple interaction mechanism is the basis of dye protein association. It is because of the chemical structure that matrix bound dye can engage in ionic hydrophobic or charge transfer interaction with the protein (Janson & Ryden 1998).

The advantages of the dye-based ligands are that they are cheap, available in plenty, reusable, highly stable, high protein binding capacity to the matrix column. Being group specific ligand, flexibility of a particular column matrix is very high, strength of binding of the protein to the matrix is medium and coupling technique is through chemical coupling, and thus the cost of such type of ligand is lowest. Affinity chromatography is one of the biospecific chromatography processes where the elution of protein is done through the specific elution technique though it can be done with the change in pH, ionic strength or the changes in the polarity of the solvent (Schott 1984).

4.3 Analytical assay of the purity level of a protein sample: electrophoresis

After purification of the protein to identify the level of purity, electrophoresis is done. Electrophoresis is basically the separation of any charged molecule under the influence of electrical field. There are two types of gel available for a protein chromatography process, native gel electrophoresis and SDS-page electrophoresis. In case of SDS-PAGE, multiphasic buffer system is used to achieve the best resolution of the protein. In this case, the ionic constituent making up the buffer is same in both stacking and resolving gel, but the pH is different, whereas the constituent of the electrode buffer is totally different from the gel buffer, which is present in the electrode compartment. Glycine buffer is used as electrode buffer; in gel compartment tris-HCl is used. To explain the separation process under such multiphasic buffer system, the two ionic species, Cl^- and glycinate ions, which migrate in the same direction (i.e. from cathode to anode) as the sample molecules are called leading and trailing ions, respectively. At the beginning of the experiment, the leading Cl^- ion present in both the phases leads, trailing the glycinate ion as soon as the electric current is switched on. The leading ions attempt to migrate away rapidly from the other ionic species leaving behind a zone of lower conductivity. Since the specific conductivity is inversely proportional to the field strength, there is a gradient of voltage formed, which accelerates the trailing ion so that they migrate immediately behind the leading ions at the same velocity and during this process they form a boundary, which is called Kohlrausch boundary. The leading ion then reach the transition of the stacking and the resolving gel as the pH and the condition of the gel such as pore size are changed abruptly from that of a stacking gel, it takes some time to adapt themselves to the new changed conditions. In this situation the buffer in the separation gel the ionization of the trailing ions increased and its velocity is equal to that of the leading ion. The trailing ions then overtake the leading ions and thus the separation of the macromolecules within the gel moves. Because of this reason the separation of protein molecule with in the gel is maximum (Andrews 1988).

5. AN EXAMPLE OF PROCESS FOR THE PRODUCTION AND PURIFICATION OF KINASES

Finally, below is an example showing various steps involved in isolating and purification of an enzyme produced by a microbial culture.

Culture of *Bacillus sp.* through submerged fermentation

↓

Separation of cell biomass from the fermented broth by filtration/centrifugation

↓

Extraction of intracellular substances through cell disruption with the addition of suitable buffer in controlled amount

↓

Intracellular products + Cell debris

↓

Separation of cell debris through Centifugation/Filtration

↓

One can go for further precipitation to 80% ammonium sulphate/Aqueous two phase extraction for further concentration

↓

Separation through blue Sepharose CL-6B dye affinity column

↓

Elution with 5mM NAD+ to 20mM NAD+ concentration to elute the desired protein

↓

Separation through Gel filtration chromatography

↓

Electrophoresis to identify the purity of the protein

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Applications of Industrial Enzymes

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1 INTRODUCTION

Enzymes are special proteins, which catalyze chemical reactions with great specificity and rate enhancements. These reactions are the basis of the metabolism of all living organisms, and provide tremendous and economical biocatalyst conversions (Godfrey & West 1996). First half of the last century saw a rapid development in enzyme chemistry. Commercial exploitation of microbial enzymes began much before their nature and properties were worked out. For centuries, extracts of plants had been used to bring about hydrolysis of polymeric materials. However, these sources of enzymes were unreliable and expensive too, hence search for alternative sources began. Largely, this was found in the microbial cultures (Menon & Rao 1999). The first enzyme produced industrially, as the fungal amylase, was amylase takadiastase, employed as a pharmaceutical agent (for digestive disorders) in the United States in 1894. Otto Roehm´s patented "laundry process for any and all clothing via trypic enzyme additives" was announced in 1915. Later on, more researches focused on discovering the primary, secondary and tertiary structures of enzyme polypeptides, as well as the mechanism of enzyme action. The Enzyme Commission, set up by the International Union of Biochemists (1965), has published a system of enzyme classification, which is universally adhered to throughout biochemistry (Menon & Rao 1999). Although about 25,000 natural enzymes are speculated to exist, only about 2,100 have been recognized by the International Union of Biochemists. This means about 90% of the reservoir of biocatalysts still remains to be discovered and characterized.

The 1950's saw an important growth in the industrial enzyme production and use of microbial enzymes. By 1969, 80% of all laundry detergents contained enzymes, mainly proteases. Other enzymes such as lipases, amylases, pectinases and oxidoreductases were used experimentally in the detergent industry. Due to the occurrence of allergies among production workers and consumers, the use of proteases in detergents was drastically reduced in 1971 and the world sales fell from $ 150 million to one-third. "Only when special processing techniques, such as microencapsulation were developed, dustless protease preparations be produced that were risk-free to workers and consumers" (Crueger & Cureger 1982). The development of amylases and amyloglucosidases for the production of glucose from starch led to a new industrial application of enzymes. Also, the use of glucose isomerase for the production of fructose has become widespread since 1970.

The Organization for Economic Cooperation and Development (OECD) has recognized enzymes as an important component of sustainable industrial development (OECD 1998) as their applications range from straightforward industrial processes to pharmaceutical discovery and development. Table 1 presents

Table 1. Industrial applications of enzymes

Enzyme class	Industry	Application
Acetolactate decarboxylase	Beverage	Maturation of beer
Acylase	Organic synthesis	Synthesis of semisynthetic Penicillin
Amylase	Detergent	Starch stain removal
	Starch and fuel	Starch liquefaction and saccharification
	Baking	Bread softness and volume, flour adjustment
	Beverage	Juice treatment, low calorie beer
	Textile	De-sizing
	Pulp and paper	Starch-coating an deinking
Amyloglucosidase	Starch and fuel	Saccharification
Amyloglusidase	Personal care	Antimicrobial
Beta-glucanase	Animal feed	Digestibility
	Beverage	Mashing
Catalase	Textile	Scouring
Cellulase	Detergent	Cleaning, color clarification
	Textile	Denim finishing
	Pulp and paper	De-inking drainage
Cyclodextrin-Glucotransferase	Starch and fuel	Cyclodextrin production
Glucose isomerase	Starch and fuel	Glucose to fructose conversion
Glucose oxidase	Personal care	Bleaching, Antimicrobial
	Baking	Dough strengthening, bread whitening
Laccase	Beverage	Clarification, flavour
	Textile	Bleach termination
Lactase	Food	Lactose removal (milk)
Lipase	Food	Cheese flavor
	Baking	Dough stability and conditioning
	Pulp and paper	Pitch control, contaminant control
	Fats and oils	Trans esterification
	Organic synthesis	Resolution of chiral alcohols and amides
	Leather	De-pickling
	Detergent	Lipid stain removal
Lipoxigenase	Baking	Biscuits, cookies
Mannanase	Detergent	Mannan stain removal
Nitrilase	Organic synthesis	Synthesis of enantiopure carboxylic acid
Pectinase	Beverage	De-pectinization, mashing
	Food	Fruit based products
Pectin methyl esterase	Food	Firming fruit- based products
Peroxidase	Personal care	Antimicrobial
	Textile	Bleaching

Enzyme class	Industry	Application
Phospholipase	Baking	Dough stability and conditioning
	Fats and oils	De-gumming, lyso-lecithin production
Phytase	Animal feed	Phytate-digestibility,phosphorous release
Protease	Detergent	Protein stain removal
	Food	Milk clotting, infant formulas, flavor
	Baking	Dough strengthing
	Pulp and paper	Biofilm removal
	Leather	Unhearing, bating
Pullulanase	Starch and fuel	Saccharification
Transglutaminase	Food	Modify visco-elastic properties
	Baking	Laminated dough strengths
Xylanase	Starch and fuel	Viscosity reduction
	Baking	Dough conditioning
	Animal feed	Digestibility
	Pulp and paper	Bleach boosting

Modified from: Kirk et al. 2002

some examples of enzyme applications in different industrial sectors.

2. INDUSTRIAL ENZYMES PRODUCTION AND THEIR TRADITIONAL MARKETS

Gross world sales for enzymes in 1977 were $150 million (Crueger & Crueger 1982). In 2000, worldwide enzyme sales amounted $1.5 billion (McCoy 2000). The predicted annual growth rate ranged from 2% in the leather industry to 15% in paper production and 25% in feed enzymes (OECD 1998). Fig 1 shows sector-wise distribution of enzymes in various industries.

2.1. General enzyme applications

The role of enzymes in many processes has been known for a long time. Their existence was associated with history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making, etc. With better knowledge and purification of enzymes the number of applications has increased many folds, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged (Haki & Rakshit 2003). Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan et al. 2001). Advances in this area have been possible with the isolation of large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them.

One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Elevated process temperatures include higher reaction rates due to a decrease in viscosity and increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reaction (Kumar & Swati 2001).

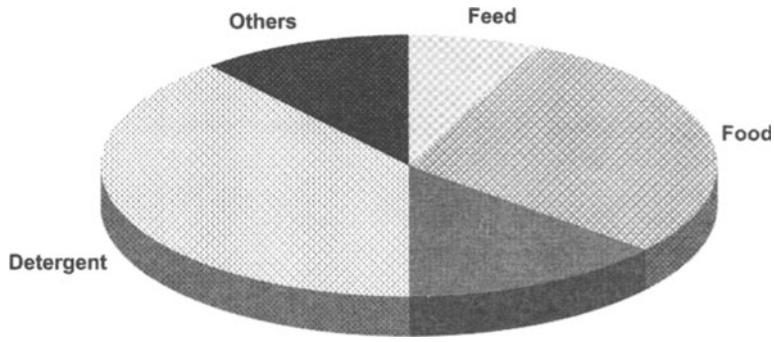

Figure 1. Segmentation of the industrial enzyme market

Many enzymes are available commercially and numerous industrial applications have been described. An overview was recently published by Pszczola (2001) about the new developments in the food sector. The food, feed, agriculture, paper, leather and textile industries are well suited for enzyme technology because products as well as raw materials consist of biomolecules, which can be produced, degraded or modified by enzymatic processes (van Beilen & Li 2002). The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and baking industries, represent the second largest group (Godfrey & West 1996). This growth, however, has stagnated in some of the major technical industries, first of all the detergent industry. The fastest growth over the past decade has seen in the baking and animal feed industries.

In the pulp and paper industries, enzymes are increasingly used for cleaner production processes. Thus, the resulting market value of biotechnology for clean production in the paper industry (US$ 31-62 million) is very significant (OECD 1998, 2001). Another example is the use of laccases for bleaching or catalase to remove hydrogen peroxide in textiles production. These enzymes cause a significant reduction in the use of raw materials and production of waste.

3 ENZYMES APPLICATIONS IN THE NON-FOOD INDUSTRY

3.1 Animal feed

The use of enzymes as feed additives is well established. Some examples of enzyme used for this purpose are xylanases, β-glucanases, phytase and cellulases, which can be used in cereal based feed for monogastric animals which, contrary to ruminants are unable to fully degrade and utilize plant-based feeds containing high amounts of cellulose and hemicellulose (Kirk et al. 2002). In cereals such as barley, arabinoxylans form the major non-starch polysaccharide. Arabinoxylans constitute 4 to 8% of the barley kernel and they represent ~25 and 70% of the cell wall polysaccharides of the endosperm and aleurone layer, respectively. The arabinoxylanases are partly water-soluble and result in a highly viscous aqueous solution. This high viscosity of cereal grain water extract might be involved in brewing problems (decrease rate of filtration or haze formation in beer) and is a negative parameter for the use of cereal grains for animal feed (Devilly et al. 2001, 2002).

Currently, phytase is considered as one of the most potent feed enzyme (Pandey et al 2001, Lei & Stahl 2000), in particular from fungal sources, which show much higher specific activities than previously

reported (Lassen et al. 2001). Plant feedstuffs are the major constituents of poultry diets. About two-thirds of phosphorus of feedstuffs of plant origin is presented as phytic acid in the form of phytate. Under most dietary conditions, phytate P is unavailable to poultry. In addition, phytate P chelates several important minerals and thereby reduces their availability. Monogastric animals such as humans, chickens and pigs produce little or no phytase in the intestine. This requirement of phosphorous is met by supplementing soy bean meal with relatively inexpensive rock phosphate, which provides the animal with this necessary nutrient. The availability of phosphorus can be improved by adding microbial phytase to the feed or by using phytase-rich cereal diet. The enzyme minimizes the need for supplementation with inorganic phosphorus due to improvement in the utilization of organic phosphorus in poultry, and thus markedly reducing the excretion of phosphorus in manure. Phytase hydrolyzes phytate, and the addition of phytase to feed (250 to 1000 U/kg) can fully replace phosphorus supplementation (Pandey et al 2001, Vohra & Satyanarayana 2003).

3.2 Paper and pulp industries

Pulp and paper mills are beginning to use enzymes to solve problems in their manufacturing processes (Reid & Ricard 2000). The public concern on the impact of pollutants from the paper and pulp industries, which use chlorine as the bleaching agent, act as strong driving force in developing biotechnology-aided techniques for novel bleaching that is biobleaching (Subramaniyan & Prema 2002). Xylanases and pectinases can be used in biobleaching. They are preferable to cellulases as they easily result in the hydrolysis of cellulose, which should be the main recovered product in paper industry. Cellulase is then used to remove fibrils from the surfaces of recycled fibers, promoting faster drainage (Pommier 1990). Lipase is used to hydrolyze triglycerides released during mechanical pulping and prevent pitch deposition problems (Hata et al. 1996). The key characteristic of these enzymes for pulp and paper applications is their ability to recognize and selectively transform specific substrates in the presence of other chemically similar materials (Ried & Ricard 2000).

The most common pulping process is the Kraft process where cooking of wood chips is carried out in a solution of Na_2S/NaOH at about 170°C for 2 h resulting in degradation and solubilization of lignin. The resulting pulp has a characteristic brown color, which is primarily due to the presence of residual lignin and lignin derivatives. To obtain pulp of very brightness and brightness stability, all lignin must be removed from the pulp. For that, chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin, which has to be removed by bleaching process. This residual lignin is difficult to remove due to its covalent binding to the hemicellulose and perhaps to cellulose fibers. Ligninases and hemicellulases (xylanases) were tested for biobleaching. The use of hemicelluloses was first demonstrated by Viikari et al (1996), which resulted in the reduction of chlorine consumption.

Two types of phenomena are involved in the enzymatic pretreatment. The major effect is due to hydrolysis of re-precipitated and re-adsorbed xylan or xylan-lignin complexes that are separated during the cooking process. As a result of the enzymatic treatment, the pulp becomes more accessible to the oxidation by bleaching chemicals (Subramaniyan & Prema 2002).

Papermaking is essentially a continuous filtration process in which a dilute suspension of fibers, fiber fragments (fines), and inorganic filler particles, such as clay or $CaCO_3$ are formed into a sheet. The need for rapid water drainage leads to use of a filter fabric with holes large enough to allow passage of the fines and filler particles. In modern papermaking, retention aids are added to the pulp to keep fines and

filler parcels in the paper sheet and to speed the drainage of water. Cationic polymers of various structures are commonly used as retention aids (Horn & Linhart 1996). Treatment with the enzyme pectinase has been reported to lower the cationic demand of thermomechanical pulp (TMP) bleached with alkaline peroxide. Dynamic drainage jar experiments showed that the enzyme treatment improves the effectiveness of several cationic polymers to increase retention of fines and filler particles (Reid & Ricard 2000).

3.3 Textiles and leather industries

The use of enzymes in textile processing dates back to middles of 19th century when malt extract was used to remove amylaceous sizes from textiles before printing. During the processing of cotton, a huge amount of water is consumed (in scouring step) industry. This step consumes high energy too. Both of these (high water and energy) are undesirable. High consumption of water leads to the proportionate generation of wastewater. Use of pectate lyase has led to the development of an eco-friendly process, which utilizes much less water and is carried out at much lower temperature (Tzanov et al. 2002). Similarly in other steps also such as de-sizing, stonewashed process, bleaching and finishing processes, enzymes have been introduced in the processing of cotton. The positive environmental impact of the new process was recognized by a grant of the United States Presidential Green Chemistry Challenge Award in 2001 (Kirk et al. 2002). Some examples of enzymes, which are used in the textile processing are presented in Table 2 (adapted from Senai 2003).

The natural sizes, which are of starch origin, are the most frequently removed by amylases, which is advantageous in the sense that they do not cause any physico-chemical damage to cellulosic materials. The enzymes are used in their natural modified state and they can destroy any type of starch turning into

Table 2. Examples of enzymes and their action in textile products

Enzymes	Action
α–amylases	Remove the starch warp sizing from fabric, leaving fabric softer and ready for the consumer
Cellulases and hemicellulases	De-gumming of CM Effects on cellulose fibers Used for the removal of surface hairs from garments for increased comfort and fashion.
Pectinases	De-gumming of vegetal fibers Used as a bioscour – an environmentally friendly alternative to caustic scouring.
Proteases	De-gumming of animal fibers Wool characteristics alteration
Lipases	Lipids removal
Peroxidases	Natural pigments oxidation
Catalases	Used to remove peroxide after bleaching to improve the dyeing process

Modified from SENAI, 2003

water-soluble products without affecting the cellulose.

Cellulases are increasingly being used in the textile industries. Their most successful application is in producing the stonewashed look of denim garments. Increased use is also being made of cellulases in domestic fabric washing products where they are claimed to aid detergency and to clean fibre surfaces, improving appearance and colour brightness (Cavaco-Paulo 1997). Now a days, these finishing and washing effects represent the largest market for cellulase enzymes worldwide. The finishing and washing effects delivered by cellulases are always applied in processes (rotating drum washers and jets) where strong mechanical action on the fabric is provided (Cavaco-Paulo 1998).

The whitening of textiles is achieved with different oxidizing or reducing agents, capable of destroying the natural pigments and matter present in the fibers. Nowadays the hydrogen peroxide, due to its biodegradability, almost entirely replaced the conventional chlorine oxidizing chemicals. Hydrogen peroxide precursors, such as perborates and percarbonates are incorporated in a major part of commercially available detergent compositions. Alternatively, hydrogen peroxide could be produced enzymatically by glucose oxidase catalyzed conversion of glucose in the presence of oxygen in aqueous solutions. However, no bleaching process using enzymatically-produced peroxide is industrially available because enzymes are still quite expensive products. Their recycling and multiple use could be ensured using the existing enzyme immobilization techniques (Tzanov et al. 2002).

Another possibility of enzyme application in textiles is the bioscouring, which is the scouring of cotton using pectinases. Effective removal of pectin and wax from raw cotton substrate with the enzyme under mild conditions provides high quality products for subsequent dyeing and finishing processes with less energy consumed under safer conditions. In addition, modification of wool by an enzyme is also a favorable process. Wool fabrics have a disadvantage to felt by rubbing under wet conditions. It is generally well known that felting of wool fabrics is caused by tangle between the cuticles at external surface of wool fiber. Moderate removal of external surface region, therefore, will prevent such shortcomings. The effectiveness of bioscouring was evaluated by measuring weight loss of cotton, analyzing pectin and cotton wax remaining and by wetness testing. Pectinase enzyme showed excellent activity even in organic media, and the effectiveness of scouring was equivalent or better than that achieved by conventional alkaline process or bioscouring in aqueous media (Sawada & Ueda 2001.)

The application of enzymes such as proteases, lipases, protein disulphide isomerase and transglutaminase on the modification of wool properties has been reported (Heine & Hocker 1995, King & Brockway 1987, Cortez et al. 2002). In particular, proteases such as subtilisins have been employed in the industrial treatment of wool goods to impart desirable properties, such as improving handle properties and imparting shrink-resistance. A variety of chemical methods to produce shrink proof wool goods are known and widely used commercially. The most common methods involve are acid chlorination of the wool goods or the application of permonosulphuric acid (PMS), followed by a polymer application. Such method achieve a significant level of shrink-resistance to wool textiles, but may affect adversely the handle properties, as well as generating damaging substances that may be released into the environment. The deficiencies in performance, cost and environmental friendliness of wool processing methods currently used in the textile industry reveal the need for a new process that imparts improvements in shrink-resistance, softness and resistance to pilling without a decrease in tensile and bursting strength. The use of the protein cross-inking enzymes transglutaminases, as biocatalysts in the processing of wool textiles

offers a variety of exciting and realistic possibilities, which include reducing the propensity of wool fabric shrink and maintaining or increasing of fabric strength (Cortez et al. 2003).

3.4 Detergents

The use of proteases as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value. Proteases and different hydrolases such amylase, cellulases and lipases are introduced to provide various benefits, such as the removal of specific stains (Kirk et al. 2002). Today, proteases have been joined by amylases and lipases in increasing the effectiveness of detergents, especially for household laundry and industrial cleaning operations.

Microbial alkaline proteases added to laundry detergents enable the release of proteinaceous material from stains. The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, nonphosphate detergents. In addition to improved washing efficiency, the use of enzymes allows lower wash temperatures and shorter periods of agitation, often after a preliminary period of soaking (Kumar & Takagi 1999). Ideally, proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels (0.4-0.8%) and should also be compatible with various detergent components along with oxidizing and sequestering agents. They must also have a shelf life (Ward 1985). Proteases are also used in dishwashing detergents. The in-place cleaning of ultrafiltration (UF) and reverse osmosis (RO) membranes forms one of the most important aspects of modern dairy and food industries. The UF and RO membranes are put to a variety of uses, including concentration, fractionation, clarification and/or sterilization of liquid foods such as milk, whey, egg white, fruit juices, wines and other beverages (Glover 1985, Cheryan 1986). Another possibility is the use of a cocktail of proteases and lipases to degrade and solubilize protein and fat foulants have also proven beneficial.

Amylases are finding great application in laundry and dishwashing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergent with α-amylases optimally working at moderate temperatures and alkaline pH can help solving this problem (van der Maarel et al. 2002).

Cellulases contribute to cleaning and to overall fabric care by rejuvenating or maintaining the appearance of washed garments (Novozymes 2003).

3.5 Personal care

Some brands of toothpaste and mouthwash already incorporate glucoamylases and glucose oxidase. These enzymes have a positive effect in preventing plaque even though people normally brush their teeth for only 2-5 minutes. In the field of contact lens cleaning, proteases and lipases can be used. The residual hydrogen peroxide used for disinfections is neutralized by catalase before the lens is placed back in the eye (Novozymes 2003). Lipases are also used in personal care products (Benjamin & Pandey 1998, Pandey et al 1999a).

3.6 Energy (Fuel alcohol)

The use of enzymes for the production of fermentable sugars from starch is also well established. In the

last ten years there has been an increasing interest in fuel alcohol as a result of increased environmental concern, higher crude oil prices. These days intense efforts are currently being undertaken to develop improved enzymes that can enable the utilization of cheaper and partially utilized substrates such as lignocellulose to make bio-ethanol more competitive with fossil fuels (Zaldivar et al. 2001 and Wheals et al. 1999 cited by Kirk et al. 2002). Current work focuses both on the development of enzymes with increased activity and stability as well as on their efficient production. In the United States, huge governmental programs have been started by the department of energy to support these developments in order to reduce pollution and the need to work towards fulfilling the Kyoto protocol (Kirk et al. 2002).

3.7 Industrial waste treatment

Traditionally, waste treatment processes have been evaluated in terms of reducing gross indicators of pollution, such as biochemical oxygen demand (BOD), chemical oxygen demand (COD) or total organic carbon (TOC). Today, there is an increased emphasis on the removal of specific pollutants from waste mixtures and on the multimedia (air, land and water) effects of treatment processes. The use of enzymes in waste treatment applications was first proposed in the 1930's. However, the concept of using enzymes to destroy individual pollutants in waste mixture was not developed until the 1970's (Aitken 1993). Enzymes catalyze chemical reactions that would otherwise be too slow at ambient temperatures to be interest and, therefore, can achieve efficiently with conventional chemical treatment processes. More importantly, enzymes are highly utilizing any required chemical reactants with very high stoichiometric efficiency. Therefore, enzymes have the potential to combine the advantages of selectivity with the simplicity, reliability and predictability of conventional chemical treatment systems (Aitken & Irvine 1987).

Development of any commercial application of enzymes requires more than a demonstration of technical feasibility. Cost is obviously a major consideration and some enzymes have properties that would preclude their development based on potential cost alone. Several enzymes have been studied specifically for their potential application in waste treatment processes *in vitro*. A review on these enzymes has been presented by Aitken (1993). The most studied enzymes in terms of work directed specifically at waste treatment applications are the organophosphate pesticide hydrolases and phenol oxidizing enzymes (laccase, peroxidases and polyphenol oxidase).

3.8 Therapeutic enzymes

The manufacture or processing of enzymes for use as drugs is an important facet of today's pharmaceutical industry (Cassileth, 1998). Attempts to capitalize on the advantages of enzymes as drugs are now being made at virtually every pharmaceutical research center in the world. Therapeutic enzymes have a broad variety of specific uses: as oncolytics, as anticoagulants or thrombolytics, and as replacements for metabolic deficiencies. Additionally, there is a growing group of miscellaneous enzymes of diverse function. At present, the most successful applications are extracellular: purely topical uses, the removal of toxic substances and the treatment of life-threatening disorders within the blood circulation.

Among different industrial enzymes, collaginase, protease, deoxyribonuclease, lysozyme, hyaluronidase and superoxide dismutase have considerable therapeutic applications. In contrast to the industrial use of enzymes, therapeutically useful enzymes are required in relatively tiny amounts but at a very high degree of purity and (generally) specificity.

4. ENZYME APPLICATIONS IN THE FOOD INDUSTRY

The use of enzymes in food preservation and processing predates modern civilization. Fermentation of substrates such as fruits, vegetables, meat and milk provide a diverse array of food in the human diet. Beer, wine, pickles, sausage, salami, yogurt, cheese and buttermilk are fermented products. These fermented foods are result of the enzymatic modification of constituents in the substrate. The use of enzymes in the food industry also involves a wide range of effects including the production of food quality attributes such as flavors and fragrances, and control of color, texture and appearance. Some examples of enzymes used in the food industry are amylases, anthocynases, cellobiases, glucoamylases, invertases, lactases, lipases, pectinases, proteases, inulinases and pullulanases (Menon & Rao 1999, Benjamin & Pandey 1998, Pandey et al. 1999a, b).

4.1 Sweetener production

The most widely used thermostable enzymes are the amylases in the starch industry. Other applications are in various stages of development. The starch industry began using enzymes at an early date. Special types of syrup that could not be produced by conventional chemical hydrolysis were the first compounds made entirely by enzymatic processes. The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this raw material. The starch polymer, like other such polymers requires a combination of enzymes for its complete hydrolysis. These include alpha-amylsases, glucoamylases or beta-amylases and isoamylases or pullulanases (Pandey 1995, Pandey et al 2000a, b).

The enzymatic conversion includes gelatinization, which involves the dissolution of starch granules thereby forming a viscous suspension liquefaction, which involves partial hydrolysis and loss in viscosity and saccharification, involving the production of glucose and maltose via further hydrolysis. Gelatinization is achieved by heating starch with water, and starch is water-soluble only at high temperatures, which are dependent on the source (Rakshit 1998). One of the concerns of the starch industry is the calcium requirement of the alpha-amylase enzyme and the formation of calcium oxalate, a substrate that may block process pipes and heat exchangers. Besides, such accumulation in some products like beer is not acceptable.

4.2 Baking products

For decades, enzymes such malt and alpha-amylases have been used in bread making. Rapid advances in biotechnology have made a number of exciting enzymes available for bread making. Some examples of this are proteases, glucose-oxidases, lipases, lipoxygenases, xylanase and hemicellulases. Table 3 shows how these enzymes act.

The main component of wheat flour is starch. Amylases can degrade starch and produce small dextrins for the yeast to act upon. There is also a special type of amylase, alpha amylase, which modifies starch during baking to give a significant anti-staling effect. Staling is associated with a loss of freshness in terms of increased crumb firmness and decreased crumb elasticity. Bread staling is responsible for significant financial loss for both consumers and bread producers. The alpha-amylase used in bakery modifies the starch at the temperature where most of the starch starts to gelitinize. The resulting modified starch granules remain more flexible during storage. Bread produced with this enzyme has a softer and more elastic crumb than bread produced with distilled monoglycerides as emulsifiers.

Gluten is a combination of proteins, which forms a large network during dough formation. This network

Table 3. Typical uses of enymes in baking

Enzyme	Source	Effect
Amylase	*Aspergillus oryzae* *Aspergillus niger*	Maximizes the fermentation processes to obtain an even crumb structure and a high loaf volume Provides a concentrated and controlled substitute for additives such as malted barley
Maltogenic-alpha-amylase	*Bacillus stearothermophilus*	Improves shelf life
Glucose oxidase	*Aspergillus niger* *Penicillium*	Removes glucose or oxygen from food stuffs to improve their storage capacity
Lipase	*Aspergillus niger*	Dough conditioning by producing more uniform, smaller crumb cells and a silkier texture and whiter crumb color
Xylanase	*Aspergillus niger*	Dough conditioning. Easier dough handling and improved crumb structure
Protease	*Aspergillus oryzae* *Bacillus subtilis*	Weakens the gluten to provide the plastic properties required in doughs for biscuits

Modified from : Novozymes, 2003

holds the gas in during dough proof and baking. The strength of this gluten network is very important for the quality of all bread raised using yeasts. Enzymes such as hemicellulases, xylanases, lipases and oxidases can directly or indirectly improve the strength of the gluten network and so improve the quality of finished bread (Novozymes 2003).

Addition of proteases to doughs improves their handling properties and increases loaf volume, thereby, producing more symmetrical loaf with good crumb and curst texture. Normally, proteases are present in low levels in wheat but are increased by malting. Wheat gluten is not susceptible to attack of flour proteases, thus necessitating supplementation of protease. However, excess of protease produces sticky loaf, hence the enzymes treatment has to be controlled. Fungal proteases from *Aspergillus oryzae* are normally used in bread production due to their lower temperature stability and amylase content, which also contributes to the baking process. Both fungal and bacterial proteases have application in the manufacture of cracker and biscuit dough. Bacterial neutral proteases improve the extensibility of cracker dough allowing it to be rolled very thinly without tearing and reducing bubbling during baking. This enzyme is also used in high protein doughs for cookie, pizza and biscuit production (Menon & Rao 1999).

4.3 Dairy products

Since the origin of cheese production, the manufacturing process has been adapted to properties of calf rennet. Rennet, also called rennin, is a mixture of chymosin and pepsin. The enzyme coagulates milk quickly at neutral pH value with little further degradation of the milk proteins. Unfortunately, the enzyme can only be extracted from the fourth stomach of the unweaned calf, which is not

viable economically. Microbial enzymes have therefore been introduced as alternatives to calf rennet (Menon & Rao 1999). The properties of microbial enzymes are similar to those of calf rennet but with differences. Microbial rennets are produced by submerged fermentation of selected strains of fungi such as *Rhizomucor miehei*, and have properties similar to those of chymosin. The ratio between milk coagulating activity and proteolytic activity is lower for microbial enzymes than for pure calf rennet. In practice, only slight modifications must be made to the cheese-making process, as a result of using these types of enzymes (Novozymes 2003).

Proteases are also used for accelerating cheese ripening, for modifying the functional properties of cheese and for modifying milk proteins to reduce the allergic properties of dairy products (proteins). Other components of milk are also allergens such as lactose. Cow's milk contains 5% of lactose. The enzyme lactase (beta-galactosidase) is used to hydrolyse lactose in order to increase digestibility or to improve solubility or sweetness of various dairy products.

4.5 Enzyme-modified cheese

The main alternatives to use of the natural cheese in processed consumer foods requiring cheese flavor are high-intensity cheese flavor concentrates, such as enzyme-modified cheese (EMCs), cheese powders and cheese flavours (Missel 1996). This subject was recently reviewed by Kilcawley et al. (1998). Cheese has been treated enzymatically to enhance the flavor or significant portion of the flavor profile of that cheese is considered to be an EMC and it provides the food manufacturer with a strong cheese note in a form that is cost effective, nutritious and natural (Moskowitz & Noelck 1987).

EMCs are used in food recipes to fulfill several roles, e.g. as the sole source of cheese flavour in a product, to intensify an existing cheesey taste or to give a specific cheese character to a more bland-tasting cheese product (Anon 1996). They have approximately 15-30 times the flavor intensity of natural cheese and are available as pastes or spray-dried powders (Freund 1995). The production of EMCs is an important industrial activity, which has been increasing use to a greater demand for convenience foods together with the health-related concerns regarding the amounts of fat, cholesterol and cholesterol-producing saturated fat in traditional dairy products which has resulted in the inclusion of EMCs in no-fat and low-fat products replacing the functional and flavoring characteristics of fats previously derived from natural cheese (Anon 1993, Freund 1995). The addition of intense cheese flavors creates the desired flavor without an increase in fat content, as some can be added at levels of 0.1% (w/w) and contribute less than 0.07% fat 2.28 calories) per 100 g (Buhler 1996). Most new applications are targeted at texture and old and provide rich mellow tones, pleasant flavor-enhancing effects, fatty mouth-feel, flavor masking, rounding-off of sharp spicy notes and harmonization of other ingredient flavors (Buhler 1996).

EMCs are ideal in frozen cheese type as the proteins from a natural cheese tend to coagulate an produce a grainy texture; since the proteins in EMCs have been hydrolysed to more soluble peptides and amino acids, these problems are overcome (Missel 1996). EMC flavours available include Cheddar, Mozzarela, Romano, Provolone, Feta, Parmesan, Blue, Gouda, Swiss, Emmental, Gruyere, Colby and Brick. These cheese flavours have a wide range of applications in salad dressings, dips, soups, sauces, snacks, crisps, pasta products, cheese analogues, frozen foods, microwave meals, ready-made meals, canned foods, crackers, cake mixes, biscuits, quiches, gratins, cheese spreads, low-fat and no-fat cheese products and cheese substitutes (Buhler 1996).

The basis of EMC technology is the use of specific enzymes acting at optimum conditions to produce typical cheese flavours from suitable substrates. These enzymes consist of proteinases, peptidases, lipases, esterases. Lipases are used in cheese ripening. They are used in blue and Italian cheeses to develop their piquancy, which is primarily due to short-chain fatty acids.

5 CONCLUSIONS AND PERSPECTIVES

Although enzymes in general have favorable turnover number or biocatalyst capacity, most enzymes are molecules with only one active site, hence the productivity per unit of catalyst mass is sometimes low. Besides, the typical enzymes are not particularly stable. Moreover, significant technological sophistication is usually required to produce industrial scale quantities of biological catalysts at reasonable cost. These are some barriers that must be overcome. New technologies for enzyme discovery are changing the rules of the game for industrial biocatalysts. Different enzymes are available with higher thermostability, for instance, and their costs are coming down. This fact shows the great evolution capacity of these biocatalysts.

Acknowledgements

SB acknowledges the grant of a Junior Research Fellowship to her by CSIR, New Delhi

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Immobilisation of Enzymes

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1. INTRODUCTION

Enzymes are attractive tools in biotechnology for example for the production of complex organic compounds, in chemical analysis and medical diagnostics for the quantitative determination of specific compounds in complex matrices, but also in bioelectrochemistry as components of biofuel cells. In all application areas the use of immobilised enzymes is preferred over the use of enzyme solutions, as this allows multiple uses and easy handling of the enzyme. The results are bioreactors with immobilised enzymes and enzyme sensor systems, respectively.

According to these application areas, enzymes are immobilised on polymeric, organic or on inorganic carriers, which are beads, membranes, capillaries or surfaces of transducers in sensor systems (Mulchandani and Rogers 1998, Sleytr et al. 1993, Barker 1987, Hartmeier 1986). Beads are suspended in bioreactor liquids, entrapped in small flow-through columns as part of automated analytical systems (Weigel 1996) or embedded in a polymeric matrix on top of a transducer surface. Membrane reactors are described for enzyme catalysed synthesis (Délécouls-Servat et al. 2002), but the major application area are enzyme electrodes with the membrane fixed on top of the transducer surface. The immobilisation of enzymes gets increasing importance also for miniaturised analytical systems, in which all components required for automated biochemical analysis are integrated on a chip. At present, the favourite material for chip fabrication, and thus the carrier for enzymes in microsystems, is glass, however, also gels and beads are entrapped within capillaries. Integration of enzyme reaction and signal transduction is achieved by enzyme sensors with the enzyme immobilised directly on the transducer surface. The most important materials are carbon, graphite, platinum and gold.

Immobilisation of enzymes can be achieved by different methods ranging from physical adsorption on a surface and entrapment in or behind polymers to chemical cross-linking of proteins and chemical binding to surfaces (Mulchandani and Rogers 1998, Sleytr et al. 1993, Barker 1987, Hartmeier 1986). The choice of the immobilisation method and the particular immobilisation protocol influence the total amount of immobilised protein, the amount of protein in intimate contact with the solid support, the flexibility of the composition of the immobilisation matrix and the thickness of the protein layer and of layers on top of the enzyme. These properties influence characteristics of the enzyme preparation: The reaction rate of the enzymatic reaction increases with the amount of enzyme so that a high enzyme loading is desirable with respect to high analytical signals or a rapid turnover. However, an increasing load with enzyme increases the thickness of the enzyme layer, if the amount exceeds the amount

required for monolayer coverage. This may reduce the transportation rate of the enzyme substrate and the amount of accessible and used enzyme. Moreover, in enzyme sensor systems, in which the detector is covered by a too thick enzyme layer, the product of the reaction may diffuse out into the solution instead of reaching the detector. Thus, at low enzyme loadings the analytical signal or the enzymatic turnover is limited by the rate of the enzyme reaction (kinetic control), whereas at high enzyme loadings the transportation rates of the substrate and / or product are limiting (diffusion control) (Pfeiffer et al. 1992, Scheller et al. 1989). For a number of applications diffusion control of the turnover is desirable, because these preparations proved to be more stable due to the excess of enzyme. On the other hand, diffusion barriers prevent a rapid response of the enzyme, which is to be achieved at least in enzyme sensors.

Thus, the immobilisation method has to be chosen considering requirements of the desired application, and a protocol has to be developed, which takes specific properties of the target enzyme into account, such as the stability of the enzyme towards UV-light or chemical modifications.

2. PROPERTIES OF ENZYMES INFLUENCED BY IMMOBILISATION

Depending on the chosen immobilisation procedure, kinetic or thermodynamic parameters of the enzyme reaction are influenced:

i) Most immobilisation methods result in a statistical orientation of the enzyme with respect to the carrier surface. Thus, the active centre of some enzyme molecules may be sterically blocked by the solid surface so that they are not accessible for the enzyme substrates and the amount of active enzyme is reduced. This phenomenon is independent on the immobilisation method.

ii) If immobilisation is based on physical forces, the structure of the enzyme usually remains unchanged, so that the activity of an enzyme molecule per se is not affected (besides the above mentioned steric effects). However, if chemical bonds between the protein, substrate surface, component of a surrounding polymer or other proteins are involved, these may distort the enzyme structure and thus affect the enzyme activity (Gerard et al. 2002).

iii) The entrapment of the enzyme within a dense polymer can enhance its stability during storage and use by stabilizing the conformation of the proteins. Unfolding as the first step of denaturation of an enzyme is hindered and the following irreversible reactions to complete inactivation like aggregation, dissociation of prosthetic groups or peptide bond hydrolysis cannot occur (Gupta 1991). Optimisation of the polymer composition by addition of different components can lead to further enhancement of the stability. This will be discussed in more detail in the corresponding chapters.

iv) Immobilisation can alter not only stability but also the pH optimum, temperature optimum, energy of activation, linear range, and K_m-value (Sung & Bae 2003, Andreescu et al. 2002, Pundir et al. 1999, Fágáin 1997), because the local environment of the immobilised enzyme is different from the environment of the enzyme in solution. This is due to the chemical composition of the immobilisation matrix and the reduced exchange rates for components of the catalysed reaction.

A number of enzyme reactions lead to the formation of acids and, consequently, to a reduced pH. In a buffered aqueous enzyme solution this is readily compensated, whereas in an immobilisation matrix

maintenance of a constant pH is influenced by accessible base groups of the immobilisation matrix and acid / base exchange to the liquid. Thus, the local pH of the enzyme may be lower than the pH of the surrounding solution shifting the pH-profile of the enzyme activity to higher pH-values (Cho et al. 1999). On the other hand, denaturation may be prevented due to the entrapment leading to broader applicable pH - and temperature ranges. Due to restricted diffusion also the substrate concentration may be lower at the site of the immobilised enzyme leading to higher apparent K_m-values.

3. PRINCIPLES AND EXAMPLES

3.1 Adsorption

3.1.1. Adsorption on planar surfaces

The simplest immobilisation method is the adsorption of the enzyme on the surface of the carrier. Suitable materials are graphite, gold or synthetic polymers and binding occurs through van der Waals and / or ionic forces (Andrade 1985) (Fig. 1). Binding is achieved by just incubating the carrier with the enzyme solution for a sufficient period of time. Gold surfaces are usually carefully cleaned, for example by immersion in Piranha solution (Ferapontova & Gorton 2002), and, as graphite electrodes, polished

Fig. 1. Enzyme layer resulting from adsorption of enzyme on a surface and binding due to van der Waals or ionic forces. Covalent coupling results also in a monolayer-coverage, if cross-linking of enzyme molecules is avoided

with fine emery paper (Haghighi et al. 2003) prior to the application of the enzyme solution. Because of the mild procedure conformational changes of the proteins are negligible and the activity is conserved. Moreover, as there is intimate contact between enzyme and electrode a direct electrical communication between the enzyme and a conducting support (graphite or gold) may occur, as observed for example for peroxidases (Ferapontova & Gorton 2002) or laccase (Haghighi et al. 2003, Tarasevich et al. 2003). Tachikawa et al. (1994) adsorbed alcohol dehydrogenase on polymetallophtalocyanine (PmePc) films, which were created on glassy carbon electrodes. The conducting polymers facilitated amperometric measurements due to their electrocatalytic behaviour for redox reactions of the cofactor NAD/NADH. Gavalas et al. (1998) adsorbed horseradish peroxidase and glucose oxidase on the inner surface of porous carbon rods. The enzymes were precomplexed with diethylaminoethyl-dextran (DEAE-dextran) leading to an increased operational stability of such sensors. Miao et al. (1999) adsorbed glucose oxidase in polypyrrole (PPy) nanotubules by polarizing the electrode surface covered with PPy. L-Lactate dehydrogenase genetically modified with a poly(histidine) tag could be adsorbed on poly(aniline)-poly(vinyl sulfonate) films without the addition of Ni^{2+}-ions, probably because the histidine tag provided sufficient positive charges to allow ionic binding to the negative sulfonate groups (Halliwell et al. 2002). The increased enzyme loading compensated the reduced specific activity of the mutant enzyme compared to the wild type enzyme.

Ionic interactions between enzymes and polymers were used for immobilisation by Mizutani et al. (1998). Solutions of poly(4-styrenesulfonate), enzymes and poly-L-lysine were placed step-by-step onto a glassy carbon electrode leading to entrapment of enzyme in the network formed by the oppositely

charged polymers. Lactate oxidase and alcohol oxidase sensors were manufactured showing reduced influence of interfering substances. The redox polymer poly((vinylpyridine)Os(bpy)$_2$Cl), introduced by Gregg and Heller (1991) for wiring the active centre of enzymes to electrodes, also allows binding of the enzyme by mainly electrostatic interactions (Heller 1990), as the polymer is cationic, whereas most of the relevant enzymes are anionic at their optimal pH. In the original work the redox polymer was adsorbed on clean graphite surfaces. Narváez and co-workers constructed enzyme sensors with fructose dehydrogenase, horseradish peroxidase and a combination of horseradish peroxidase and alcohol oxidase (Narváez et al. 2000). They used the strength of gold-sulfur-bonds (Au-S-bonds) to obtain negatively charged Au-surfaces from the adsorption of 3-mercapto-1-propane sulfonic acid. By electrostatic interactions first the Os-redox polymer was deposited followed by the negatively charged enzyme (modified horseradish peroxidase) or by the anionic polymer poly (styrene sulfonic acid) (PSS) and the positively charged enzyme (fructose dehydrogenase or native horseradish peroxidase). Though the amount of immobilised enzyme increased with the PSS-layer due to stronger electrostatic interactions the electronic coupling to the electrode decreased due to the increased distance between enzyme and electrode. Due to the specific binding of thiol-compounds to gold surfaces a similar approach could be used for the fabrication of gold microenzymeelectrodes (Revzin et al. 2002). Negatively charged electrode surfaces were obtained by self-assembling of 11-mercaptoundecanoic acid, on which an allylamine-copolymer of the Os-redox polymer was deposited followed by anionic enzymes, such as glucose oxidase, lactate oxidase or pyruvate oxidase.

However, binding to the support is based only on physical forces and the enzymes might desorb due to changes of the composition of the solvent. Moreover enzymes can be washed out during application in fresh, enzyme-free solutions due to the balance of adsorbed and desorbed proteins.

3.1.2 Carbon paste and carbon composite electrodes

As the amount of enzyme adsorbed on a planar surface is limited, desorption results in a continuous decrease of enzymatic turnover and of the sensitivity of an enzyme sensor, respectively (Xue & Shen 2002). To increase the amount of adsorbed protein, the surface area has to be enlarged, e.g. by using graphite particles or carbon powder instead of graphite rods. The particles are incubated with the enzyme solution and dried to obtain an enzyme-loaded powder, which is mixed with oil to result in a carbon paste, which is pressed into a suitable holder and electrically contacted by an inserted wire. The oil was replaced by other binding materials, such as polyvinylpyrrolidone (Bilitewski et al. 1992), hydroxyethylcellulose (Kulys & D´Costa, 1991), Teflon (Guzmán-Vázquez de Prada et al. 2003) or wax (Wang & Naser 1995), resulting in screen-printable pastes (Bilitewski et al. 1992) or more rigid composite electrodes. The advantage of this type of enzyme electrode is the modification of the bulk of the electrode with enzyme (see Fig. 2), leading to an increase in the amount of immobilised enzyme and the possibility to generate a fresh surface just by removal of the upper layer of the electrode. The proper choice of the ratio of enzyme to graphite powder in solution gives particles with a difined enzyme loading. The time-dependent measurements of the enzymatic activity of the supernatant in a mixture of enzyme and graphite powder showed that the adsorption was a relatively fast process (Fig. 3).

This basic principle can be modified regarding different aspects: The graphite particles can be incubated with a mixture of enzymes, thus developing bienzymatic systems. Electrodes based on the respective oxidase and horseradish peroxidase were prepared for a number of compounds, such as alcohol and

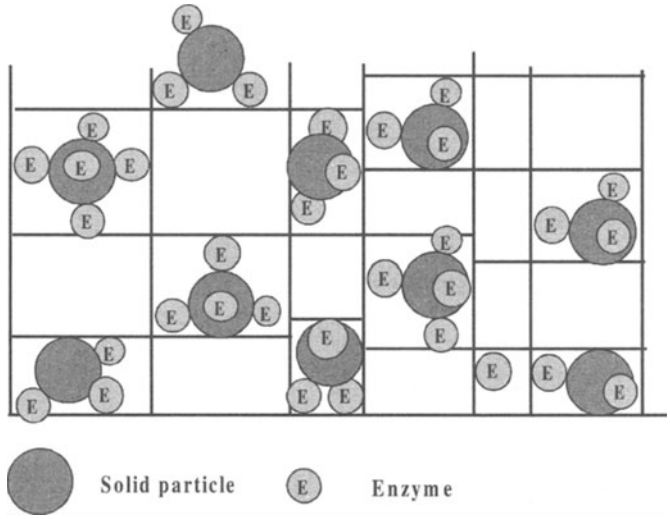


Fig. 2. Enzymes were adsorbed on particles, such as graphite or carbon powder, and embedded in a matrix. The matrix can be oil, Teflon, wax, a sol-gel or an organic polymer resulting in carbon paste or carbon composite electrodes, or carbon-inks suitable for screen-printing

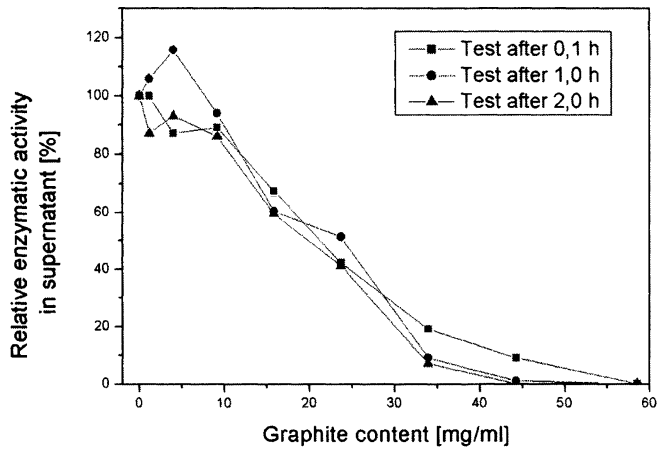


Fig. 3. Adsorption of horseradish peroxidase on graphite particles in solution. The time-dependent measurements of the enzymatic activity in the supernatant indicates the adsorption capacity of the particles whereas the enzymatic activity itself remains constant

amino acids (Gorton et al. 1996). Charged polymers, such as polyethylenimine (PEI) or Gafquat (a copolymer of vinylpyrrolidone and dimethylamino-ethyl-methacrylate) were used as further additives to improve binding efficiency, direct electron transfer, and better operational stability of the sensor. Moreover, the electrode material can be modified by the addition of organic mediators (Wang et al. 1999, Wring & Hart 1992, Bilitewski et al. 1992) or metallic particles, such as finely divided platinum (Bilitewski et al.

1993), rhodium (Wang et al. 1997), ruthenium (Wang et al. 1994), iridium (Wang et al. 1996) or copper, to transfer electrons from the enzyme to the electrode or to catalyse the electrochemical reaction (Miscoria et al. 2002, Patel et al. 2003, Pandey et al. 2003). Leakage of the mediator into the medium is prevented or at least reduced with mediators immobilised or entrapped in beads, as described by the use of vinylferrocene, which was integrated in a polymer (Patel et al. 2003), the entrapment of ferrocene in organic modified silica beads (ormosils) (Pandey et al. 2003) or the use of polymeric mediators, such as the redox polymer poly((vinylpyridine)Os(bpy)$_2$Cl) (Pravda et al. 1995a). The composition of the paste, i.e. the type and degree of modification of the carbon particles, the binder and type and amount of further additives, strongly influences selectivity, stability and sensitivity of the resulting electrodes.

3.1.3 Adsorption on membranes

Due to their 3-dimensional structure and at least partly hydrophobic surface enzymes can be immobilised by adsorption also on membranes. Suitable membrane materials are nitrocellulose (Park et al. 2003, Cui et al., 2001), polyethersulfone (Boshoff et al. 1998), nylon 6,6 (e.g. Biodyne and Immunodyne membranes) (Xie et al. 1990, Mazzei et al. 1992), polyvinylidenefluoride (PVDF) (e.g. Immobilon membranes) (Mazzei et al. 1992) or chitosan (Edwards et al. 1999) of which the nylon membranes are available with carboxyl and amino groups on the surface so that binding is based also on electrostatic interactions. The advantage of the method is its simplicity, as again only incubation of enzyme and membrane material is required. Moreover, membranes can be used in a number of formats, allowing their versatile application. Thus, nitrocellulose membranes were combined with screen-printed electrodes (Cui et al. 2001), but also with microfluidic systems made from polydimethylsiloxane (Park et al. 2003), whereas the polyethersulfone membrane and the chitosan membrane were part of bioreactor systems for the removal of polyphenols (Edwards et al. 1999; Boshoff et al. 1998).

3.2. Entrapment behind membranes

The stability of systems based on adsorbed enzymes can be improved by additional membranes with pore sizes sufficiently small to prevent the dissolution of the enzyme. The first step in the immobilisation protocol is the incubation of the carrier in the enzyme solution, followed by coverage with a membrane. Thus the 3-dimensional structure of the enzyme is not affected and intimate contact between electrodes and enzymes are maintained. Kinetic parameters of the enzyme reaction are only changed, if transportation rates of the enzyme substrate become limiting, which is influenced by the pore size of the membrane. Thus, by choice of the membrane the linear range and sensitivity of the sensor is influenced, as smaller pore sizes, i.e. increased diffusion limitation, leads to increased linear ranges and reduced sensitivities (Bilitewski et al. 1993). The membranes used can be polycarbonate (Bilitewski et al. 1993) or dialysis membranes (Ferapontova et al. 2003; Tkac et al. 2002; Schuhmann et al. 1992) or membranes, which are formed directly on the surface of the carrier, such as a sol-gel/chitosan-membrane (Wang et al. 2003), or membranes electropolymerised from phenolic compounds (Craig & O´Neill 2003, Valdés García et al. 1998) or pyrrole (Mailley et al. 2003). The pyrrole monomer was mixed within the paste so that the polypyrrole membrane not only covered the electrode surface, but also was part of the carbon paste resulting in additional diffusion barriers and preventing loss of enzyme even after 72 h of immersion in solution. Moreover, response times lower than 20 s were obtained.

Physical retention of lactate dehydrogenase onto an amperometric graphite electrode through a dialysis membrane showed a clear preservation of the catalytic activity (Schuhmann et al. 1992). When the

small cofactor NAD was coupled with polymers like PEG the complexes were enlarged, so that even the cofactor could be entrapped (Kulys et al. 1991).

In another approach a cavity is utilised as a reservoir for different enzyme solutions acting as an enzyme reactor with plunged electrodes. The sample solution is pumped through a dialysis tube guided through this reservoir and low molecular weight analytes are able to pass this membrane. The enzymes are not immobilised but free in solution under retention in the cavity (Böhm et al. 2001).

The Yellow Springs Company (YSI Inc., Yellow Springs, Ohio, USA) offers combined membranes in sandwich construction usable in bioprocess and food analyzers. The first polycarbonate layer limits substrate diffusion. The next layer consists of the enzyme followed by a cellulose acetate membrane, which excludes electrochemical interferents from the electrode surface. The combination of alcohol oxidase or glutamate oxidase with diethylaminoethyl-dextran and lactitol, a sugar alcohol, increased the shelf life of the enzyme membrane significantly and allowed dry storage for more than one month even at 37°C (Gibson et al. 1992).

3.3. Entrapment in matrices

Due to the strong adsorptive capacity of graphite or carbon materials, these are preferentially used as carriers for adsorbed enzymes. Thus, though the procedure of enzyme immobilisation via adsorption is rather simple, the preferential choice of graphite together with the limited stability of enzyme preparations limits the wide-spread application of this method. The simplicity of the method together with a wide range of possible modifications are maintained when enzymes are immobilised in matrices, which are formed as network in the presence of the enzyme. The enzyme and required additives are mixed with precursors, which form a network by polymerisation or gelation entrapping the protein. Hence, the enzyme is immobilised mainly due to the presence of diffusion barriers created by the matrix (scheme according to Fig. 4). However, depending on the precursors and the principle of polymerisation the enzyme may be damaged so that a reduced activity is observed in addition to steric effects. Networks with entrapped enzymes are formed either from inorganic or organic precursors and adhere without any further aid to a carrier or are fixed to a support by suitable holders.

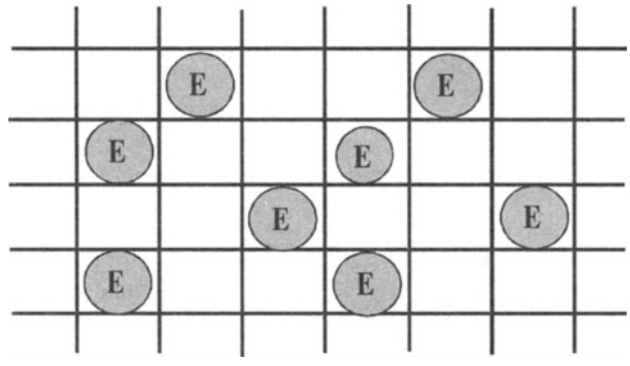


Fig. 4. Enzyme entrapped in a polymeric network, which is formed either from a polymer solution or in the presence of the enzyme by polymerisation of precursors (monomers or oligomers)

3.3.1 Entrapment in inorganic hydrogels

Enzymes are immobilised in hydrogels created by polycondensation of silicates (Dave et al. 1996). They are mixed into the precursor solution and entrapped in the matrix formed by acid silicate hydrolysis. Widely used are TMOS (tetramethyl orthosilicate) and TEOS (tetraethyl orthosilicate) as precursors. Alternatively, alkoxysilanes with different organic residues, such as phenyl- or methyl trimethoxysilane, are used, from which methanol or ethanol is produced during hydrolysis (Xu et al. 2000a, b; Lev et al., 1995; Tsionsky et al. 1994). Recently, enzyme electrodes utilizing sol-gels based on other metal oxides were described, such as vanadium pentoxide (Glezer & Lev 1993), ZrO_2 (Liu et al. 2003) and titanium oxide (Chen & Dong 2003). Problems associated with pure inorganic sol-gels are the brittleness, the harsh conditions during gel formation (use of acids and production of alcohols) and (depending on the precursors) the hydrophobic nature of the material. Organic additives contribute to the formation of defect-free membranes and make them less brittle. Thus, polymers, such as Nafion (Kim & Lee 2003), chitosan (Chen et al. 2003) or poly(vinyl alcohol) (Chen & Dong 2003) were included in the gelation mixture. Damage of enzyme was reduced by preparation of sol-gel stock solutions to which the enzyme was added (Kim & Lee 2003, Kim et al. 2001). Further modifications of the properties of sol-gels were achieved by carbon composite electrodes comprising a dispersion of graphite powder, enzyme and sol-gel (Gun & Lev 1996; Tsionsky et al. 1994).

Sol-gels can be formed on metal or graphite working electrodes as part of an enzyme electrode. Due to the transparency of sol-gel materials also optical detection is possible. The activity of alcohol dehydrogenase was observed by the fluorimetric detection of the reduced cofactor NADH which is soluble in the hydrogel (Williams & Hupp 1998). As sol-gels have no pre-determined form they were also used for enzyme immobilisation in microstructured networks (Kim et al. 2001).

Catalytic activities of enzymes immobilised in sol-gels differed from those of the soluble form. For immobilised horseradish peroxidase the pH activity profile was shifted to extreme pH values at which the enzyme in solution was almost inactive (Cho et al. 1999). Studies on the stability of different flavoprotein oxidases showed that immobilised glucose oxidase retained almost 60% of its activity even after heating to 63°C for 20 h, whereas the half-life of the dissolved enzyme was only 6.5 min. (Chen et al. 1998). Lactate oxidase and glycolate oxidase, however, lost almost completely their activity after being heated to 63°C for 20 – 40 min.. This could be improved by the addition of cationic additives, such as polyethylenimine (PEI) or poly(1-vinylimidazole) (PVI) leading to an extension of the half-life compared to the free enzyme. The stabilising effect was explained on the basis of the protein structures (Heller & Heller 1998). Glucose oxidase contains positively and negatively charged amino acid residues at the entrance of the active site, whereas lactate oxidase and glycolate oxidase carry highly conserved positive arginines the orientation of which may be disturbed by the interaction with polysilicate anions. Precomplexing with polycations like PVI or PEI can create a shield which preserves the active site from the influence of silicates.

3.3.2 Entrapment in polymers

Enzymes can be entrapped in polymers forming thin films or membranes when spread as solution on planar surfaces and allowed to dry. Usually these films are sandwiched between cellulose, cellulose acetate, polycarbonate, polyester or polyethylene membranes (Woodward 1998, White et al. 1994, Pfeiffer et al. 1992) and placed in membrane holders which are connected to the working electrodes of enzyme analysers. However, the polymer layer adhered directly on the surface of graphite rods (White et al. 1994), modified glassy carbon electrodes (Karyakin et al. 1996) and screen-printed platinum electrodes (Olschewski et al. 2000). Examples for suitable polymers are hydroxyethylcellulose (Hart & Collier 1998,

Hart et al. 1996, White et al. 1994), gelatin (Scheller et al. 1989, Lisdat et al. 1997), polyvinylalcohol (Szeponik et al. 1997, Lisdat et al. 1997), polyurethane (Szeponik et al. 1997, Pfeiffer et al. 1992, Scheller et al. 1989), poly(carbamoyl)sulfonate (PCS) (Olschewski et al. 2000, Kwong et al. 2000, Schneider et al. 1996), Nafion (Karyakin et al. 1996) and regenerated silk fibroin (Liu et al. 1997).

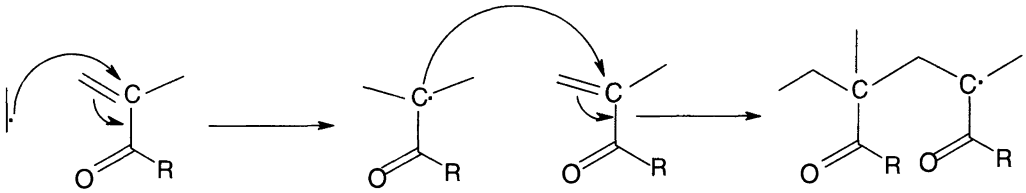
Enzymes are only mixed with the polymer solution, spread or screen-printed on a suitable carrier, such as a dialysis membrane or the electrode surface, dried and covered with a second membrane, if required. Sometimes prior to the addition of enzyme heating and cooling of the polymer solution is required to obtain a homogeneous solution. One advantage of this immobilisation method is, besides its simplicity, the possible wide variation of the amount of enzyme allowing the determination of the optimal enzyme loading (Pfeiffer et al. 1992, Scheller et al. 1989). Moreover, the permeability of the enzyme membrane system can be adapted to practical requirements by further additives, such as polyethyleneglycol (Liu et al. 1997), or by adapting the porosity of covering membranes (Pfeiffer et al. 1992).

Immobilisation of enzymes can also be done in Nafion, which is known as cation exchanger and can be emulsified with aqueous enzyme solutions. However, it was found that Nafion membranes were more stable, when they were prepared from a homogenous solution, so that the enzyme was suspended in a 90% alcohol solution and mixed with Nafion (Karyakin et al. 1996). Membranes were created by dropping the mixture on the working electrode followed by evaporating of the solvent. The activities of alcohol dehydrogenase and glucose oxidase in these membranes were dependent on the amount of polymer, showing an optimum for alcohol dehydrogenase, whereas for glucose oxidase signals decreased with increasing Nafion content. Nafion was obtained as sulfonic acid and had to be neutralised before use, as in particular glucose oxidase was inactivated by the low pH of the polymer solution. Sensors based on Nafion membranes were protected from fouling and negatively charged interfering substances were repelled from the electrode surface due to the negative charge of the polyelectrolyte.

Though enzymes entrapped in polymers are mainly used as enzyme membranes in enzyme analysers, the formation of corresponding microspheres is also possible by chosing appropriate process parameters. Li et al. (2000) could preserve the activity of glucose oxidase as a model enzyme encapsulated in beads of 1-10 µm diameter made of poly-DL-lactide-poly(ethylene glycol). Encapsulation of glucose oxidase into polyelectrolytes of opposite charge was achieved by keeping the enzyme in solid state in a high salt solution (Trau & Renneberg 2003). Microparticles with glucose oxidase in the centre covered by alternating layers of poly-(sodium-4-styrenesulfonate) and poly-(allylamine-hydrochloride) were obtained and entrapped in a poly (carbamoyl) sulfonate membrane on top of an electrode.

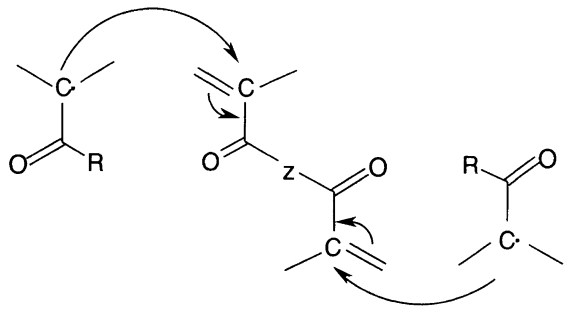
3.3.3. Radical polymerisation

Polymer networks, in which enzymes are entrapped, can be obtained by polymerisation of monomers in the presence of the protein. Acrylates are among the frequently used monomers, because several derivatives of acrylic acid are commercially available allowing the adaptation of hydrophobic / hydrophilic properties of the polymer and the introduction of different functional groups (Kennedy & Cabral 1987, Gooding et al. 1997). Polymers are obtained by radical polymerisation of the monomers (Fig. 5), which is initiated by ammonium peroxodisulphate (Gooding et al. 1997), azobisisobutyronitrile (AIBN) (Yang et al. 1995) or irradiation with UV-light (Rohm et al. 1995) using a photoinitiator, such as dimethoxyphenyl acetophenone (Brahim et al. 2002, Jiménez et al. 1995). Irradiation with UV-light allows the generation of patterned enzyme layers, e.g. for the production of enzyme microelectrodes (Jiménez et al. 1995).



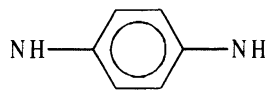
Polymerisation of acrylate through radical initiation

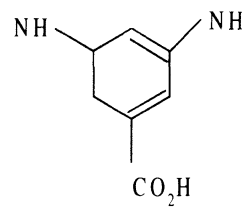
R= NH_2	acrylamide
OH	methacrylic acid
OMe	methyl methacrylate



Polymerisation of acrylate through radical monomer and cross-linker

Z = NH- CH_2-CH_2 –NH	O-CH_2-CH_2 –O
N, N'-methylenebisacrylamide	ethylene glycol dimethacrylate





N, N' - phenylenediacrylamide

3, 5-bis (acyloylamido) benzoic acid

Fig. 5: Polymerisation mechanisms of acrylate through radical initiation respectively radical monomers and cross-linker. Various derivatives as suitable reaction partners are shown

Polymer networks result from cross-linking of linear polymers by oligofunctional acrylamides, such as N,N´-methylene bisacrylamide or tetraethyleneglycol diacrylate. As polymerisation is based on the reaction of radicals in the presence of the enzyme, the enzyme can also be attacked by radicals leading on the one hand to a covalent attachment to the polymer backbone, on the other hand to partial inactivation of the enzyme. If polymerisation is initiated by UV-light, the stability of the enzyme during UV-light exposure is to be considered. However, mixtures of acrylates are commercially available, and they form the basis not only of slab gels comparable to those used in gel electrophoresis (Pribyl et al. 2003) but also of screen-printable pastes. The latter allows the utilisation of the screen-printing process not only for the

fabrication of the electrode used as transducer but also for enzyme immobilisation. Mechanically stable enzyme layers were obtained by UV-irradiation of the printed layer (Rohm et al. 1996). Not only the mechanical stability of the polymer films is influenced by the polymerisation reaction and the composition of the precursor solution, but also the film continuity, structure and hydrophobicity (Gooding et al. 1997). It was shown that not only the chosen monomers influenced these properties, but also the presence of the enzyme (Gooding et al., 1997), the addition of polymers (Mersal et al. 2004, Schumacher et al. 1999, Hall et al. 1999, Rohm et al. 1996) or of particles (Mersal et al. 2004, Rohm et al. 1995). Polymers such as poly(vinyl alcohol) (Hall et al. 1999), DEAE-dextran (Rohm et al. 1996), poly-L-lysine, Gafquat (Mersal et al. 2004, Schumacher et al. 1999), polyethyleneimine (Schumacher et al. 1999) increased the activity of the enzyme layer which is indicated by increasing signals. This effect is dependent on the amount of additive and also on the type of enzyme (Schumacher et al. 1999). It can be explained by an improved diffusion of enzyme substrates to the enzyme entrapped within the polymer layer together with improved diffusion of the reaction product, usually hydrogen peroxide, to the underlying electrode (Gooding et al. 1997, Hall et al. 1999, Rohm et al. 1996). Depending on the enzyme, the polymeric additives also improved the stability of the enzyme electrode during storage and use (Schumacher et al. 1999). A glucose oxidase electrode with the additives polylysine and BSA in the photopolymerizable acrylate paste could be used as glucose sensor in an automated flow-injection analysis system. The resulting currents for standard solutions are related to the concentration and enable the autocalibration of the system. They remained constant during several days of continuous use, as the changes of the signal heights were due to the alternating tempetatures at day and night (Fig. 6). The enzymatic activity remained constant when stored even for weeks. A good long-term stability was also obtained when the photopatterned gel was cross-linked with glutaraldehyde (Jiménez et al. 1995).

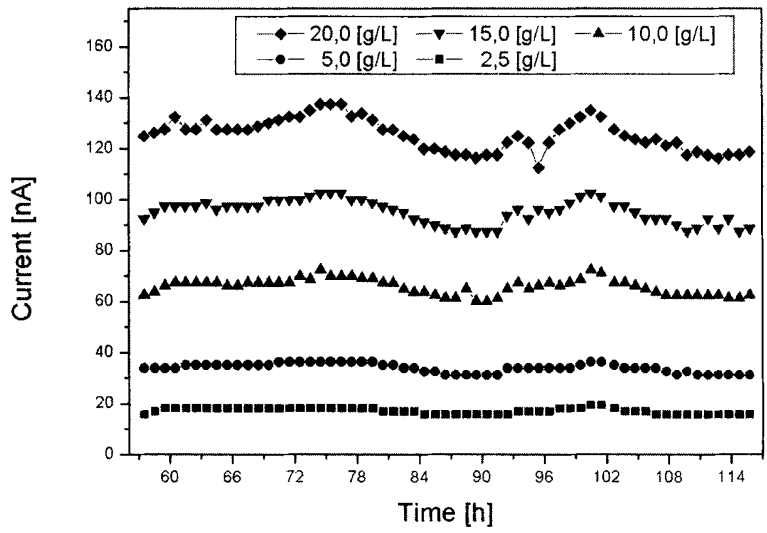


Fig. 6: Signal heights of a glucose oxidase electrode as a response for standard solutions of glucose. The enzyme was entrapped in an acrylate paste screen-printed onto a thick-film electrode which was inserted in a FIA-system used for bioprocess monitoring. Due to the alternating temperature at day and night the signals showed regular changes although the enzymatic activity did not alter

The adhesion of the acrylate hydrogel on the surface was improved, when the surface was first functionalised with 3-(trimethoxysilyl)propyl methacrylate (Jiménez et al. 1995), as the acrylate residues were on the one hand covalently attached to the surface and on the other hand incorporated into the acrylate polymer. Incorporation of enzymes into the acrylic polymer was achieved by covalent attachment of polyethylene glycol acrylate to subtilisin and thermolysin (Yang et al. 1995). This improved the solubility and activity of the enzymes in organic solvents used for polymerisation of the acrylates. The incorporation of the enzymes in the polymer enhanced the stability of the enzyme and tolerance toward heat. Even electron mediators were integrated in the polymer, thus simplifying the preparation of mediated enzyme electrodes and reducing leakage of the mediator. This was achieved by copolymerisation of vinyl ferrocene (Bu et al. 1995) or by an acrylamide-based Os-redox polymer to which the enzyme was cross-linked (de Lumley-Woodyear et al. 1995).

3.3.4 Electrochemical polymerisation

The electrochemical synthesis of organic polymer films is initiated by the electrochemical oxidation of the monomer to a cation radical followed by the polymerisation reaction. The synthesis can be performed by application of a constant current (galvanostatic), a constant potential (potentiostatic) or by potential scanning or cycling (sweeping methods) (Gerard et al., 2002). The thickness of the polymer layer and its degree of oxidation are controlled by the charge and electrode potential used for polymer deposition. These films can be used to cover and encapsulate enzymes adsorbed on the surface (García et al. 1998) or to entrap enzymes which are present in the monomer solution during growth of the film (Cosnier 1999, Bogdanovskaya & Tarasevich, 1996, Pravda et al. 1995b). One of the drawbacks of this method is the requirement of high concentrations of monomer (0.05 to 0.4 mol/L) and enzyme (0.2 to 3.5 mg/mL) (Cosnier 2003).

The majority of work done in the field of enzyme electrodes was carried out with redox or electronic conducting polymers, using monomers such as pyrrole (Gros & Bergel 1995, Lindfors et al. 1997), N-methylpyrrole (Bogdanovskaya & Tarasevich 1996), aniline or thiophene, as the polymer backbone can act as a molecular wire enabling electron hopping inside the membrane. But also phenol (Pravda et al. 1995b), *o*-aminophenol (García et al. 1998), *o*-phenylenediamine or *p*-aminophenol were used. A suitable monomer is water soluble and polymerises at biological pH-values.

Swann et al. (1997) used electroadsorption to attach fructose dehydrogenase onto a platinum surface with subsequent electropolymerisation of pyrrole. Amperometric measurements showed an increasing current by the use of the polymer which was explained by improved electron transfer through the conducting polymer. Moreover dehydrogenases offer the advantage that no hydrogen peroxide is formed as reaction product which can damage the polypyrrole film. For oxidases this is achieved by the introduction of mediators, such as ferrocene. Covalent attachment of ferrocene to glucose oxidase lead to an effective coupling of the deeply buried active center of glucose oxidase to the surrounding matrix of polypyrrole (Schuhmann 1995). In this study ferrocene was linked to the protein via a flexible spacer of defined length. The linkage circumvents the problem of leaching of mediators during use. The electrons can be transported directly from the active center to the electrode enabling the regeneration of the enzyme. A similar example with immobilised glucose oxidase was given by Koide et al. (1999) using crosslinked polyallylamine with covalently attached ferrocene. A relatively high signal could be measured in oxygen-free solution indicating electron transfer via the polymer. The applied potential at the working electrode could be reduced to minimize the influence of interfering substances.

As during polymerisation only a low amount of enzyme is immobilised and activity may be damaged a two-step immobilisation of enzymes has been established (Cosnier 1997, Cosnier et al. 1999a). First the enzymes are simply adsorbed onto clean surfaces by soaking in enzyme solutions. In the next step they are embedded in the conducting polymer by electropolymerisation of the monomers. As a result the membranes show an improved reproducibility while the required amount of enzyme is reduced. Li et al. (1998) coupled glucose oxidase onto a platinized platinum wire and covered the enzyme with pyrrole and 4-(3-pyrrolyl)-4-oxobutyric acid. As a result the signals of interfering substances were minimized beneath 1 and 2% respectively. The polymer membrane may not only perform electrical wiring but also the electrochemical reaction of the enzymatically created product hydrogen peroxide (Rikukawa et al. 1997). Also shown is the electrocatalytic behaviour of electrodeposited films of 3,4-dihydroxybenzaldehyde and phendione complexes with transition metals for the oxidation of NADH as the cofactor of aldehyde dehydrogenase and alcohol dehydrogenase (Lorenzo et al.,1998).

Amphiphilic derivatives of pyrrole were tested to investigate the influence of the hydrophobicity onto the properties of the resulting layer. Experiments with horseradish peroxidase, galactose oxidase, polyphenyl oxidase, glucose oxidase, and xanthine oxidase showed that the extent of immobilised enzymes rises with decreased hydrophobicity of the monomers (Coche-Guerente et al. 1995). The sensitivity of the electropolymerised membrane depended onto its permeability.

The enzyme loading in polypyrrole layers is precisely controlled by covalent attachment of the enzyme (glucose oxidase) to this polymer followed by mixing with carbon powder (Kojima et al. 1997). Losada & Armada (2001) created potentiostatically a chloranil-N-aminopyrrole film and coupled glucose oxidase via the chinone-group of the chloranil onto the membrane followed by deposition of a Nafion layer. Copolymerisation of pyrrole and pyrrole-biotin leads to a conducting thin film with outstanding biotin groups feasible for later immobilisation of biomolecules which are coupled to avidin (Bidan et al. 1999, Cosnier et al. 1999b). Spacer between the pyrrole and the biotin moiety improved polymerisation and the access of the biotin for the enzyme to be immobilised (Cosnier et al. 1999b).

3.3.5 Influence of additives

The activity of enzymes usually decreases with time, with the rate of activity loss being dependent on the enzyme and chemical and physical conditions it is exposed to (Fágáin 1997). Generally the denaturation of enzymes is accelerated at elevated temperatures because unfolding of the protein structure is facilitated. Immobilisation can enhance the lifetime of an enzyme by stabilising its structure, but also the addition of certain compounds (Fágáin 1997). Additives such as sugars (Gibson et al. 1993), polyelectrolytes (Gibson et al. 1996) or proteins (BSA) (Gouda et al. 2001) are known to have a positive effect on the stability of the enzymes during immobilisation, storage, freeze-thawing, or use.

The mechanisms of enzyme stabilisation by polymers are still not understood in detail. However, the formation of protein-polyelectrolyte complexes in aqueous solutions could be shown for glucose oxidase supplemented with DEAE-dextran and the methacrylic co-polymer Gafquat (Gibson et al. 1996). Storage stability of glucose oxidase sensors greatly depended on the concentration of DEAE-dextran (Gavalas et al. 2000). Obviously the charged amino acid residues on the protein enable the attachment of polymers with oppositely charged side chains leading to a kind of wrapping the protein by the polymer. Stabilising effects of Gafquat could also be observed for horseradish peroxidase (Schumacher et al. 1999), whereas for lactate oxidase and malate dehydrogenase DEAE-dextran together with the polyhydroxyl compound

lactitol showed the best effects (Schumacher et al. 1999, Gibson et al. 1996, Hart et al. 1996). Treatment of alcohol oxidase with the Gafquat and DEAE-dextran showed intermolecular complexing due to electrostatic interactions whereas the uncharged dextran T-500 did not form complexes (Gibson 1996). Unfolding of the enzymes as the first step to irreversible denaturation is therefore hindered and the integrity of the proteins prolonged.

The concentration combination of additives indeed is crucial for stabilising effects. Studies with alcohol oxidase showed the positive influence of combinations of lactitol and DEAE-dextran on the shelf life of the membrane-entrapped enzyme (Gibson et al. 1992). Lactitol enhanced the lifetime of such enzyme membranes whereas DEAE-dextran did not. Surprisingly the combination of both additives gave much higher stability. The degree of long term stability depended on the concentrations of the single additives respectively on their ratio. The activity of dried horseradish peroxidase could be completely preserved by the combination of lactitol and Gafquat 755N compared to the absence of additives or just the addition of one compound (Gibson et al. 1993). Similar effects were observed for other enzymes, such as lactate oxidase. The addition of the cationic form of Gafquat preserved the activity of immobilised lactate oxidase best, whereas hydroxyethyl cellulose, diethylaminoethyl-dextran (DEAE-dextran), and the form of Gafquat no beneficial effects (Hart et al. 1996). From various mixtures of the enzyme acetyl-cholinesterase with polymers, the addition of Gafquat and lactitol had the best influence on the stability (Hart & Collier 1998).

However, additives influence not only the stability of enzyme preparations, but as mentioned already in earlier chapters, also the permeability of the enzyme layer is affected. This, in turn, influences the accessibility of the enzyme for its substrate and thus the apparent activity of the enzyme (Schumacher et al. 1999). Moreover, the access of the electrode surface for interfering compounds and thus the selectivity of the sensor system is affected (Mersal et al. 2004). Hence, performance of an immobilised enzyme preparation can be optimised to a large degree by variation of the composition of the matrix, in which the enzyme is embedded.

4. CHEMICAL COUPLING OF ENZYMES

Immobilisation of enzymes by physical principles relies on van der Waals and ionic forces between enzyme and carrier and on diffusion barriers created by a matrix embedding the enzyme. The strength of van der Waals and ionic forces between the enzyme and the carrier is influenced by the composition of the solution, such as pH, ionic strength or presence of amphiphilic compounds. Diffusion barriers prevent the dissociation of the enzyme from the carrier, however, they may also decrease the diffusion rate of the substrate or product within the matrix and thus increase response time and decrease the efficiency of the enzymatic reaction. Chemical binding of the enzyme to the support eliminates the sensitivity of the binding for changes of physical parameters, as it is based on covalent binding of the enzyme to the support. Chemical bonds between the enzyme and the support are only possible, if suitable reactive functional groups are available, which are delivered on the one hand from the side chains of the amino acids forming the enzyme and on the other hand from the carrier material. Thus, covalent binding leads to a chemical modification of the enzyme, which may reduce the enzyme activity, as the 3-dimensional structure of the enzyme or the accessibility of the active site can be influenced. Due to the chemical inertness of most carriers these usually have to be pretreated to generate the respective functional groups, and bifunctional reagents are used to couple or cross-link the enzyme to

the support. Usually chemical coupling of the enzyme to the support is based on the reaction between amino, carboxyl or aldehyde groups leading to Schiff bases or amide bonds.

4.1 Coupling via glutaraldehyde

One of the most common cross-linking reagents is glutaraldehyde, a bifunctional aldehyde, which readily reacts with primary amino groups of the protein and of the carrier (Fig. 7). The amino groups of the protein are delivered from lysine residues, amino groups on the surface of the carrier are usually created by silanisation with an amino-functionalised silane (aminopropyltrimethoxysilane (APTMS), aminopropyltriethoxysilane (APTES)). Due to the reactivity of the aldehyde and amino groups the proteins are mixed with glutaraldehyde and an aliquot of the mixture is deposited onto the carrier surface. An enzyme membrane is formed by cross-linking between proteins and between protein and surface. Frequently bovine serum albumine (BSA) is added to the enzyme as an inert protein, to reduce the chemical modification of the active protein and thus preserve its activity. The composition of the protein-glutaraldehyde mixture has to be optimised for each enzyme, as the density of the network is dependent on the amount of protein, of cross-linker and the number of available amino groups (Chemnitius & Bilitewski 1996, Schmidt et al. 1994, Bilitewski 1994). The thickness of the enzyme layer can be reduced to almost a monolayer, if cross-linking is done in a two-step procedure. In the first step aldehyde groups are introduced to the carrier surface by incubation of the amino-groups functionalised carrier with glutaraldehyde, and in the second step the enzyme is coupled to the carrier (Fig. 7) (e.g. Okuma and Watanabe 2002, Pundir et al. 1999, Mayer et al. 1996). This procedure avoids cross-linking between enzyme molecules and thus, a 3-dimensional network (result according to Fig. 1). Alternatively, the enzyme is first adsorbed on the support followed by cross-linking with glutaraldehyde (Fig. 8) (Dremel et al. 1989), e.g. by exposure to glutaraldehyde vapour (Li et al. 1999b, Bachmann & Schmid,

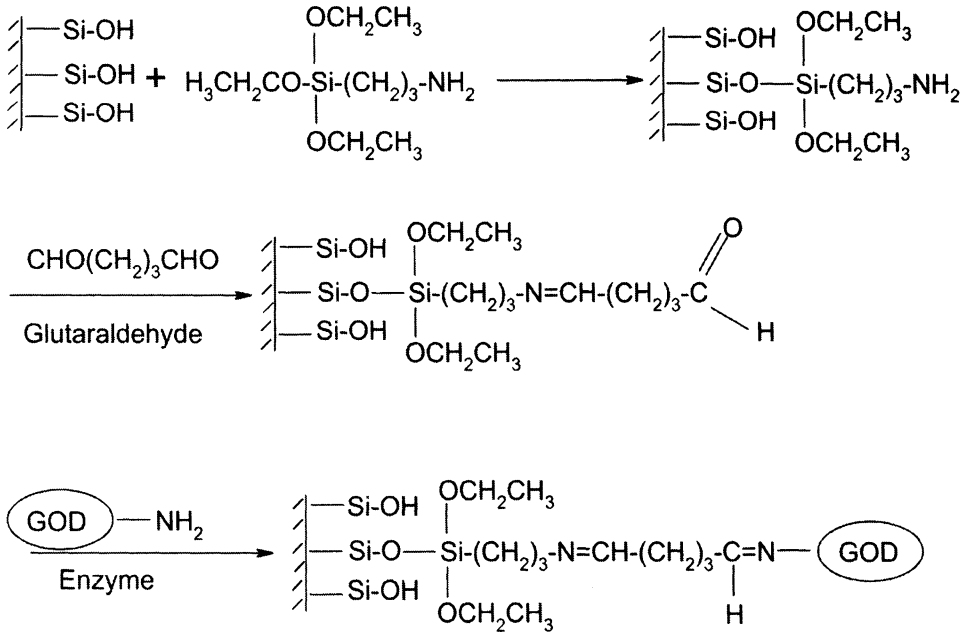


Fig. 7: Reaction sequence of glutaraldehyde cross-linking

1999, Poyard et al. 1998). In this case, glutaraldehyde is used for cross-linking of only protein molecules to improve the stability of the adsorbed enzyme layer and no covalent binding to the carrier is achieved.

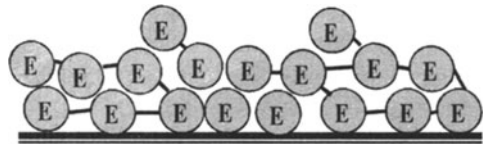


Fig. 8: Enzyme adsorbed on a surface followed by cross-linking of proteins with bifunctional reagents. The thickness of the layer depends on the amount of adsorbed protein

Suitable materials for covalent binding to the carrier are (amino-group functionalised) platinum or graphite/carbon electrode surfaces, but also glass, gels (Arenkov et al. 2000) or other supports delivering amino groups on the surface. Glass is used for optical fibres, controlled pore glass beads (CPG), which are entrapped in flow-through columns (Weigel 1996) or bead microchambers (Richter et al. 2002), porous silica supports in capillaries (Wilson et al. 2000) and for the fabrication of microfluidic chips (Xiong & Regnier 2001). Usually it is first treated with APTES or APTMS to generate amino groups on the surface, followed by incubation of glutaraldehyde. Amino groups are also delivered from polymers. As an example Tang et al. (1998) utilized polyethylenimine-coated (PEI) activated carbon rods for the immobilisation of glycerol dehydrogenase and diaphorase via glutaraldehyde as a detection system for glycerol. The enzymes were bound covalently and the intermediate layer of PEI prevented the electrode from fouling and enhanced the lifetime of the enzymes.

Where glutaraldehyde is used for cross-linking of proteins and no covalent attachment to the carrier is aimed at, all supports are suitable allowing finally the adhesion of the protein layer. Thus, glutaraldehyde-cross-linked protein membranes were also created on polymeric membranes, which were used for removal of interferences or extension of linear ranges (Matsumoto et al. 2001, Palmisano et al. 2000, Poyard et al. 1998, Kyröläinen et al. 1997). Willmer (1998) used a combination of affinity binding followed by immobilisation of crosslinking via glutaraldehyde. First a PQQ-NAD^+ monolayer was attached to a gold electrode. The enzyme lactate dehydrogenase bound by an affinity reaction its cofactor NAD and was fixed by crosslinking with glutaraldehyde. The same approach was applied to microperoxidase and nitrate reductase. These layers may even comprise only an inert protein, such as BSA or gelatin (Khan & Wernet 1997) and are used as diffusion barriers for the protection of adsorbed enzymes.

4.2 Coupling via carbodiimide

Covalent binding via activation of carboxylic groups is achieved by incubation with N-hydroxysuccinimides (NHS) in the presence of carbodiimide derivatives, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Yang et al. 2003, Kapustin et al. 2003, Yoshimoto et al. 1995). Coupling occurs by formation of amide bonds from primary amino groups of the reaction partner. Usually the carboxylic groups are delivered from the carrier, such as oxidised graphite, and the amino groups from lysine residues of the protein. The coupling of lactate oxidase, malate dehydrogenase and glutamate oxidase to modified graphite was shown by Gibson et al. (1998), whereas Yang et al. (2003)

and Kapustin et al. (2003) immobilised glucose oxidase on graphite particles which were embedded in composite electrodes (Kapustin et al. 2003) or in a sol-gel matrix (Yang et al. 2003). Besides graphite coupling of glucose oxidase to polypyrrole having carboxylic groups was described (Kojima et al. 1997). The polymer was prepared by oxidative polymerisation of 1-(2-cyanoethyl)pyrrole followed by hydrolysis of the cyano group. Control experiments showed that the addition of a carbodiimide derivative was required to achieve binding of glucose oxidase. However, Yoshimoto et al. (1995) used capillary surfaces functionalised with amino groups as carrier and described the activation of carboxylic groups from the enzyme β-galactosidase by *m*-maleimidobenzoic acid N-hydroxysuccinimide ester. This capillary was used as component in high-performance capillary electrophoresis. The amount of required enzyme and enzyme substrate was much smaller than in conventional analytical systems.

4.3 Other methods

There are a number of other chemical reactions which are used for covalent coupling of the enzyme to a solid support. Usually amino groups from the enzyme react with different functional groups supplied by the carrier. Quinone sites were created on a chitosan–HEMA (poly(hydroxyethylmethacrylate)) copolymer (Ng et al. 1998) and on an N-aminopyrrole membrane (Losada & García Armada 2001) by activation either with *p*-benzoquinone or with cloranil. Coupling of glucose oxidase occurred by incubation of the electrode covered with the polymer in the enzyme solution at room temperature.

Matsumoto et al. (2003) compared different beads with covalently bound α-glucosidase in a flow system, in particular aminopropyl-controlled pore glass activated with glutaraldehyde (see above) and isothiocyanate-controlled pore glass with the same pore diameter and particle size. They found that almost 92% of the enzyme was coupled to the isothiocyanate-glass, whereas the coupling efficiency was only 51 % for the glutaraldehyde-activated glass.

Epoxide groups provide a convenient covalent immobilisation site as they also react readily with amino groups from proteins. Eupergit C is a versatile carrier, on to which proteins are coupled via oxiran groups. It can be used in preparative enzyme catalysis as it can be used in stirred reactors, but it is also applicable to enzyme analysis. Thus, it was chosen for multivalent covalent attachment of chymotrypsin and penicillin G acylase (Mateo et al. 2000). However, epoxide groups can be introduced on other carriers by silanisation with 3-glycidoxypropyltrimethoxysilane (GOPS) (Polzius et al. 1996), but also by glycidyl methacrylates as components of an acrylate polymer (Hall & Hall 1995). Moreover, with poly(oxyethylene)bis(glycidyl ether) a bifunctional polymer is available, which was used for cross-linking of the Os-redox polymer and the enzyme tyrosinase (Daigle & Leech 1997, Sapelnikova et al. 2003).

If enzymes are to be immobilised in microfluidic systems in which flow is controlled by electroosmosis, the availability of surface charges has to be considered. Thus, immobilisation in acrylamide gels is not feasible for this application, because acrylamides suppress the electroosmotic flow. Lee et al. (2003) suggest coating of microchannels by a 3-dimensional layer of poly(maleic anhydride-alt-α-olefin), as the highly reactive anhydride groups allow the efficient immobilisation of the enzyme (soybean peroxidase and others were used) together with the formation of carboxylic groups, which maintain the negatively charged surface. Thus, compared to systems with enzymes

immobilised via glutaraldehyde approximately ten fold higher flow rates were possible. Xie et al. (1999) and Peterson et al. (2002) used a mixture of acrylamide with 2-vinyl-4,4-dimethylazlactone, 2-hydroxyethyl methacrylate and ethylene dimethacylate, in which the azlactone groups allowed covalent attachment of the protein. Fluid was transported by pressurized flow, as the electroosmotic flow was significantly reduced. However, the chips could be used for on-chip protein digestion by immobilised trypsin.

Though in most cases the protein delivers amino groups for chemical reactions, there are also some other approaches. It was already mentioned that carboxylic groups can be activated by carbodiimide and used for coupling to amine groups on the carrier. They were also used without further activation for coupling to activated glass beads (Pundir et al. 1999). Zirconia coated arylamine glass was activated in a diazotization reaction by sodium nitrite, and horseradish peroxidase was immobilised. Comparison to glutaraldehyde activated alkylamine glass showed a much higher efficiency, as approximately 77% of the initial activity were retained on the arylamine glass, whereas only 38% were retained on the alkylamine glass. However, the Vmax was decreased, whereas it was unchanged for the alkylamine-glass bound enzyme. Both enzyme preparations showed higher stability in cold compared to the free enzyme.

A combination of several binding principles was suggested for the immobilisation of glycoproteins by Abad et al. (2002). Carbohydrate residues show a binding affinity to boronic acids, which form cyclic esters with saccharides. Thus, on the surface of a gold electrode a self assembled monolayer was created from alkane thiols modified with boronic acid residues and epoxide groups. Thus, the affinity between the boronic acid and the carbohydrate residues from the glycoprotein leads to binding of the enzyme on the electrode surface, which is then strengthened by the chemical reaction between the amino groups from the enzyme and epoxide groups from the monolayer on the transducer surface. Comparison of native horseradish peroxidase to the recombinant, non glycosylated form clearly showed the contribution of the carbohydrate residues.

5. IMMOBILISATION VIA BIOAFFINITY REACTIONS

Besides physical and chemical reactions immobilisation of enzymes is also possible using specific binding properties of proteins. A sequence of six histidine residues (*his* tag) shows high affinity to Ni^{2+}-ions, which can be used for protein purification but also for protein immobilisation. *His* tags can be introduced in recombinant proteins either at the C- or at the N-terminus and Ni^{2+}-ions are bound to a matrix via chelating agents (e.g. nitrilo triacetic acid, NTA) (Andreescu et al. 2002) or complexing by carboxyl residues (Davis et al. 1999). The NTA-groups were present on commercially available silica particles, which were embedded in a screen-printed hydroxyethylcellulose matrix. Binding occurred after deposition of the enzyme solution on the screen-printed layer. Sensors with immobilised acetylcholinesterase showed a reduced sensitivity for the enzyme substrate acetylcholine, but an improved sensitivity for enzyme inhibitors compared to electrodes with the enzyme embedded in a sol-gel (Andreescu et al. 2002). Davis et al. (1999) incorporated carboxylic groups in a polypyrrole film by appropriate pyrrole derivatives and could show binding of Ni^{2+} and of a *his*-tagged alkaline phosphatase. They observed a significant difference to the wild-type enzyme proving the suitability of the immobilisation concept. However, Simon et al. (2002) could immobilise a *his*-tagged protein to poly(vinylsulfonate) film without the addition of Ni^{2+}-ions and attributed this to electrostatic interactions.

The affinity between biotin and biotin-binding proteins, such as streptavidin, is sufficiently strong to allow permanent immobilisation of proteins. Usually, the surface of the support is biotinylated allowing binding of streptavidin (Yao et al., 2003, Mao et al., 2002, Ayyagari et al. 1995). As streptavidin has 4 binding sites for biotin, it can be used as a bridge between a biotinylated enzyme and the biotinylated surface (Yao et al. 2003, Mao et al. 2002). Alternatively, streptavidin-enzyme conjugates can be immobilized (Ayyagari et al. 1995). Biotin-groups are created on surfaces of capillaries by coating with a biotinylated polymer (Ayyagari et al. 1995), entrapment in lipid bilayers by biotinylated fatty acid derivatives (Mao et al. 2002) or by covalent binding to an activated surface, such as a chitosan membrane (Yao et al. 2003) or a screen-printed electrode (Fernández Romero et al. 1998). In all cases the systems proved to be applicable with a sufficient reliability.

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Protein Engineering of Industrial Enzymes

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1. INTRODUCTION

Scientists have known the role of DNA as a storage and retrieval medium of genetic information since the 1940's. The molecular structure of DNA was published in 1953. Inventions of genetic engineering tools like recombinant DNA technology and polymerase chain reaction have made it possible to overproduce enzymes and to improve their properties towards a desired target. This has resulted in a development of new enzyme technologies and applications. In the future bioinformatics technologies such as statistical analysis of protein structures will make it possible to predict structure-function relationships.

Many large scale applications of enzymes have been known for decades. Interest in biocatalysis in chemical production is increasing. Enzymes are attractive because they function in mild conditions and are typically regio-, chemo- and enantioselective. The availability of enantiopure compounds is critical in the pharmaceutical industry. Enzymes can be used to produce compounds that are not easily available by chemical synthesis. Chemical production by enzyme catalysis might also result in higher yields with lower waste generation due to the specificity of enzyme reactions. However, enzyme properties must often be modified in order to achieve commercially viable reaction rates, specificities, stabilities and yields. With protein engineering methods it is possible to create enzymes with properties sufficient to meet these demands. Some engineered enzymes are already able to tolerate temperatures above the boiling point of water (Arnold 1998). This chapter describes some protein engineering targets, methods and achievements for industrially used enzymes.

2. TARGETS AND RESULTS FOR INDUSTRIAL ENZYMES

Enzymes isolated from nature are optimal in their own context but often not in a given industrial process. In such a case the first choice might be to try to find a better enzyme from nature. Such a search has been going on systematically by many research groups and enzyme companies resulting in some commercial applications. Organisms which inhabit hot or alkaline environments are expected to have thermo- or alkalitolerant enzymes and some such enzymes have found commercial applications. However, often the enzymes found in extremophiles may not be functional in the desired conditions. It may also be difficult to overproduce these enzymes in a suitable host. Another alternative is to engineer a commercially available enzyme by rational design or random methods to be a better industrial catalyst. In the following we present some examples of targets and results of protein engineering of industrial enzymes.

2.1 Detergent enzymes

Proteases, amylases, lipases, cellulases and peroxidases are used in laundry products. Proteases are used to enhance cleaning of protein-containing soils, amylases to remove starch-containing soils (particularly food) and lipases to remove greasy foods, cosmetics and body soils. Cellulases are used for cleaning and color maintenance and fabric restoration of cotton and cotton blends. Cellulases improve the quality of faded garments by removing pills and fuzz. Peroxidases can be used to bleach the dyes that have been released from the colored garments. These enzymes have to be stable at high temperature, alkaline pH, and tolerate surfactants, oxygen bleaches, calcium sequesters and proteases.

Enzymes suitable for detergents have been found from nature, but also protein engineering has been used to develop better enzymes for laundry applications (Showell 1999). Serine protease subtilisin has been a major model system for protein engineering studies and subtilisin has been subjected to a huge number of mutations in order to study or modify its catalytic mechanism, folding mechanism, stability, activity and substrate specificity (Bryan 2000). Stability has been improved considerably by protein engineering, especially stability and activity in detergents, including oxidizing conditions, has been a subject for mutagenesis studies. The susceptibility of subtilisin to oxygen bleaches has been decreased by replacement of a methionine residue near the catalytic serine with alanine. Because oxidative attack on this methionine largely inactivates the enzyme, the alanine mutant is stable toward oxygen bleach. Activity of subtilisin is dependent on calcium. Removal of the calcium by calcium sequesters has been decreased by increasing the affinity of calcium to the enzyme. This has been achieved by introducing more negative charge close to the calcium binding site. Also, calcium-free subtilisin mutants have been produced.

Because lipases are most active at the water-substrate interface, anything that interferes the adsorption of lipase to this interface, such as surfactants, can strongly inhibit the substrate hydrolysis. The stability of lipases in detergents has been increased by several approaches. Modifying the protease cleavage site has decreased sensitivity to proteases. The anionic surfactant compatibility of *Fusarium solani pisi* cutinase was decreased by introducing more positive charge onto the protein surface, whereas introducing more negatively charged residues resulted in an enzyme with increased stability against the anionic surfactant lithium dodecyl sulphate. Altogether, many lipase variants have been created, that function in various detergents and are independent on calcium for activity (Svendsen 2000).

Other detergent enzymes have also been improved by protein engineering. Because most α-amylases found in nature are sensitive to oxygen bleach, protein engineering has been used to develop α-amylases that are resistant to oxygen bleach. Alkaline activity and detergent compatibility of cellulases have been targets for the improvement. Because there is a trend towards lower wash temperatures, improved activity of enzymes at lower temperature is required. Another important goal for washing enzyme development is a better fabric care.

2.2 Starch hydrolysis and fructose manufacturing

Production of fructose from starch is a large scale enzymatic industrial process. Starch is hydrolysed to maltodextrins (liquefaction) by thermostable α-amylase and then saccharified to glucose by glucoamylase. The glucose is converted by glucose isomerase (ie. xylose isomerase) to fructose, which is used to make high fructose corn syrup (HFCS) in sweetener industry. Because these enzymes function optimally at different conditions, the improvement of their performance has received much attention in industrially

related research (Crabb & Shetty 1999, Nielsen & Borchert 2000, Sauer et al. 2000). Ideally the whole process could be carried out in a single step but in practice the process is done in three separate steps due to the different demands of each enzyme as shown in Figure 1.

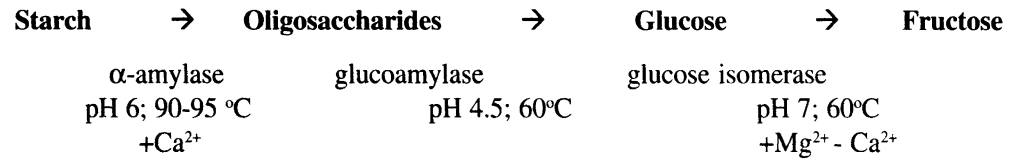

Figure 1. Enzymes and conditions needed for starch hydrolysis to glucose and isomerization of glucose to fructose

A highly thermostable *Bacillus licheniformis* α-amylase and its engineered derivatives are widely used in starch liquefaction industry (Cherry & Finantsef 2003). α-amylases are used at temperatures as high as 90-95 °C. The need for engineering the α-amylase or finding new α-amylases from nature comes from the requirement to use high levels of Ca^{2+} in the enzyme reaction and adjustment of pH to ~6 from the acidic pH of the starch slurry (pH ~4). The added calcium has to be removed prior to later processing steps and the pH lowered to ~4.5 for saccharification with glucoamylase. For these reasons the engineering efforts have focused on producing an α-amylase that can function in the absence of Ca^{2+} and has a lower pH optimum (pH 4.5). There are reports about engineered α-amylases with a decreased requirement for calcium (Crabb & Shetty 1999). Furthermore, the thermostability of *Bacillus licheniformis* α-amylase has been increased 23°C by seven point-mutations (Machius et al. 2003). This was achieved by introducing hydrophobic side chains to the solvent-exposed surface.

Glucoamylase is used at 60°C (pH 4.2-4.5) and the reaction requires much time (36-96 hr), and therefore the improvement of thermostability and reaction efficiency are major targets. The thermostability of glucoamylase has been increased by mutagenesis (Allen et al. 1998). Furthermore, the decreased production of a byproduct, isomaltose, in process conditions, has been achieved by redesigning the active site, yet retaining the hydrolytic activity on maltose (Fang et al. 1998).

Both the thermostability of xylose (glucose) isomerases and activity in acidic conditions has been improved by site-directed mutations. The goals have been the streamlining of the enzymatic process from starch to fructose and the conducting of the isomerization process at higher temperature, which favors the accumulation of the product. The currently used thermostable enzymes are already very effective. One kilogram of xylose isomerase can be used to produce 20 tonnes of HFCS.

2.3 Animal feed enzymes

The nutritional value of animal feed is commonly increased by the addition of hydrolysing enzymes, such as xylanases, glucanases, proteases and phytases. Animal feed manufacturing applies extrusion in high temperatures (temporarily 65-95°C). In such a process mesophilic enzymes may suffer severe losses of activity. Thus, the enzymes should be stable for a short time at high temperature, while the enzyme functions at lower temperature. *Trichoderma reesei* xylanase II (TRX II) is a widely used feed enzyme. It brings nutritional benefits, although a large part of the enzyme activity is lost during the preparation of the feed. TRX II is a good example how a protein engineering by rational design of a

mesophilic enzyme increased tremendously its thermostability and industrial applicability. The thermostability of TRX II has been increased by engineering of disulphide bridges and multiple single amino acid mutations, including introduction of five arginines into the protein surface (Turunen et al. 2001, 2002, Sung 2003, Fenel et al. 2004). The stabilization of the protein aminoterminus increased the apparent temperature optimum by over 10°C, whereas stabilization of the α-helix increased only thermostability but did not increase the apparent temperature optimum. The overall thermostability is increased by over 20°C by a combination of disulphide bridges and other mutations and even without any disulphide bridges. Chen et al. (2001) used error-prone PCR to mutate the *Neocallimastix patriciarum* xylanase. Beneficial mutations found after two rounds of error-prone PCR were combined using site-directed mutagenesis. One of the composite mutants was more thermostable and alkalophilic than the wild-type xylanase and therefore better adapted for conditions found in paper pulp bleaching processes.

Aspergillus fumigatus phytase is more thermostable, but has a lower specific activity than *A. niger* phytase. By site-directed mutagenesis, the interaction of phytate with the active site of *A. fumigatus* phytase was weakened, leading to a remarkably increased specific activity. This suggested that the product release could be a rate-limiting step (Tomschy et al. 2000). Increase of domain flexibility may also increase the catalytic efficiency. *E. coli* phytase has a higher specific activity than *Aspergillus* phytases, but it is thermolabile. Thus a major interest is to increase the thermostability of this enzyme. Furthermore, by applying a consensus stabilization method, an artificial biosynthetic phytase has been created on the basis of a group of mesophilic phytases. After some refining the thermostability of the consensus phytase was 15-26°C higher than that of the starting enzymes (Lehman & Wyss 2001).

2.4 Xylanases in pulp bleaching

Xylanases are used in pulp bleaching to aid the removal of lignin and consequently, reduction in the consumption of chlorine chemicals in the bleaching process (Buchert et al. 1994). Pulp from the chemical pulping process is hot and alkaline. The enzymes encounter temperatures up to 90°C and pH up to over 10. Thus the targets for enzyme engineering are high alkali- and thermostability together with high activity in these conditions. High catalytic rate is also important since the time for enzymatic bleaching is not very long. Thus far, there are no optimal xylanases for pulp bleaching conditions, and thus, the process conditions have to be adjusted suitable for xylanases. Literature describes pulp bleaching testing for a number of thermophilic xylanases. There are no great breakthroughs. The usefulness of thermophilic enzymes depends essentially on the abililty to produce them cost-effectively. The industrial strains of *Trichoderma reesei* can achieve production levels of extracellular proteins close to 100 g/l. Family 11 xylanases are small in size and function thus better in pulp bleaching than family 10 xylanases having a higher size. The most stable known family 11 xylanase is *Dictyoglomus thermophilum* xylanase (Morris et al. 1998). It appears that a major property still requiring improvement is the alkali-tolerance of the pulp bleaching xylanases.

2.5 Chemoenzymatic synthesis

The use of enzymes is expanding from the traditional areas to a wide variety of new applications in biocatalysis. The use of enzymes in biocatalysis is an area with great promise. Enzymes accept a wide array of complex molecules as substrates, they show unparallel chiral and positional selectivities, have only few by-products and there is no need for tedious blocking and deblocking steps. Especially, in food and pharmaceutical industries high reaction selectivity on complex substrates is important. Enantiomerically

pure amino acids, aspartame, rare sugars, semisynthetic penicillins, acrylamide and several other chemicals are produced commercially by enzymes. Often the enzymes are not stable in the reaction mixtures containing e.g. organic solvents. Thus, stability, tolerance to organic solvents and catalytic activity are targets of protein engineering. Because the active site of enzymes can be quite flexible allowing reactions with different kinds of substrates, this enables researchers to use protein engineering, especially random methods, to create new uses for the enzymes. Substrate specificity can be modified or expanded and even new enzyme activities can be created. Often, the side activity of an enzyme can be strengthened remarkably, offering a possibility to develop new enzymatic production processes.

3. RATIONAL DESIGN METHODS

Rational design is based on a detailed knowledge of the protein structure and the catalytic mechanism of enzymes. The three-dimensional structure and the location of the residues responsible for substrate binding and catalysis must be known in order to design rational changes to them. The analysis of the proteins for rational design involves sequence comparison of the protein family, which gives information about the structurally important amino acids and variability at each amino acid site. When the protein structure is known, computer simulations can be used to study the active site properties, substrate binding, thermostability and unfolding of the enzymes. Simulations provide information that is useful in planning mutations. For example, molecular dynamics simulations have been used to identify flexible regions in proteins, and subsequently, the protein stability has been increased considerably by introducing a disulphide bridge into such a region. Engineered disulphide bridges in flexible regions may contribute more to protein's thermostability than bridges in rigid regions. Based on modelling, active sites can be modified to accomodate new substrates or improve the reaction rate of slowly reacting substrates by reshaping of the active site.

When planning the mutations, one tries to find the key determinants in the protein structure that define the property that needs to be modified by site-directed mutagenesis. In engineering the thermostability, the comparison of amino acid sequences, amino acid contents and crystal structures between mesophilic and thermophilic enzymes gives information about the key factors behind the elevated stability. When engineering the enzyme reaction, deep understanding of the catalytic mechanism, rate limiting steps and structure-function relationships in the active site are necessary. A new semirational method to improve enzyme stability is to make a consensus sequence to the protein family (Lehman & Wyss 2001). This approach is based on the assumption that conserved amino acid properties have been selected in nature due to their impact on protein stability. Improvement of even 20 - 30°C in the thermostability has been achieved by this method.

The ultimate goal of rational protein design would be to design *de novo* catalysts, that could fold into structures, which could identify substrates and transition states as well as convert these to products at a high turnover and with high specificity. Some success has recently been reported in designing proteins, that fold into well-defined tertiary structures and follow saturation kinetics (Baltzer & Nilsson 2001). However, at the moment there is not enough knowledge about the structure-function relationship of proteins to design catalysts as efficient as those found in nature.

3.1 Site-directed mutagenesis

Site-directed mutagenesis was originally used for studying the effects of particular amino acid residues on kinetic properties of enzymes (Wilkinson et al. 1983). Today the method is applied largely also for

creating improved enzymes. Presently, two main methods are used for site-directed mutagenesis. The principles of these methods are presented in Figure 2. The two-round PCR method uses two rounds of PCR (Figure 2A). Two heteroduplex forming products are made in the first round. The second round of PCR then amplifies only the heteroduplex with recessed 3'-ends. This results in a double-stranded product with the desired mutation.

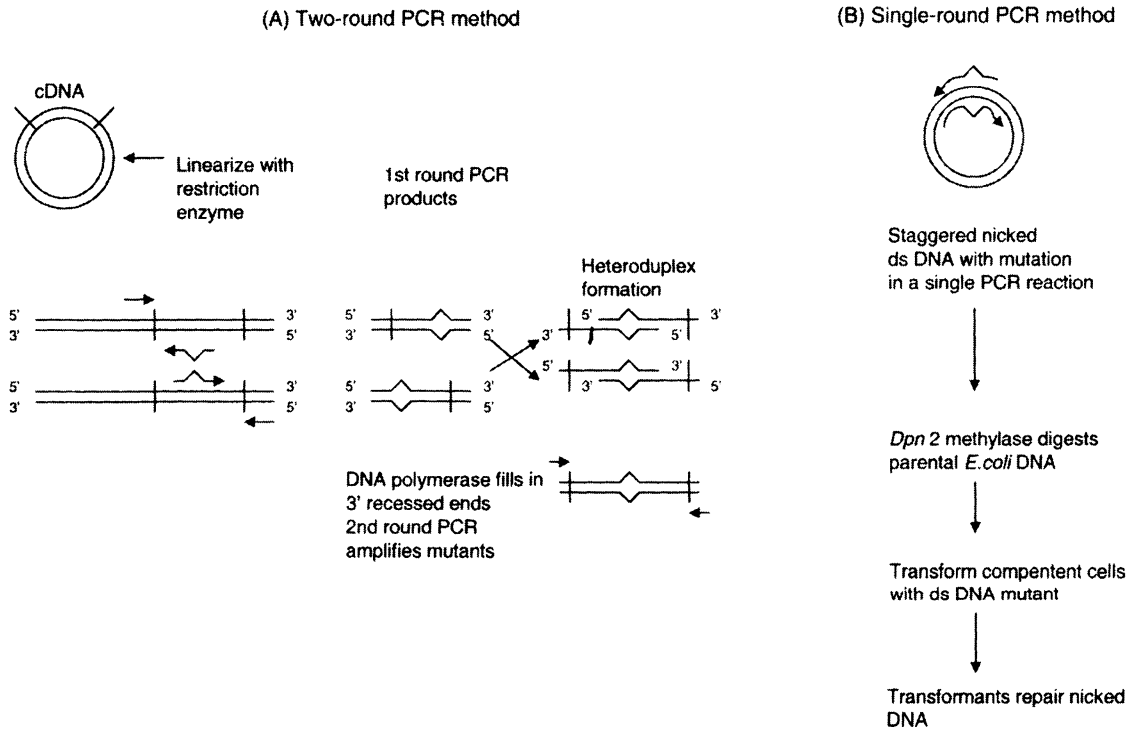

Figure 2. Principle of the two main site-directed mutagenesis methods. (A) The two-round PCR method; (B) the single-round PCR method. Modified from Penning & Jez (2001).

The second, single-round PCR method (Figure 1B), is becoming more popular. It uses forward and reverse primers, which contain the desired mutation. These primers are used to amplify the gene to be mutated directly from a plasmid template, which is digested after PCR with *DpnI* restriction enzyme, leaving the remaining nicked plasmid with the desired mutation.

3.2 Chemical modifications and unnatural amino acids

The use of site-directed mutagenesis is limited to the naturally occurring amino acids. In some specific cases, however, incorporation of unnatural amino acids to modify the properties of enzymes is possible by expressing the protein in the presence of unnatural amino acids. Even then the unnatural amino acids must closely resemble natural ones. In order to examine the effects of unnatural amino acids on catalysis, Parsons et al. (1998) incorporated 5-fluorotryptophan into the rat M1-1 glutathione transferase, which contains two tryptophan residues in the active site. The modified enzyme had a two-fold improved catalytic efficiency toward 1-chloro-2,4-dinitrobenzene and a nearly four-fold improved turnover number k_{cat}. The problem of having to use unnatural amino acids, which resemble natural ones, can be overcome

by using chemical modification of the amino acid sidechains. This strategy enables the introduction of unlimited unnatural sidechain structures. Chemical modification can be done by altering existing amino acids (Häring & Schreier 1998) or by combining site-directed mutagenesis and subsequent modification of the introduced amino acids (Berglund et al. 1997).

4. RANDOM METHODS

Regardless of the advances in the area of protein engineering, understanding of the structure-function relationship in proteins is still far from extensive. Despite some successes in altering enzyme activity, it is often very difficult to attain the results predicted by rational design. Corey & Corey (1996) list several failed attempts to produce *de novo*-designed biocatalysts.

4.1 Sequence space

Amino acids can be arranged into a protein sequence in an enormous number of different combinations. This astronomous sea of possibilities is called a sequence space. A 100-amino acid protein can be built in 20^{100} (1.3×10^{130}) different ways. This is a staggering number. We can compare this figure to the mass of Earth that is about 6×10^{27} g. If this mass would be made of different 100 amino acid proteins we would have about 3×10^{47} sequences. This amount of different proteins is only a small fraction of all the possible 100-amino acid long alternatives. When we think about the possibilities to form proteins by random processes we have to ask the question: how common are functional proteins in the sequence space? Evidence which seems to indicate that functional proteins are very rare in the sequence space is accumulating presently. Random methods can thus improve enzymes within the existing fold, but it is unlikely that new activities can be formed very easily if that involves a change of many amino acids in a single step.

Random or directed evolution methods are assumed to mimic the Darwinian evolution, but in practice this is true only to a limited extent within the existing fold. In directed evolution methodologies the gene of interest is diversified through mutation, and the created library of mutated genes is then tested based on a specific selection pressure, for example a particular property of a protein. The best variants are then iteratively subjected to a new round of mutations. The selection is directed towards a desired activity, hence the name directed evolution. The diversity of the library can be produced with random point mutations, recombination of genes or a combination of both.

Obvious limitation of rational protein engineering is the necessity to know the three-dimensional structure. This knowledge is not required by random methods. Furthermore, mutations leading to improved properties, such as substrate specificity or stability, are often located away from the active site in areas that would not be rationally predictable. The practical experience shows that directed evolution can produce changes that are not achieved by rational design. However, these methods have their own limitations. When the desired change involves a simultaneous change in several amino acids it is not likely to be reached by the random approach. In such a case a combination of design and random techniques may be useful.

Several methods exist for creating genetically diverse libraries. Random mutagenesis of the gene and recombination using a method called DNA shuffling have been common directed evolution strategies. Recombination-based methods have gained ground as methods for creating highly diverse libraries with high fitness (Kurtzman et al. 2001). Common to all of these techniques is that they must be coupled to

a sensitive selection method, which will detect changes in the desired property of the protein. Some of the recently introduced directed evolution strategies are presented in the following.

4.2 Methods for mutagenesis

4.2.1 Error-prone PCR

In error-prone PCR, mutations are deliberately introduced into DNA during PCR. There are two methods for error-prone PCR. The first method is a modification of the normal PCR protocol. Error-prone DNA polymerases such as *Taq* DNA polymerase are commonly used because they lack proof reading activity and are inherently error-prone. Conditions such as variable concentrations of Mg^{2+} or Mn^{2+} or unbalanced dNTP concentrations increase the error rate of *Taq* DNA polymerase (Cline & Hogrefe 2000). Enzymes that make errors have also been developed. In the other method, mutation-inducible nucleosides are used. These nucleosides can be for example 8-oxo-D-guanine, which pairs with either adenine or cytosine or 3,4-dihydro-8H-pyrimido-[4,5-c]oxazine-7-one, which as a tautomer binds either adenine or guanine (Penning & Jez 2001).

4.2.2 Saturation mutagenesis

In random mutagenesis experiments the mutation rates must be kept low to obtain beneficial mutations and to avoid harmful ones, which would disrupt the structure of a mutant with the evolved desired activity. This can be achieved by controlling the number of cycles in the error-prone PCR. However, error-prone PCR generates only single base mutations leading to conservative amino acid substitutions. Thus, only an average of 5.7 amino acid substitutions out of 19 are possible with single base substitutions. With saturation mutagenesis, it is possible to generate multiple base substitutions in a single codon, resulting in nonconservative mutations (Miyazaki & Arnold 1999). In saturation mutagenesis, DNA codons are randomized using a set of degenerate oligonucleotides. The principle is shown in Figure 3. The use of oligonucleotides makes the method site-specific and therefore is a mixture of rational design and directed evolution.

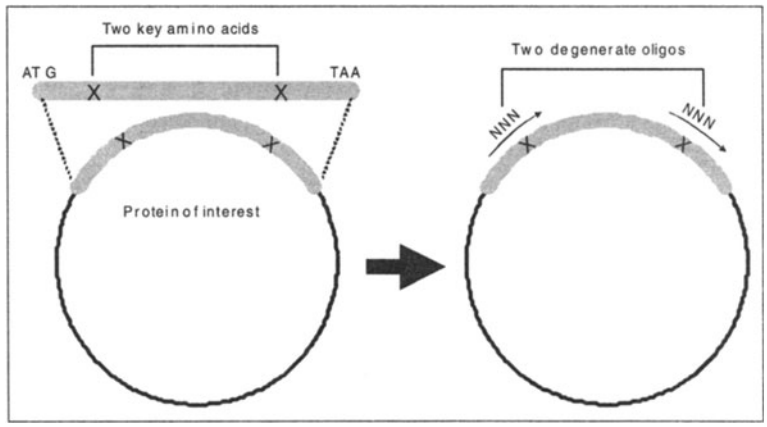

Figure 3. A method for generating a site-specific saturation mutagenesis library. Two oligonucleotides containing degenerate nucleotides in the codons of the key amino acids are used to randomise the amino acid side chains. Modified from (Anonym, 2002).

The oligonucleotides are synthesized so that two or three bases of the codon are randomly mixed from all four bases A, G, C and T. When the resulting oligonuceotides are used as primers in PCR, they randomize the DNA sequence of the target codon.

4.2.3 Mutator strains

The study of mutations in procaryotic organisms has revealed genes, whose function is to increase the rate of mutations in other genes. In *E. coli*, for example, mutator genes such as *mutY*, *mutM*, *mutD*, *mutT*, *mutA* and *mutC* affect proteins, which are necessary for accurate DNA replication. Mutagenesis of these genes has been used to find necessary components in the DNA replication machinery, which keeps DNA base pairing accurate. Mutator genes can also be used to mutate DNA that is brought into the "mutator strain is Stratagene's XL1-Red strain of *E. coli*." in a plasmid or a phage. An example of a commercial mutator strain is the *Epicurian coli* XL1-Red. The XL1-Red strain lacks the *mutS*, *mutD* and *mutT* DNA repair systems. The *mutS* mutator gene operates a methylation–directed mismatch repair system. When the newly synthetised DNA is not methylated, the errors in it can not be edited (Childs et al. 1997).

4.3 Methods for recombination

Homologous recombination is assumed to be an important process in natural evolution. It combines beneficial mutations and creates functional diversity by recombining genes, which encode different functions and share DNA sequence homology (Kurtzman et al. 2001; Zhao et al. 1998). *In vitro,* recombination has been found to be a very useful tool for directed evolution of enzymes. It can quickly generate genetic diversity that may contain significantly new and improved activities. Computer simulations of protein evolution have revealed that it is not possible to obtain novel folds by a process including mere amino acid substitutions. Instead, recombination of amino acid stretches is more likely to produce a new fold from the parental protein sequence, although the basic fold of the protein family in question still sets limits to the eventual diversity that is possible to obtain by recombination methods. However, in practical biocatalysis, the goals are usually within the limits of the protein family in question and, thus, can be achieved effectively by these methods. The method developed for *in vitro* recombination was called DNA shuffling (Stemmer 1994). The principle is depicted in Figure 4.

DNA shuffling can combine beneficial mutations from different genes, for example a pool of random mutagenesis genes or a pool of related genes. Random mutagenesis accumulates non-lethal mutations, which are deleterious to the activity. When DNA shuffling is done recursively, i.e. when improved variants generated in the first rounds are backcrossed with the wild-type, non-contributing and deleterious mutations can be eliminated. However, DNA shuffling has its drawbacks. It often reassembles parental genes. This is because both strands of DNA in the self-priming PCR reaction can come from the same parental sequence, which lowers the amount of crossovers and causes parental sequences to appear in the selection phase. Generation of too many point mutations in the reassembly PCR reaction is undesirable. The quality of PCR is inversely proportionate to DNA length, which means that DNA shuffling is not very suitable for long genes. The method relies on sequence homology, which limits the diversity that can be reassembled. DNA shuffling methods have not succesfully created crossovers in sequence identity regions shorter than 15 bases. The total amount of crossovers in shuffled sequences has also been low: the highest number of crossovers reported has been four per round of shuffling (Coco et al. 2001).

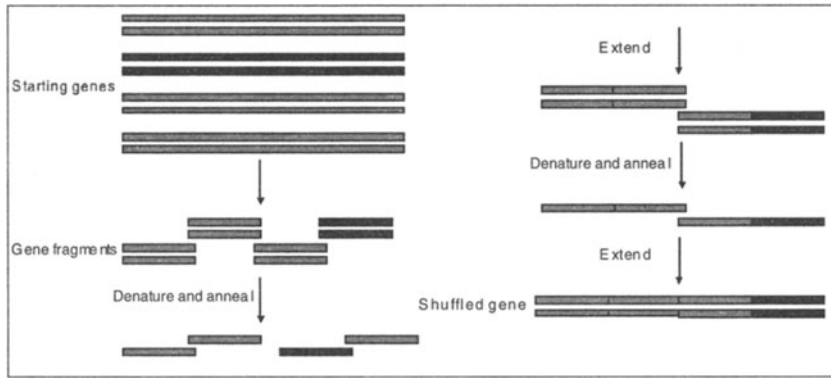

Figure 4. The principle of DNA shuffling. The starting genes are fragmented using e.g. DNase I and used for self-priming PCR. The fragments with homologous regions of sequence will anneal to each other like primers and can be extended. The product is a full-length shuffled gene, which can be amplified using normal PCR. Modified from Minshull & Stemmer (1999).

Several modifications of the method have been introduced to address these problems. To overcome the problem of homoduplex formation between parental sequences in the shujjling reactions, (Kikuchi et al 2000) designed a method using single-stranded DNA. Abécassis et al. (2000) developed a technique for DNA family shuffling of eucaryotic genes called combinatorial libraries enhanced by recombination in yeast (CLERY). CLERY combines PCR based DNA shuffling with *in vivo* recombination in yeast. Coco et al. developed a new recombination method called random chimeragenesis on transient templates RACHITT. The principle of RACHITT is depicted in Figure 5.

RACHITT may be technically challenging because ssDNA containing uracil is not easy to obtain (Pelletier 2001). To overcome this problem and to improve the recombination efficiency of RACHITT, Coco et al. (2002) introduced partially scaffolded (PARSed) DNA shuffling. Using PARSed DNA shuffling, they generated a library of five mammalian genes differing in amino acid sequence by 58% to 84%. The number of crossovers per gene ranged from 6 to 18, the average being 11, with 3.69 crossovers per gene between adjacent codons compared to 2.45 crossovers per gene in regions of sequence identity shorter than five base pairs generated by RACHITT. Hence, PARSed DNA shuffling generated crossovers at very high densities and the libraries had no bias or parental sequences, making this method perhaps the most efficient recombination method at present.

Arnold and colleagues introduced two methods to improve DNA shuffling. Zhao et al. (1998) presented a method called staggered extension process (StEP), which is a variation of the normal PCR reaction and is based on extremely short annealing/extension performed at conditions, that are optimized to limit polymerization. Random-priming *in vitro* recombination is an improvement of DNA shuffling and StEP (Shao et al. 1998). In the random-priming *in vitro* recombination method several short, random sequence $dp(N)_6$ primers are annealed to the parent templates. The primers are extended by a polymerase at 22°C to obtain short DNA fragments of 50-500 kilobases, which can prime one another based on sequence homology, are purified and reassembled into full-length genes (Arnold & Volkov 1999). However, the use of polymerases causes point mutations in the DNA fragments, which may be undesirable.

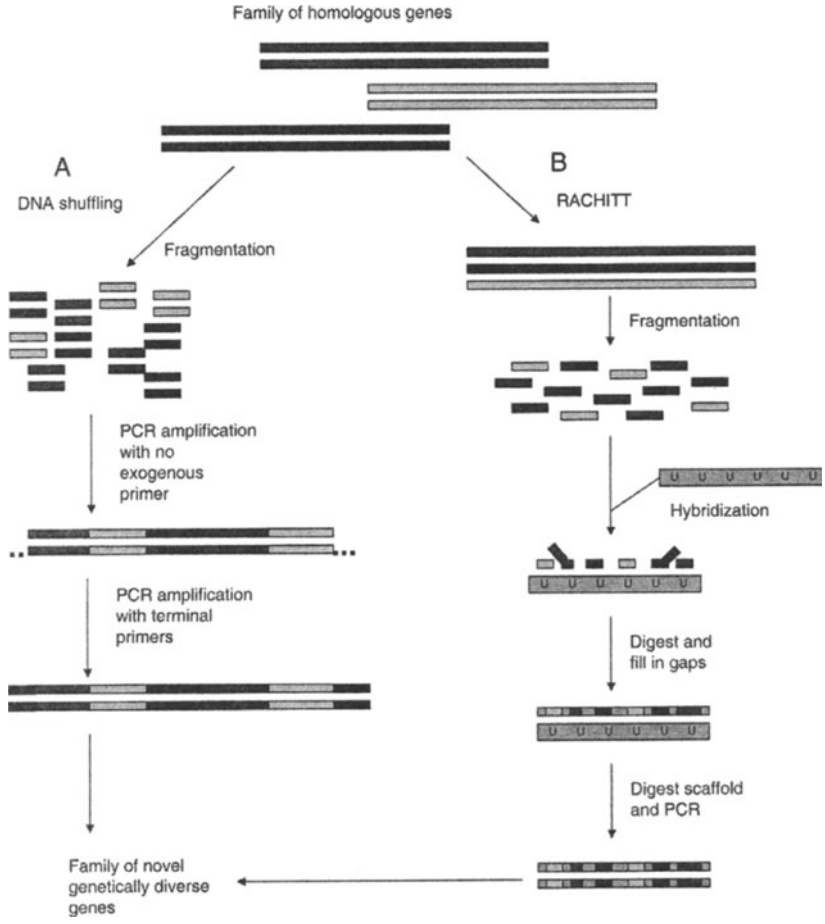

Figure 5. The principle of RACHITT compared to "classical" DNA shuffling. (A) DNA shuffling; (B) RACHITT. Modified from Pelletier 2001.

PCR-based *in vitro* recombination methods, such as DNA shuffling require random fragmentation of the DNA and synthesis of large amounts of DNA during the PCR recombination. The efficiency of PCR amplification decreases markedly for very long sequences. In practice, *in vivo* shuffling can be easier to perform since it requires no fragmentation and is less mutagenic. This is because the sequence amplification is done by the high-fidelity recombination and replication mechanism in yeast (Cherry et al. 1999) or bacteria (Zhang et al. 1998). Volkov et al. (1999) described a hybrid *in vitro-in vivo* DNA recombination method for recombining large genes and entire operons. The method is called heteroduplex formation, and is based on the ability of host cells to repair mismatched nucleotides (Figure 6). The method is proposed for recombining DNA sequences too big for PCR based DNA recombination methods.

4.4 Sequence homology independent recombination

Protein sequence alignments have shown, that structurally similar proteins often catalyze similar reactions and have similar active sites. It has also been found, that enzymes with hardly any DNA sequence homology

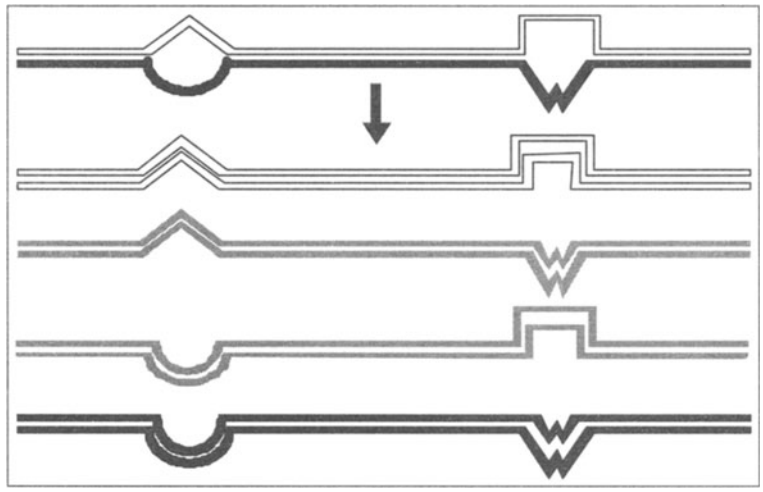

Figure 6. When regions of non-identity are repaired independently, *in vivo* repair of heteroduplexes creates a library of parent (black and white) and recombined (line patterns) sequences. Modified from Volkov et al. 1999.

can have high structural homology. In order to create new activities it is important to search large areas of sequence space. This can be done by shuffling gene families with similar three-dimensional structures. However, DNA shuffling can only be used for genes with high genetic homology. Ostermeier et al. (1999) have developed a method for creating libraries of either hybrid enzymes with low homology or of highly homologous genes with crossovers at regions of non-identity. The method is called incremental truncation for the creation of hybrid enzymes (ITCHY) and involves slow, directional, controlled digestion of DNA using Exonuclease III. In ITCHY, it is possible to create all possible single base-pair deletions of a DNA sequence. To improve ITCHY, Lutz et al. (2001a) introduced an alternative called THIO-ITCHY, based on incorporating nucleotide triphosphate analogs, such as α-phosphothioate dNTPs into the target gene. The phosphothioate internucleotide linkages protect the DNA from Exonuclease III digestion and therefore cause variation in the truncation length. The principle of the method is shown in Figure 7. Although THIO-ITCHY is useful for proteins with low sequence homology, it can only be used for combining two genes at a time, and produces only one crossover per gene. Lutz et al. (2001b) developed a method called SCRATCHY, which combines ITCHY and DNA shuffling. The method can combine genes with low sequence homology and can access extended sequence space unattainable by DNA shuffling.

ITCHY or THIO-ITCHY create random-length fragments of the target genes, thus the target genes are mostly recombined at structurally unrelated sites. As a result, only a small fraction of the crossovers connect the two target genes at sites of sequence alignment. Sieber et al. (2001) introduced a method for sequence homology-independent protein recombination (SHIPREC), which retains the correct sequence alignment. Sequence length, instead of sequence similarity, is used to create crossovers at structurally related positions. The method resembles ITCHY and can be used for creating libraries of single-crossover hybrids of two sequences with low homology. A very promising new method is synthetic shuffling, in which functional protein diversity is introduced into the desired gene by a series of synthetic

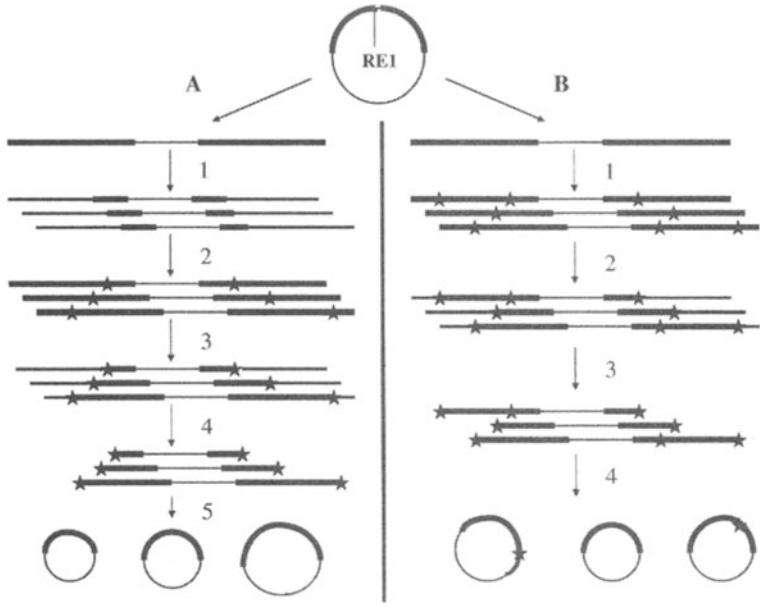

Figure 7. Incorporation of α-phosphothioates into DNA with THIO-ITCHY using (A) incorporation by primer extension or (B) incorporation by PCR amplification. (A1) The linear plasmid is treated with Exonuclease III to make single-stranded overhangs. (A2) α-phosphothioates (⋆) are randomly incorporated into the DNA in a polymerase fill-in reaction. (A3) A second Exonuclease III treatment truncates the genes up to the α-phosphothioate blocks. (A4) ssDNA is removed with mung bean nuclease. (A5) Blunt-ended plasmids are recircularized. (B1) The linear plasmid is amplified with PCR in the presence of α-phosphothioates. (B2) Exonuclease III treatment creates single-stranded overhangs, which are removed with mung bean nuclease (B3). (B4) Blunt-ended plasmids are recircularized. Modified from Lutz et al. 2001a.

oligonucleotides. The method allows the formation of amino acid variability not achieved by random mutagenesis or traditional gene shuffling (Ness et al. 2002).

In nature, many proteins consist of modules, which have a specific function in the whole protein. By mimicking nature and recombining modules, which already have a specific function such as catalysis or binding, new proteins could be generated. Several methods have been introduced to enable module shuffling. When similar restriction sites exist between the modules, they can be exchanged by digesting the genes with a restriction enzyme and ligating the fragments corresponding to the module of interest. Another approach is using homologous *in vivo* recombination (Harayama 1998). In a third approach, inteins could be used to combine modules from different proteins. Most inteins contain sequences similar to homing endonucleases, which have been identified in introns (Figure 8).

Scott et al. (1999) used *trans* – splicing inteins to catalyze head-to-tail ligation of proteins *in vivo* by split intein-mediated circular ligation of peptide and proteins (SICLOPPS). Such post-translational cyclization of proteins could make them more thermostable and resistant to proteolysis. Although the method may be useful for creating enzymes with improved stability, some enzymes might not fold properly with a linker between the amino and carboxy termini. In an *in vitro* module shuffling method described recently,

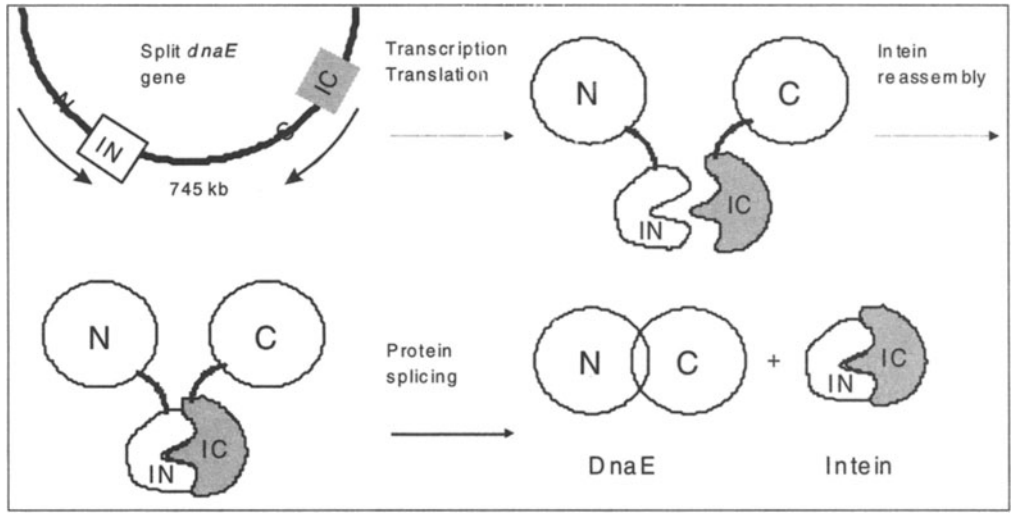

Figure 8. *Trans*-splicing of the *Synechocystis* sp. PCC6803 DnaE fragments. N and C are parts of the DnaE protein. IN and IC are intein fragments. Modified from Perler 1999.

homologous domain-encoding exons from related genes are PCR amplified using chimeric oligonucleotides. These oligonucleotides determine which modules are spliced together. The pre-made PCR fragments are reassembled into a full-length gene in a self-priming overlap PCR reaction (Kolkman & Stemmer 2001). Several other PCR-based module shuffling methods also exist as listed by Hiraga & Arnold (2003). They introduced recently a method called sequence-independent site-directed chimeragenesis (SISDC), which can be used to swap modules from different parental enzymes. The method is based on inserting *Bae* I –recognition sequences into the recombination sites in the parental genes. After digestion specific sticky ends are created between specific modules. This way it is possible to recombine known modules from different parents.

4.5 Screening and selection

In order to apply the principles of natural enzyme variability in the laboratory, random systems must have four components: a method for generating genetic diversity, a method for linking the genotype to the phenotype, a mutational pathway to new structure through functional variants and a selection for the desired biological activity. At present, the success of protein engineering experiments relies heavily on the screening of polypeptide libraries but methods for creating genetic diversity are much more developed than methods for selecting enzymatic activity. The difficulty of finding a good screening method was well illustrated in an example by Cherry & Fidantsef (2003). Selection can be accomplished in one of two ways. Indirect selection uses binding to select catalysts whereas in direct selection catalytic activity is tested for. This is the most effective way of evolving catalysts.

In selection the gene of the target enzyme is removed from a cell and the library of mutated genes is inserted into the auxotroph. Active enzymes then complement the missing activity and are selected in living cells. Screening for enzymes means displaying the generated mutant libraries on a defined matrix and assaying them for the desired activity as individual clones. The matrix can be a 96-well plate or an agar plate with the

substrate for the enzyme and an indicator of product formation, for example for pH changes. Positive clones are selected by detecting a product either manually or by a robot. The benefit of screening is that testing for enzyme activity can be performed in the conditions the enzyme is evolved for. In spite of technological development in screening techniques and robotics, screening libraries larger than 10^6 clones in a day remains difficult. Another drawback is the necessity of a reaction product, which is readily detectable at low levels, often in the mM to nM range. This means that modified substrates resembling the target of the selection must be used (Griffiths & Tawfik 2000). This could lead to enzymes adapting to catalysing the modified substrate instead of the actual target (Bornscheuer & Pohl 2001). In other words, "you get what you screen for". The advantages and disadvantages of screening and selection are compared in Table 1.

Screening large libraries of mutants can be made much easier by creating a direct physical link between a gene, the protein it encodes and the evolved function (Chen & Georgiou 2002). In *in vivo* selections both genotype-phenotype linkage and selection take place *in vivo* and cells function as compartments in a way resembling nature, keeping the genes, the proteins coded by them and the products of the proteins together. *In vivo* libraries are currently in the size range of 10^5 to 10^{10}

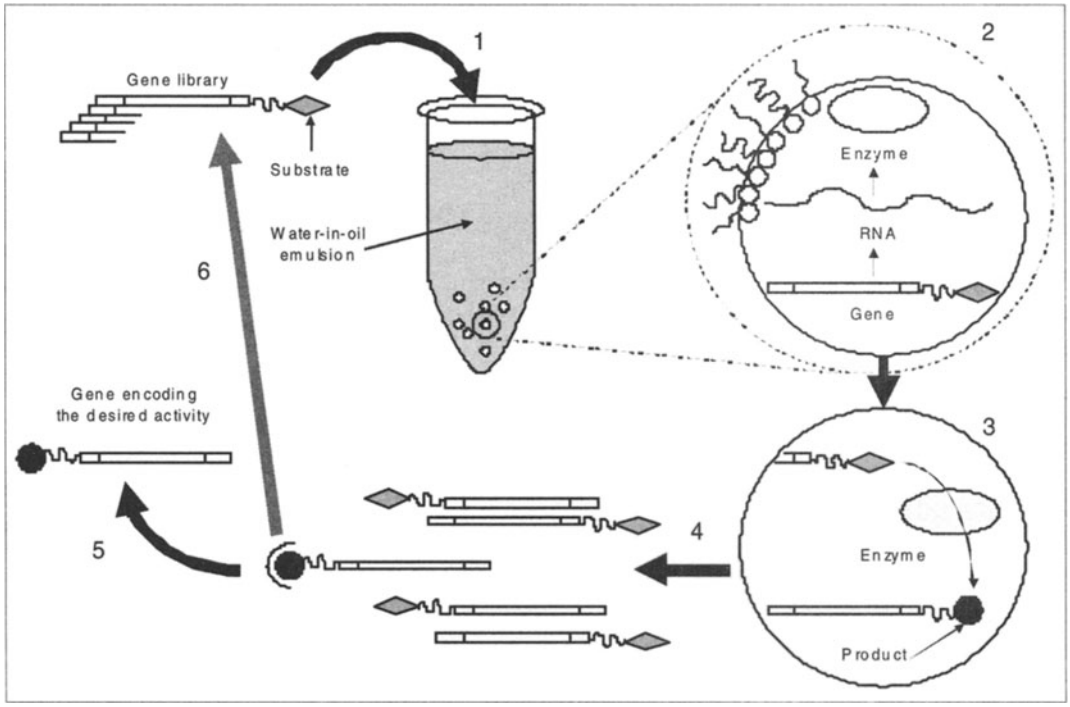

Figure 9. The principle of gene selection in a compartmentalised *in vitro* system. (1) A reaction mixture of genes linked to a substrate of the selected reaction is dispersed to form a water-in-oil emulsion. (2) The genes are transcribed and translated. (3) The protein (or RNA) with enzymatic activity converts the substrate into a product linked to the gene. (4) The emulsion is broken and all reactions are stopped. (5) Genes linked to the product are enriched and amplified. (5) The selected gene can be sequenced or (6) linked to the substrate for further selection. Modified from Griffiths & Tawfik 2000

Table 1. Comparison of screening and selection for searching libraries of evolved enzymes Brakmann 2001.

Method	Requirements	Advantages	Disadvantages
Selection	Linkage between desired activity and cell survival	Enrichment of positives and elimination of unwanted mutant, large libraries (10^{10} mutants)	False positives (viable cells with undesired mutants), indirect measurement, complex and nontrivial assay
Screening	Individualization of mutant clones; sometimes isolation from competing cellular activities, fluorogenic or chromogenic substrates often needed	Direct testing of each clone for the desired activity, assays in nonnatural environments (organic solvents, artificial substrates...), qualitative and quantitative assay of one or more parameters	Multiple pipetting/ washing/transfer steps, low throughput (10^6 mutants a day maximum at present), detectable minimum change and details of analysis determine throughput

members, whereas ribosome display libraries can be up to 10^{15} members. A larger and more diverse library can sample a greater portion of sequence space and increase the likelihood of finding very rare sequences. In partially *in vitro* selections the genotype-phenotype linkage is performed *in vivo* in cells but the selection step is *in vitro*. In totally *in vitro* selections both genotype-phenotype linkage as well as selection are done *in vitro*. Griffiths & Tawfik (2000) introduced a method called GENESCIS (Figure 9).

GENESCIS (gene selection in a compartmentalised *in vitro* system) combines genotype-phenotype linkage in a compartment and *in vitro* selection. The system uses water-in-oil emulsions, where a single gene can be isolated into an aqueous compartment. A method for *in vitro* genotype-phenotype linkage and selection called ribosome display was reported by Hanes & Plückthun (1997). Using the method a 10^8 –fold enrichment in five rounds of selection was observed. However, the method was used for evolving binding instead of catalysis. Whereas the conditions of the translation mixture may be approriate for binding, the specific conditions needed for *in vitro* enzyme activity assays may be too harsh for the ribosome –scFv complex, leading to dissociation of the genotype-phenotype linkage. Roberts & Szostak (1997) presented another method for *in vitro* genotype-phenotype linkage and selection. Covalent fusions between the protein and the mRNA encoding are generated using synthetic mRNAs with puromycin at the end. Puromycin is an antibiotic, which mimics the aminoacyl end of tRNA and acts as a translation inhibitor (Figure 10) .

A library of 10^{12} fusions between mRNA and a random 27 amino acid sequence was created in a 10-ml translation reaction. With improvements the library size could be increased to 10^{15} fusions, which is much larger than *in vivo* library sizes and would enable searching a larger portion of sequence space. Also, in comparison to ribosome display, the conditions of the selection procedure are not limited due to the covalent fusion of mRNA and the protein. However, the maximum length of the protein must be defined, since protein modules alone can be 40 to 100 amino acids in length (Fuchs & Buta 1997).

Phage display is a very popular method of screening for ligand binding. In the most widely used system

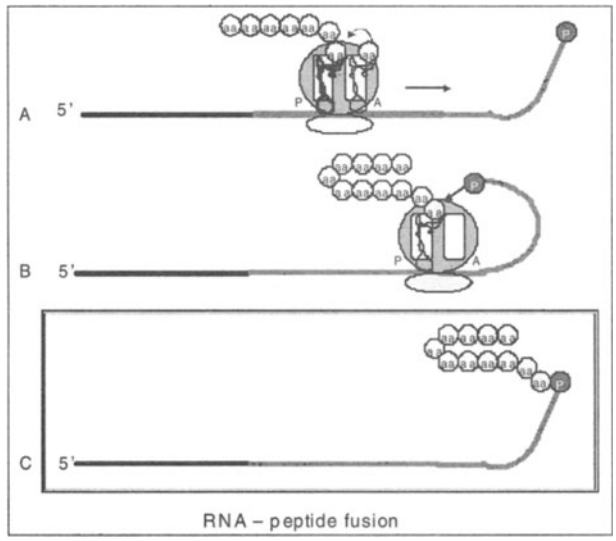

Figure 10. The proposed mechanism for the formation of the covalent mRNA-protein fusion. (A) The ribosome translates the synthetic mRNA. (B) The translation pauses at the junction between mRNA and the DNA linker. The puromycin (P) enters the A site of the ribosome and is attached to the nascent protein. (C) The covalent mRNA-protein can be purified using affinity chromatography. Modified from Roberts & Szostak 1997.

of filamentous phage display, the gene encoding mutated protein is fused to the gene encoding the phage pIII coat protein. When the phage is used to transfect cells, the generated phages display the mutated protein on their coat protein. The great benefit of this system is that the displayed protein is available for interactions with substrates and can be screened based on binding or catalytic activity. Phage display techniques can create relatively large libraries of approximately 10^9 variants and are a simple method for screening libraries (Chen & Georgiou 2002). However, it is difficult to use phage display for evolving catalysts efficient in turnover. Activity selection is based on single-turnover events so enzymes with a slow turnover may then also be selected.

In a method for high-throughput screening addressing some of the problems of phage display the proteins are displayed on cells instead of phage. However, incorporation of foreign proteins in the outer membrane can be toxic for the cell. The exposure of surface-anchored proteins to their substrates may be limited due to steric effects caused by the lipopolysaccharide layer of the outer membrane. In some cases the steric hindrances can be avoided by fusing the evolved protein onto components protruding far away from the cell surface, such as flagella. Still, as in phage display, most proteins are unsuitable for this (Chen & Georgiou 2002). The benefit of cell-surface display is that it enables the use of fluorescence-activated cell sorting (FACS), a very high throughput screening technology. In principle, enzyme reactions convert substrates with a cationic anchor to fluorescent products, which are captured by the cell surface due to the negative net charge of the surface. Hence a physical link is created between a fluorescent product and the cell expressing the respective enzyme (Figure 11).

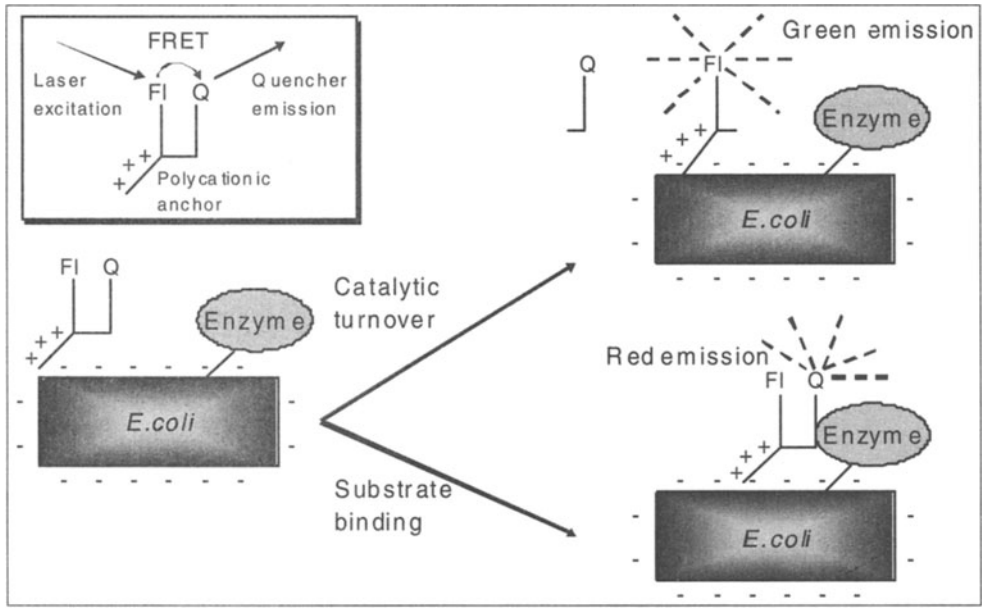

Figure 11. FACS – based enzyme evolution using FRET-substrates. FRET –substrates contain a fluorescent moiety (Fl) and a quencher moiety (Q), which emit different wavelenghts, and a cationic anchor. Enzymes evolved for improved catalytic activity will cleave the bond between Fl and Q and display green fluorescence. Enzymes evolved for improved binding will bind the quencher moiety and display red fluorescence. Cells can thus be sorted based on k_{cat} or K_m. Modified from Olsen et al. 2000.

Fluorescence resonance energy transfer (FRET) substrates contain a fluorescent moiety and a quencher moiety. When cells containing these substrates are excited with laser, they emit different wavelenghts depending on whether the quencher moiety has been cleaved off by the enzyme displayed on the cell surface. By using FRET-substrates, mutated enzymes can be distinguished based on k_{cat} or K_m (Olsen et al. 2000). Although useful, application of FRET–substrates is limited to enzymes, which can be expressed on a cell and for which a FRET-substrate can be constructed, thus making it a rather specialized screening approach (Farinas et al. 2001).

As a summary, Kurtzman et al. (2001) compared the use of random mutagenesis and high homology DNA family shuffling and estimated the amounts of variants with a certain improvement in activity, that can be obtained with different selection methods (Table 2).

5. CONCLUSIONS

During the last two decades protein engineering methods, both rational design and directed evolution methods, have produced remarkable results in stabilizing enzymes and modifying their activity. Design and random techniques both have their benefits and drawbacks. Random methods quickly create a large pool of enzyme variants, which is a benefit compared to design techniques. Often, desired changes in enzymes that cannot be made by rational design are achieved by directed evolution methods. Achieving the optimal result may sometimes require the use of both rational design and directed evolution methods.

Table 2. Comparison of the estimated number of variants with a 4-fold improved single activity or two 4-fold improved activities obtainable with different selection methods after random mutagenesis or high homology DNA family shuffling (Kurtzman et al. 2001).

		Clones with a single activity improved four sfold		Clones with two activities improved four fold	
Screen/ selection	Screening capacity	Random mutagenesis	High homology family shuffling	Random mutagenesis	High homology family shuffling
Cell-based assay	10^3	0	10	0	5
Surface display	10^6	10	10^4	0	10^2
Phage display	10^9	10^2	10^6	0	10^3
Ribosome display	10^{12}	10^4	10^8	10	10^4

New protein engineering tools are becoming available continuously and will speed up the process. We can expect remarkable improvements in substrate specificity and reaction rates in the case of minor side activities of existing enzymes. One of the areas, in which new progress is expected to occur, is the development of efficient screening methods for new protein variants. With present systems many potentially interesting proteins may not be found at all.

Several spectacular examples of changes in substrate specificity, pH-optimum and catalytic rate are available. The changes are, however, limited to existing protein folds. A problem outside the established protein folds can be that once the basic 3-D structure that holds the ordered form of the proteins together is destroyed, the resulting proteins are no more properly folded, precipitate in the cell and are quickly destroyed (Balance et al. 1999). The dream of protein engineer is the creation of a completely unknown and non-natural enzyme, for example to degrade man-made toxic compounds. This dream is still far from reality due to the enormous sequence space of possible protein structures and our inability to reliably predict the protein structure and function. It is not known how common functional structures are in this space. Calculations (Axe 2000) and direct experiments (Blanco et al. 1999) however seem to indicate that active proteins are very rare. On the other hand, this unexplored universe of protein structures forms an ocean of opportunitites for a protein engineer.

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Thermozymes

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1. INTRODUCTION

Life is known to thrive in the most challenging environments once thought to be too hostile to permit the survival of living organisms. Microbial communities known as extremophiles are found in conditions of extremes of salinity (halophiles), alkalinity (alkalophiles), temperature (psychrophiles or thermophiles), pressure (barophiles) as well as other drastic conditions. The most extremotolerant of these microorganisms are members of the domain archae, the most recently described yet least well understood domain of life (Rothschild & Manicinelli 2001). Among the extremophiles thermophiles and thermostable enzymes (thermozymes) are the most extensively studies and will be discussed in this chapter.

In 1969 T. D. Brock and colleagues discovered *Thermus aquaticus,* the source of *Taq* polymerase used in PCR techniques. *T. aquaticus* was then considered an extreme thermophile since it grew optimally at 75°C (Brock & Freeze 1969). Since then it has been found that hyperthermophiles such as *Pyrolobus fumarii* can grow at up to 113°C (Blöchl et al. 1997). This is the most thermophilic organism known at present, which grows in the temperature range of 90 to 113°C. The upper temperature at which life is possible is still unknown, but it is probably not much above 113°C. Molecules such as amino acids and metabolites become highly unstable (ATP is spontaneously hydrolyzed in aqueous solution at temperatures below 140°C) and hydrophobic interactions weaken significantly at temperatures above 110°C.

Hyperthermophiles are organisms, which grow optimally in a temperature range between 80 and 110°C. These organisms, which are either bacterial or archaeal species have been isolated from all types of terrestrial and marine hot environments, including natural and man-made environments. Enzymes from these organisms (or hyperthermophilic enzymes) developed unique structure-function properties of high thermostability and optimal activity at temperatures above 70°C. Some of these enzymes are active at temperatures as high as 110°C and above (Vieille et al. 1996). Thermophilic organisms grow optimally between 50 and 80°C. Their enzymes (thermophilic enzymes) show thermostability properties which fall between those of hyperthermophilic and mesophilic enzymes. These thermophilic enzymes are usually optimally active between 60 and 80°C. Active at high temperatures, thermophilic and hyperthermophilic enzymes typically do not function well below 40°C. The nomenclature of thermophiles and hyperthermophiles will be used interchangebly in the text. Enzymes from these organisms serve as model systems for use by biologists, chemists, and physicists interested in understanding enzyme evolution, molecular mechanisms for protein thermostability and the upper temperature limit for enzyme function.

The use of enzymes as biocatalysts till recently was from mesophilic organisms. Despite the many advantages the applications of such enzymes, their use is restricted by limited stability to small ranges of pH, temperature, ionic strength, etc. Recently there has been a burst of research interest in the use of extremophiles for the production of enzymes, which are stable at broader ranges and at industrially relevant conditions.

In the present chapter the sources of thermozymes, the likely mechanisms for their stability and benefits of these enzymes over those from mesophiles in some specific applications and the recombination of these enzymes will be discussed.

2. GEOTHERMAL SITES AS SOURCES OF THERMOPHILES

The thermophiles are found in a number of exotic regions of the earth. In situ temperatures between 80 and 115°C were found to be conducive for a number of hyperthermophiles. Some examples of such locations and organisms screened are mentioned in Table 1. In the last decade, a number of hyperthermophilic archaea, the least understood domain of life (Rotschild & Manicinelli 2001) have been isolated and are able to grow around the boiling point of water. The organisms with the highest growth temperatures (103–110°C) are members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus* and *Methanopyrus*. Among bacteria, *Thermotoga maritima* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95°C, respectively (Herbert & Sharp, 1992). These properties imply potentially important industrial and biotechnological implications due to the fact that enzymes from such microorganisms can be employed for use in harsh industrial conditions where their specific catalytic activity is retained.

Table 1. Examples of sites serving as sources of microorganisms which can provide thermo tolerant enzymes

Source	Microorganism	Enzyme
Hot spring	*Thermus* sp	α-amylase
"	*Bacillus* sp. WN.11	"
Deep sea hydrothermal vent	*Staphilothermus marinus*	"
Marine solfatare	*Thermococcus litoralis*	Pullulanase
Decomposed plant samples from a lake	*Clostridium absonum* CFR-702	Cellulase free xylanase
Hot spring	*Bacillus thermoleovocans* ID-1	Lipase
Compost of fermenting citrus peels, coffee and tea extract residues	Bacillus strain MH-1	Endochitinase
Compost	*Bacillus stearothermophilus* CH-4	β-N-Acetylhexosaminidase
Korean salt fermented anchovy	Bacillus sp. KYJ96	β-amylase
Deep sea hydrothermal vent	*Pyrococcus abyssi*	Alkaline phosphatase
Sediments of hot springs	Bacillus sp. 3183	α-amylase-like pullulanase
Garbage dump	*B. circulans*	Xylanase
Compost treated with artichoke juice	*Bacillus* sp.	Inulinase

3. THERMAL STABILITY OF THERMOPHILES AND THERMOZYMES

Microorganisms, like all living things, adapt to the condition in which they have to live and survive. Some thermophiles are reported to contain proteins, which are thermostable and resist denaturation and proteolysis (Kumar & Nussinov 2001). Specialized proteins known as 'chaperonins' are produced by some of these organisms. These help proteins after their denaturation, to refold the proteins to their native form and restore their functions. The cell membrane of many thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures. The archae, which compose most of the hyperthermophiles, have lipids linked with ether on the cell wall. This layer is much more heat resistant than a membrane formed of fatty acids (De Rosa et al. 1994).

Thermozymes share the same catalytic mechanisms with their mesophilic counterparts. When cloned and expressed in mesophilic hosts, these enzymes have been found to retain their thermal properties, indicating that these properties are genetically encoded. Sequence alignments, amino acid content comparisons, crystal structure comparisons, and mutagenesis experiments indicate that hyperthermophilic enzymes are, indeed, very similar to their mesophilic homologues. No single mechanism is responsible for the remarkable stability of hyperthermophilic enzymes. Increased thermostability must be found, instead, in a small number of highly specific alterations that often do not obey any obvious traffic rules.

The molecular mechanisms involved in protein thermostabilization, which have been studied include amino acid composition, intrinsic propensity, disulfide bridges, hydrophobic interactions, aromatic interactions, hydrogen bonds, ion pairs, intersubunit interactions and oligomerization, conformational strain release, helix dipole stabilization, packing and reduction in solvent-accessible hydrophobic surface, docking of the N and C termini and anchoring of loose ends, metal binding, post-translational modifications and other extrinsic parameters. A very good review of these mechanisms and the large volume of study done in this area are given in Vieille & Zaikus (2001).

It is now accepted that these proteins are more rigid than their mesophilic homologues at mesophilic temperatures and that rigidity is a prerequisite for high protein thermostability. This hypothesis is supported by experiments with frequency domain fluorometry and anisotropy decay, hydrogen-deuterium exchange and tryptophan phosphorescence (Gerhenson et al. 2000). Protein flexibility indexes for mesophilic and thermophilic proteins, starting from normalized atomic temperature showed that flexibility decreased as thermostability increased.

Extracellular and cell-bound hyperthermophilic enzymes (i.e., saccharidases and proteases) are optimally active at temperatures above the host organism's optimum growth temperature and are highly stable. For example, *Thermococcus litoralis* amylopullulanase is optimally active at 117°C, which is 29°C above the organism's optimum growth temperature of 88°C (Brown & Kelly 1993). Also, while they are usually less thermophilic than extracellular enzymes purified from the same host, intracellular enzymes (such as xylose isomerases) are usually optimally active at the organism's optimal growth temperature. Only a few enzymes have been described that are optimally active at 10 to 20°C below the organism's optimum growth temperature. While most thermozymes are intrinsically very stable, some intracellular enzymes get their high thermostability from intracellular factors such as salts, high protein concentrations, coenzymes, substrates, activators, or general stabilizers such as thermamine.

4. BENEFICIAL ASPECTS OF THERMOZYMES IN APPLICATIONS

Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes. Some biocatalytic conversions and industrial applications requirement of such thermostable enzymes are presented in Table 2. One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient bioremediation. Other values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions. Such enzymes can also be used as models for the understanding of thermostability and thermo-activity, which is useful for protein engineering. The following sections describe specific beneficial thermostable enzymes, their sources, characteristics and requirements of high temperature resistance in the applications. Some specific thermophilic enzyme sources, optimal condition of growth and references are given in Table 3.

Table 2. Bioconversion reactions and applications of thermostable enzymes

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-amylase (bacterial)	90-100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-amylase (fungal)	50-60	Starch → dextrose syrups	Production of maltose
Pullulanase	50-60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45-65, 105[1]	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65-75[2]	Chitin → chitobiose Chitin → N-acetyl glucosamine (chitibiase) N-acetyl glucosamine → glucosamine (deacetylation) Chitin → chitosan (deacetylase)	Food, cosmetics, pharmaceuticals, agrochemicals
Cellulase	45-55,95[3]	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65-85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30-70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90-95	DNA amplification	Genetic engineering/PCR

[1]xylanase from *Thermotoga* sp.; [2] within this range enzyme activity was high; [3]cellulases from *Thermotoga* sp.

Table 3. Source microorganisms and properties of thermostable enzymes

Enzymes	Organism	Enzyme properties		References
		Optimal temperature (°C)	Optimal pH	
α-amylase	*Bacillus licheniformis*	100	6.0-6.5	Viara et al. 1993
	Pyrococcus woesei	100	6.5-7.5	Koch et al. 1991
	Thermococcus aggreganes	100	5.5	Canganella et al. 1994
	T. fumicolans	95	4.0-6.3	Estele et al. 1997
β-amylase	*Clostridium thermosulphurogenes*	75	5.5	Shen et al. 1988
Pullulanase	*Pyrococcus woesi*	100	5.5-6.0	Rudiger et al. 1995
Xylanases	*Pyrococcus furiosis*	102	-	Kengen et al. 1993
	Sulfolobus solfataricus	105	5.3	Nucci et al. 1993
	Thermotoga sp. Strain FjSS3-B1	105	6.8-7.8	Ruttersmith and Daniel, 1991
	Thermotoga sp. Strain FjSS3-B1	115	5.3	Simpson et al. 1991
Cellulases	*Pyrococcus furiosus*	102-105	-	Kengen et al. 1993
	Rhodothermus marinus	95	6.5-8.0	Hreggvidsson et al. 1996
	Thermotoga neapoltana (EndocellulaseB)	106	6.0-6.6	Bok et al. 1998
Proteolytic enzyme	*Bacillus stearothermophilus*	85	-	Rahman et al. 1994
	Thermococcus celer	95	7.5	Klingberg et al. 1991
Lipases	*Pseudomonas sp*	90	11.0	Rathi et al. 2000
	*Pyrococcus furiosus** (esterase)	100	-	Ikeda and cark, 1998
	Pyrococcus horikoshii	97	5.6	Ando et al. 2002

5. SOME INDUSTRIALLY IMPORTANT THERMOZYMES

5.1. Amylolytic enzymes

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases, β-amylases and isoamylases or pullulanases. The enzymatic conversion of starch includes gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension, liquefaction, which involves partial hydrolysis and loss in viscosity, and saccharification, involving the production of glucose and maltose via further hydrolysis. Gelatinisation is achieved by heating starch with water, and starch is water-soluble only at high temperatures, which are dependent on the source (Rakshit 1998). For hydrolysis of the starch to proceed immediately after gelatinization, hence, among other things avoiding a lot of cooling time, the enzyme has to be thermostable. This is very valuable to the maltose and glucose syrup industry.

A number of attempts have been made to isolate and characterize thermostable amylolytic enzymes from diversified sources to meet the requirements of the starch industry. Table 3 shows thermostable microbial starch-hydrolyzing enzymes and their properties. Pullulanases that were produced in earlier

work on different microorganisms were not very suitable for operation under the conditions prevailing in the industry However, many thermophilic microorganisms have since been found to produce pullulanases (Canganella et al.. 1994, Swamy & Seenayya 1996) and β-amylases (Shen et al. 1988, Nipkow et al. 1989, Erra-Pujada et al. 2001) with desirable qualities.

Termamyl and Fungamyl are two well-known amylolyic enzymes, which are now available commercially. These enzymes are used worldwide for the production of glucose syrups and syrups with different level of dextrose equivalent (DE). Hetero-oligosaccharides have been synthesized by Termamyl obtained from *Bacillus licheniformis* (Chitradon et al. 2000). In the presence of soluble starch and added non-starch sugars, oligosaccharides were produced by the reversed catalytic reaction.

Although, raw starch dominates in nature, there are few enzymes that can catalyze its hydrolysis efficiently. Saccharification of liquefied starch is carried out at low pH values. However, currently used thermostable α-amylases are not stable at such low pH (Crab & Mitchinson 1997). An economical process could be attained through the use of amylases stable at the saccharification stage. A one-step process of starch hydrolysis using amylolytic enzymes would decrease the costs of glucose production. The anticipated diversity of species of thermophiles within high temperature environments and the requirements of the new and improved technological operations for enzymes with novel and fitting characteristics have placed a challenge both for the scientific and business community.

5.2. Xylanases

The production of the pulp from the wood involves treatment at high temperature and basic pH. Treatment with xylanase at elevated temperatures disrupts the cell wall structure. This facilitates lignin removal in the various stages of bleaching. The enzymes used in such procedures should exhibit a high thermostability and activity in a broad pH range. So far commercially available xylanases can only partially fulfill these requirements. However, some xylanases have been reported to exhibit higher thermal stability and optimal activity ranging from 80 to 100°C (Morris et al.. 1998). Xylanases have been generally classified in to two families. Family 10 (EXs 10) having high molecular weights (greater than 30 kDa) and Family 11 (EXs 11) which are low molecular weight (less than 30 kDa) xylanases. Among the two families, the EX 10-fold is generally more thermostable. Due to their capacity to enhance the bleaching of kraft paper, however, EXs 11 have remained more attractive (Morris et al. 1998).

Thermostable xylanase have been isolated from a number of bacterial and fungal sources (Table 3). Members of the *Bacillus* sp., *Streptomyces* sp., *Thermoascus aurantiacus* and *Fusarium proliferatum* have been reported to produce xylanases which are active at temperatures between 50-80°C. *Dictyoglomus* sp has been described to produce xylanases operating at an optimum temperature of 90°C; *Thermotogales* sp has been reported to secrete thermostable xylanases, which can function at higher temperatures. Alkaliphilic and cellulase-free xylanases with an optimum temperature of 65°C from *Thermoactinomyces thalophilus* subgroup C and cellulase-free xylanases from *Clostridium abusonum* CFR-702 (Swaroopa et al. 2000) were also reported recently. Simpson et al. (1991) reported that *Thermotoga* sp. enzyme was active at 115°C. Apparently extreme thermophiles that are able to secrete xylanase are few and search for a thermophile with high yield of enzyme and the desired characteristics is still being pursued.

5.3. Cellulases

In the current industrial scenario, cellulolytic enzymes are employed in the color extractions of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of

biomass that contains cellulose to improve nutritional quality of forage and in the pretreatment of industrial wastes. In order to attack the native crystalline cellulose, which is water insoluble and occurs as fibers of densely packed structures, however, thermostable cellulases active at high temperature and high pH are required. Thermostable cellulases of archaeal origin include those isolated from *Pyrococcus furiousus* (Kengen et al. 1993) and *Pyrococcus horikoshi* (Ando et al. 2002). While the latter has an optimum temperature of 97°C, the enzyme from *Pyrocuccus furiosis* has shown optimal activity at 102-105°C (Table 6). *Sulfolobus solfataricus* MT4, *S. acidocaldarius* and *S. shibatae* were also described as producers of β-glucosidases. From *Thermotoga maritima* MSB8 optimally active cellulase acting at 95°C and between pH 6.0 and 7.0 was reported (Bronnenmeier et al. 1995). CelA, with the ability to hydrolyze microcrystalline cellulose, was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* (Zverlov et al. 1998) and maximal activity of this enzyme was observed at pH 5.0–6.0 and 85-95°C. A highly thermostable cellobiose (115°C at pH 6.8-7.8) was also produced from *Thermotoga* sp. FjSS3-B1 (Ruthersmith & Daniel, 1991, 1992).

It is obvious that the successful utilization of cellulose is dependent on the development of economically feasible technologies for the production of cellulase. The biopolishing process of cotton in the textile industry, for example, requires cellulase stable at high temperature close to 100°C (Ando et al. 2002). Presently used enzymes for this purpose, however, are active only at 50-55°C. Cellulase production is also found to be the most expensive step during ethanol production from cellulosic biomass, and accounted for approximately 40% of the total cost (Spano et al. 1975). In the food industry, degradation of cellulose by acids is still unsatisfactory and results in the decomposition of the sugars. Finally, even though many cellulolytic enzymes of thermophilic origin are known, their function under physiological condition remains unclear.

5.4. Chitinases

The production of chitosan from chitin involves a deacetylation reaction that is done using 40% sodium hydroxide solution. This is a highly corrosive stream which is difficult to control. Attempts are thus being made to do this conversion using deacetylase enzyme. Perhaps a thermostable deacetylase enzyme will help in increasing yields and conversion rates and allow reuse of the enzyme. Endo-acting chitin hydrolase chitinaseA, the exo-acting hydrolase chitinase B and N-acetyl-D-glucoseaminidase are responsible for chitin degradation. However, chitin is not easily accessible to chitinases and chitin deacetylases. Evidences from X-ray diffraction studies have shown that chitin is a highly ordered crystalline structure and does not dissolve in water. This property of chitin has shown the need for thermostable enzymes.

Chitinase can also be used to produce value-added chemicals from the waste generated by sea-food processing industries, i.e. shrimp and prawns. In such processes also thermostable chitinase would offer potential benefits.

5.5. Proteases

There are some reports dealing with thermophilic proteases. A protease from *Baullus stearothermophilus* was stable at 60°C (Salleh et al. 1977). *B. stearothermophilus* sp. produced an alkaline and thermostable protease which was optimally active at 85°C (Razak et al. 1997). In a chemically defined medium, thermophilic and alkaliphilic *Bacillus* sp. JB-99 produced thermostable alkaline proteases (Johnevelsy & Naik 2001). Dominant microbes producing thermophilic protease are the strains of the genera *Pyrococcus, Thermococcus and Staphylothermus.*

5.6 Lipases

Most of the industrial processes in which lipases are employed function at temperatures exceeding 45°C. The enzymes, thus, are required to exhibit an optimum temperature for activity around 50°C. Some enzymatic processes for the physical refining of seed oils have four distinct requirements. These include pH of 5.0 and optimal temperature of around 65°C, adding an enzyme solution and enzyme reaction followed by the separation of the lysophosphatide from the oil at about 75°C (Klaus 1998). These reactions, therefore, are enhanced through the utilization of thermo-tolerant lipases.

Several *Bacillus* species have been reported to be the main source of lypolytic enzymes (Luisa et al. 1997). While most of these enzymes were active at a temperature of 60°C and pH of 7.0, lipases from *B. thermoleovorans* and a thermophilic *Rhizopus oryzae* strain could moderately function at extreme pH and temperature values (Abel et al. 2000). There are a few other lipases, which are able to operate at 100°C but their half-lives are short (Rathi et al. 2000). The pH and temperature optima for the catalytic activity of some thermostable lipases are given in Table 3.

There are some thermostable lipases from archaeal origin also. Phospholipase A_2 was obtained from *Pyrococcus horikoshii* (Yan et al. 2000) and was used in crude oil degumming. This enzyme was found to optimally react at 95°C and pH of 7.0. Phospholipase A_2 has a better performance in the degumming processes of oil refineries and thereby reduces wastewater problems and running costs (Klaus 1998).

Among the desirable characteristics that commercially important lipases should exhibit, alkali tolerance and thermostability are the main requirements. In order to meet this end, there is a continuous search for sources of highly active lipolytic enzymes with specific stability to pH, temperature, ionic strength and organic solvents.

5.7 DNA polymerases

The Polymerase Chain Reaction (PCR) process has led to a huge advance in genetic engineering due to its capacity to amplify DNA. The three successive steps in this process include denaturation or melting of the DNA strand (separation) obtained at a temperature of 90-95°C, renaturation or primer annealing at 55°C followed by synthesis or primer extension at around 75°C. Development in this process has been to a large extent facilitated by the availability of thermostable DNA polymerases, which catalyse the elongation of the primer DNA strand.

In earlier PCR procedures, DNA polymerases, which were isolated from *E. coli* were utilized. These enzymes, however, lost their enzymatic activities at elevated temperatures and, thus, adding a new polymerase enzyme after each cycle following the denaturation and primer hybridization steps was necessary. This process made the thermal cycling a time-consuming and costly procedure.

Taq Polymerase from *Thermus aquaticus* was the first thermostable DNA polymerase characterized (Chien et al. 1976). Repeated exposure to 98°C in a reaction buffer had little effect on the enzyme activity and significant activity remained after exposure to 99°C. Although, Taq polymerase exhibited a 5'–3' exonuclease activity, a 3'–5'exonuclease activity was not detected. Thus, the base insertion fidelity is low as the enzyme is unable to correct misincorporated nucleotides. It had also been possible to determine the error rates of some polymerases in terms of base pairs. A variety of thermostable polymerases with 3'-5'–exonuclease-dependent proof reading activity is required from a high fidelity

polymerase. Optimization of the PCR procedure can be done by mixing a standard polymerase, such as Taq polymerase with high fidelity polymerases. A thermostable DNA polymerase having all the desirable characteristics will indeed improve the results obtained by PCR machine.

6. RECOMBINATION DNA TECHNOLOGY FOR THERMOZYMES

More than 100 genes from hyperthermophiles have been cloned and expressed in mesophiles. When the properties of the native and recombinant hyperthermophilic enzymes are compared, the majority of hyperthermophilic enzymes expressed in *E. coli* retain all of the native enzyme's biochemical properties, including proper folding, thermostability, and optimal activity at high temperatures. Thus, while a few proteins from hyperthermophiles might require extrinsic factors (e.g., salts or polyamines), or posttranslational modifications (e.g., glycosylation) to be fully thermostable, most proteins from hyperthermophiles are intrinsically thermostable, and they can fold properly even at temperatures 60°C below their physiological conditions. The fact that most hyperthermophilic enzymes are properly expressed and folded in *E. coli* has greatly facilitated their study, since they can be purified from *E. coli* rather than from an often hard-to-grow hyperthermophilic organism. Additional indirect evidence for the correct folding of recombinant hyperthermophilic proteins is the fact that crystal structures of recombinant hyperthermophilic proteins are typically similar to that of their mesophilic homologues. The idea that recombinant and native hyperthermophilic protein structures are identical has become so widely accepted that in some studies both the native and recombinant enzymes are used indifferently in crystallization studies (Aguilar et al. 1997). It is unclear whether all hyperthermophilic proteins can be expressed in a mesophilic environment, since unsuccessful experiments are typically not reported. So far, fewer than 10% of all the hyperthermophilic enzymes expressed in *E. coli* have been reported to have stability, catalytic, or structural properties different from those of the enzyme purified from the native organism.

7. CONCLUSIONS

Recent investigations have demonstrated that extremophilic archaea, bacteria and fungi have colonized environments that were believed to be inhospitable for survival. Their true diversity in fact, is not yet been fully explored. The thermostable enzymes isolated from these organisms have just started providing conversions under conditions that are appropriate for industrial applications. The conditions required by these thermostable enzymes which bring about specific reactions not possible by chemical catalysts are still mild and environmentally benign, as compared to the temperatures and pressures required for chemical conversions. However, with the availability of thermostable enzymes a number of new applications in the future are likely. Although, believed to provide tremendous economical benefits, production of the enzymes to the level required by the industries has remained a challenge.

8. PERSPECTIVES

Recent advances in genomics have made possible quick method to decipher the DNA sequence in a vast number of pro- and eukaryotes. This data confirms that genetic evolution occurred from simple archae, including many extremophiles, to multicellular organisms. With the complete sequencing of human and other genomes, it has been realized that while the blue print for protein synthesis is important, the post translational changes leading to specific 3D confirmations are important to understand its activity fully. In this area of proteomics, thermozymes will prove an invaluable tool to determine protein stability and hence go on to better protein engineering and control of enzyme based processes.

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Cold-Adapted Enzymes

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1. INTRODUCTION

1.1 Cold and extreme environments

Temperature is a critical environmental factor controlling the evolution and biodiversity of life on Earth. Microorganisms have been isolated with the capacity to grow or metabolise at temperatures as high as 121°C (Kashefi & Lovley 2003) and as low as -20°C (Carpenter et al. 2000, Christner 2002, Deming 2002, Rivkina et al. 2000, Rivkina et al. 2002, Wagner et al. 2003). The capacity to thrive under conditions of thermal extremes has led to the adoption of the term *extremophiles*, to describe these, and other organisms which require extreme environmental conditions for growth (Cavicchioli & Thomas 2000, Cavicchioli & Thomas 2004, Rothschild & Mancinelli 2001). The capacity of organisms to survive in extreme environments has led to the rapid development of this field resulting in intense academic interest (*e.g.* the third complete genome sequence was for an extremophile (Bult et al. 1996)) and biotechnological application (Demirjian et al. 2001, Hough & Danson 1999, van der Berg 2003). It has also reinvigorated the field of astrobiology (exobiology) (Cavicchioli 2002), fueled, in particular, by the discoveries of live microorganisms in ice cores taken from sea ice (Deming 2002, Thomas & Dieckmann 2002) and the surface of deep, sub-glacial lakes in Antarctica (Christner et al. 2001), and the presence of water in cold environments on Mars (Mitrofanov et al. 2003) and Europa (Chyba and Phillips 2001).

The majority (75-85%) of Earth's biosphere is cold (< 5°C), comprised of alpine regions, polar regions, the deep-sea, caves, the terrestrial sub-surface down to a few kilometers, and the upper atmosphere (Baross & Morita 1978, Cavicchioli et al. 2002a, Russell & Hamamoto 1998, Sheridan et al. 2000, Wainwright et al. 2003). In particular, the depths of the ocean below ~1000m represent a large proportion of the cold biosphere as the cold (1-5°C) water covers ~70% of the planet (Baross & Morita 1978).

1.2 Cold-adapted organisms

Cold-adapted organisms have been isolated from all cold environments examined. In addition to isolated organisms, an enormous biodiversity has been detected using molecular approaches; primarily rDNA libraries and fluorescent *in-situ* hybridisation. Virtually all forms of life have been identified, including bacteria (Margesin et al. 2003), archaea (Cavicchioli et al. 2000; Karner et al. 2001), yeast (Hakon et al. 2003, Margesin et al. 2003), algae (Loppes et al. 1996, Morgan-Kiss et al. 2002), fungi (Robinson 2001), lichens, mosses and higher plants (Pannewitz et al. 2003, Green

et al. 1999), invertebrates (Olsen et al. 1991) and fish (Fields et al. 2001). Cold-adapted organisms have also been readily isolated from refrigerated appliances and products (Russell & Hamomoto 1998, Cavicchioli et al. 2002b). Clearly, temperature is not a barrier to life in cold, natural or artificial environments.

1.2.1 Eurythermal and stenothermal psychrophiles

Cold-adapted organisms are typically described as psychrophilic or psychrotrophic / psychrotolerant. Psychrophiles have been categorised as having an optimum temperature for growth (T_{opt}) of ≤ 15°C with a maximum growth temperature of ≤ 20°C, with psychrotolerant microorganisms being able to grow at low temperatures but with a maximum growth temperature above 20°C (Cavicchioli et al. 2002a, Morita 1975, Russell and Hamamoto 1998). These terms however have been arbitarily created and may not adequately reflect the thermal growth capacities or the physiological state of these organisms. For example, the majority of microorganisms isolated from cold environments could be placed in the category psychrotolerant. It is more likely however that these organisms are "mesotolerant", in that they grow effectively and colonise cold environments, while tolerating warmer temperatures typically colonised by mesophiles. Other physiological measures of the state of the cell (*e.g.* production of stress proteins, level of enzyme secretion), also indicate that the cells are "happiest" at cold temperatures and not temperatures that produce fastest growth rates (T_{opt}) (Russell 1998, Feller et al. 1996).

More appropriate terms for microorganisms isolated from cold environments are *stenopsychrophile* and *eurypsychrophile*, which can be used to replace the terms psychrophile and psychrotolerant, respectively. These are microbial ecology terms derived from Shelford's law of tolerance (Shelford 1913) where *steno* and *eury* describe a narrow- or wide-tolerance to an environmental determinant, respectively (Atlas & Bartha 1998). In this way a *eurythermal psychrophile* (*eurypsychrophile*) describes a microorganism which "likes" permanently cold environments and will tolerate a wider range of temperatures extending into the mesophilic range, whereas a *stenothermal psychrophile* (*stenopsychrophile*) describes a microorganism which is restricted to growth in permanently cold environments and will not tolerate higher temperatures for growth. The key point to note is that organisms growing in cold environments are cold-adapted and the term "psychrophile" clearly describes this fact.

1.2.2 Mechanisms of cold adaptation

An important factor when considering adaption to the cold is whether the organism is a warm blooded animal, and therefore able to maintain a temperature above that of its environment, or whether it is poikilothermic and at thermal equilibrium with its environment. The majority of psychrophiles are poikilothermic microorganisms, and the entire cell and all its cellular machinery must be suitably adapted to the cold. In addition to thermal constraints, the cell must adapt to the other environmental factors; the most common being nutrient-limitation. As a result, throughout evolution the factors which are rate limiting and constrain growth and survival have the greatest selection pressure for change. This necessarily implies that, depending on the individual cell, different cellular properties will be key to ensuring adequate adaptation of the cell.

We have only begun to understand the variety of ways that cells adapt to the cold. This is due in part to the small sample of microorganisms that have been isolated relative to the known biodiversity, and due to our preponderance to extrapolate mechanisms of adaptation in one organism to another. A good

example of how this knowledge could be erroneously applied would be to use the knowledge gained about the cold shock response in *Escherichia coli*, and expect it to apply to indigenous anaerobic methanogens found in Antarctic lakes (Cavicchioli et al. 2000, Cavicchioli et al. 2002a). It is therefore important to separate possible mechanisms of adaptation which may be found in an individual cell, from general principles of cold-adaptation.

One of the general principles to emerge from studies of psychrophiles is that they produce enzymes which function effectively at cold temperatures. Despite the fact that cold temperatures slow all reaction rates (enzymatic or otherwise), cold-adapted enzymes have evolved high rates of catalysis at low temperature. In comparison, enzymes from mesophiles or thermophiles have little or no activity in the cold. Moreover, compared with mesophilic or thermophilic enzymes, the maximum level of activity of cold-adapted enzymes is shifted towards lower temperatures with a concomitant decrease in thermal stability. Numerous good reviews of this topic are available (D'Amico et al. 2002, Feller 2003, Lonhienne et al. 2000, Russell 2000).

This chapter focuses on the applications of these uniquely evolved, cold-adapted enzymes and describes key factors which are known to influence their activity and stability.

2 BIOTECHNOLOGY

There are a number of relatively straight forward reasons why cold-active enzymes have application in biotechnology (Cavicchioli et al. 2002b, Gerday et al. 2000, Margesin & Schinner 1999, Ohgiya et al. 1999, Russell 1998). Most of these can be appreciated without a detailed knowledge of how cold-active enzymes achieve their performance (sections 3 and 4).

Potential and present applications of cold-active enzymes are presented in Table 1. The number of present uses is low and is likely to reflect the state of the field, which, for example has not developed as rapidly as the thermophile field. With the growth of interest in psychrophiles and their applications, cold-active enzymes will represent an increasingly larger share of the industrial enzyme market in the coming years.

2.1 High activity

The most obvious, and probably the most important reason reason why cold-active enzymes have commercial value is due to their high activity at low temperature. This enables processes which usually require heating, to be performed at lower temperatures, thereby reducing energy costs. Industries which benefit from this include those employing detergents for cold-water washing. A key problem in cleaning soiled items (household or industrial) is the retention of insoluble biological material; particularly protein, starch, cellulose and lipid components. In order for these materials to become water soluble, they need to be cleaved into smaller fragments. As a result, modern enzymatic detergents contain proteases, amylases, glucanases and lipases to degrade this material.

In the food industry, reactions need to be carried out at low temperature in order to avoid changes to food ingredients caused by undesirable side-reactions that would otherwise occur at higher temperatures. Useful examples of cold-active enzymes in the food industry include, β-glucosidase used to hydrolyze lactose in milk, pectinases to clarify/reduce viscosity in fruit, vegetable juices and wine, xylanases and amylases for dough fermentation, and proteases to soften meat, particularly in fish processing (Ohgiya et al. 1999, Vilhelmsson 1997).

Table 1 Biotechnological potential of cold-adapted enzymes

Field of Biotechnology	Enzyme	Application
Medical and pharmaceutical	Protease	Cleaning medical and surgical equipment, plaque removal, debredment of necrotic tissue, food supplement to aid digestion, thrombolytic agent, chemonucleolysis
	Chitinase, chitobiase, cyclodextrin glucano-transferase, nitrile hydratase, β-lactamase, lipase	Synthesis of pharmaceuticals
	Alanine racemase, chlamysin, dihydrofolate reductase, isocitrate lyase	Anti-bacterial and anti-fungal agents
	Hydantoinase	Synthesis of D-amino acids
	Dihydrofolate reductase	Anticancer drugs
	D-amino acid oxidase	Oxidation of side chain of cephalosporin to 2-keto acid
	Cholesterol oxidase	Oxidation of steroids (*e.g.* cholesterol, pregnenolone, stigmasterol)
Food	Protease	Improvement in taste and flavor, removal of fish skin, cheese production, meat tenderizing, brewing
	Dehydrogenases, glucose oxidase, chlamysin, alanine racemase	Food preservation
	β-galactosidase	Lactose hydrolysis in milk, improvement of ice-cream and whey quality
	Levan sucrase	Synthesis of fructose polymer (levan) and 1-kestose, animal and human feed
	Lipase, transglutaminase	Protein polymerisation and geling in fish flesh, improvement in food texture
	β-glucosidase	Enhancement of wine aroma by terpene release
	Pectinase, pectate lyase, pectin methylesterase, xylanase	Clarification of fruit juices and wine
	Pullulanase, α-amylase, β-amylase, glucoamylase, xylanase	Baking and brewing
	Cellulase	Animal feed
	Endo and exo-inulinase	Dietary fibre and fructose sweetener
	Acetolactate decarboxylase	Conversion of α-acetolactic acid to acetoin in brewing

Field of Biotechnology	Enzyme	Application
	Invertase	Confectionary, sucrose hydrolysis in juices, D-fructose production from inulin, production of melibiose from raffinose
	Catalase	Removal of H_2O_2 from milk
	Lipoxygenase	Strengthening gluten proteins in dough, bleaching of flour carotenoids
Bioscience	Alkaline phosphatase, RNA polymerase, DNA polymerase, DNA ligase, Uracil-DNA glycosylase	Molecular biology
Domestic and industrial	Protease, pullulanase, xylanase, cellulase, amylase, lipase, pectinases	Detergents and cold water washing including airconditionaing towers and pulp mills
	Chitinase, cyclodextrin glucano-transferase	Cosmetics
	Laccase	Bleaching in textile industry
	Cellulase	Biopolishing and biostoning, conversion to sugars for ethanol production
	α-amylase	Desizing denim jeans
	Lipase	Perfumery
	Nitrile hydatase	Low temperature acrylamide synthesis for use as paper strengthener
	Dehydrogenases	On-line monitoring of low temperature bioprocesses
Environmental	Organophosphorus acid anhydrolase	Detoxification/decontamination of pesticides/nerve agents
	Chitinase, cellulase, ligininase, xylanase	Degradation of chitin and lignocellulosic waste
	Catalase	Waste-water treatment in paper, food, textile, semiconductor industries, detoxification
	Alkanemonooxygenase and Alkanedehydrogenase	Bioremediation, degradation of long chain alkanes
	Catechol 1,2 dioxygenase, peroxidase	Phenol removal by degradation or polymerisation, waste water treatment
Fine chemical synthesis	Various esterases	Fine synthesis of optically active alcohols or carboxylic acids ester hydrolysis, esterification or trans-esterification

Field of Biotechnology	Enzyme	Application
	Glutamate transaminases	Synthesis of unnatural amino acids from 2-keto-acids
	Dehydrogenases	Biotransformation, asymmetric chemical synthesis
	Lipases	Optically active ester synthesis
	Triose phosphate isomerase, citrate synthetase, lipoxygenase	Fine chemical synthesis
	Adenosine deaminase	Conversion of adenosine to inosine analogues
	Xylitol reductase	Xylitol synthesis
	Dihydrofolate reductase	Single carbon transfer reactions
	Hydroxynitrile lyase	Synthesis of enantiopure cyanohydrin for pyrethroids

Cold-adapted microorganisms have application in geographically or seasonally cold regions (*e.g.* cold-adapted methanogens for generating methane for use as a source of fuel). Although there are no similar uses of cold-active enzymes presently, it is conceivable that they could be used for environmental bioremediation; *e.g.* as a biodegradable means of treating an oil spill such as that which occurred by the Exon Valdese in Arctic waters. Recently, 89 psychrophilic bacterial and yeast isolates were screened for their ability to produce polymer degrading enzymes capable of hydrolysing aliphatic and aromatic petroleum hydrocarbons (Margesin et al. 2003). Similarly, cold-adapted organophosphorus acid anhydrolases have been characterised for application in the efficient detoxification of pesticides and nerve agents (Cheng et al. 1997).

2.2 Non-aqueous catalysis

Cold-active enzymes confer a high level of stereo-specificity, which has application in fine chemical synthesis and biotransformation. These reactions are typically performed in mixed aqueous-organic or non-aqueous solvents. Moreover, organic phase biocatalysis which is performed at low temperature has advantages due to improved catalysis, increased solvent choice, increased product yield, negligible degradation of reactants and products and improved oxygen solubility for oxidases. The properties of cold-active enzymes which make them suitable for these purposes include their inherently high level of flexibility, greater accessibility of their active site, high activity and resistance to cold denaturation at moderately cold temperatues (Owusu 1999, Sellek & Chaudhuri 1999). An example of the application of a cold-adapted enzyme in non-aqueous biotransformation is the use of a lipase from *Pseudomonas* strain P38 for the synthesis in n-heptane of the flavoring compound, butyl caprylate (Tan et al. 1996).

2.3 Other useful properties of cold-active enzymes

In addition to inherently high levels of activity at low temperature, cold-active enzymes are naturally thermolabile. This property enables enzymes to be inactivated by heating rather than requiring the use of

enzyme inactivating compounds or enzyme removal. An alkaline phosphatase isolated from Arctic shrimp has been marketed for this reason. The capacity to dephosphorylate DNA during recombinant DNA manipulations enables subsequent procedures to be performed after heat inactivation of the enzyme (Olsen et al. 1991).

Cold-adapted enzymes are a unique resource for commencing protein engineering studies. Their high catalytic activity and high reaction yields provides a useful basis for engineering enzymes with enhanced thermostability while retaining high specific activity (this is further discussed in section 4). This may be achieved by recombinant DNA methods, including directed evolution (Miyazaki & Arnold 1999, Taguchi et al. 1999, Gonzalez-Blasco et al. 2000, Bell et al. 2002, Miyazaki et al. 2000, Wintrode et al. 2001, D'Amico et al. 2002) and site-directed mutagenesis (Tsigos et al. 2001, Gerike et al. 2001, Narinx et al. 1997). In addition, chemical modification methods involving enzyme immobilisation have also been developed (Koops et al. 1999).

3. STRUCTURAL AND COMPOSITIONAL CHARACTERISTICS OF COLD-ADAPTED ENZYMES

A perplexing issue regarding the high activity and low stability of cold-adapted enzymes is predicting how this is achieved in any candidate enzyme, or how to engineer these characteristics into an existing or artifical enzyme. While a number of structural or compositional characteristics can be described for cold-active enzymes, it has proven difficult to predict with assurity which elements contribute significantly to the biochemical and biophysical properties of the enzymes. Similar difficulties exist with high temperature adapted enzymes. This illustrates that there are important gaps in our fundamental knowledge regarding protein structure and function that reduces our capacity to define thermal determinants of enzyme activity and stability.

Nevertheless, despite the difficulties with prediction, important advances have been made in recent years. The biggest advances have occurred due to the generation of the first x-ray crystal structures of cold-active enzymes (Aghajari et al. 1998), and through comparative analyses with genome sequences of cold-adapted microorganisms (Saunders et al. 2003). What has become clear from these studies is that individual enzymes have evolved multiple ways of achieving similar structural and functional outcomes. As a result it is perhaps not surprising that different combinations of structural features associated with cold adaptation are found in different enzyme families.

3.1 Comparative studies with individual enzymes

A total of nine crystal structures of cold active enzymes have been published. However, the original studies examining thermal adaptation were performed in the absence of crystal structures of cold-active enzymes, and relied on protein homology modelling using crystal structures from mesophilic or thermophilic enzymes. In addition to homology modelling, comparative analyses have been performed using multiple sequence alignments to identify amino acid replacements, insertions and deletions that may be important for cold adaptation. Compositional trends such as amino acid content and predicted pI have also been correlated with thermophilicity (Smalas et al. 2000).

Comparative studies have be peformed using sequences from phylogenetically diverse organisms. For example, five citrate synthases have been compared from a psychrophilic bacterium, a pig, and thermophilic

and hyperthermophilic archaea (Bell et al. 2002). Having obtained the crystal structures for all 5 enzymes it is clear that despite the phylogenetic differences the structures are sufficiently similar to enable a very useful rationalisation of thermal adaptation. At the other extreme of phylogenetic and metabolic similarity, three elongation factor 2 (EF-2) proteins (GTPases) were compared from highly related psychrophilic, mesophilic and thermophilic methanogenic archaea using protein homology modelling (Thomas & Cavicchioli 1998). The study found that thermal adaptation did not bias the rate of evolution of EF-2 amino acid sequences vs. 16S-rRNA nucleic acid sequences, demonstrating that the amino acid changes important for thermal adaptation tend to be hidden amidst all other evolutionary changes in protein sequences.

3.2 Comparative studies with genomic data sets

The approach of using closely related organisms for comparisons was extended to the use of genome sequence data from 9 methanogens spanning the growth temperature range 0°C to 110°C (Saunders et al. 2003). This study was performed to examine the correlation between organism growth temperature and the occurrence of specific structural elements in 1111 protein homology models. Analysis of the models from the cold-adapted methanogens showed a strong tendency in the solvent accessible area for more glutamine, threonine and hydrophobic residues and fewer charged residues. The use of an all methanogen sample set, the thermal extremes examined and the large size of the data set provides a level of confidence in the observed trends that can not be found with individual enzyme sets. The real value of such studies however it to provide a rationalisation to guide functional and structural studies. To be truly meaningful, a comprehensive structural genomics program needs to be initiated to generate an equivalently large number of structures for wild-type and mutant proteins. This would enable direct comparisons with structures being generated for members of the thermophilic and hyperthermophilic methanogens including *Methanobacterium thermoautotrophicum* (Hwang et al. 1999) and *Methanocaldococcus jannaschii* (Christendat et al. 2000), respectively.

3.3 Complicating cellular factors

A potential concern which overlays the ability to interpret protein adaptation, is the effect of the environment in which the enzymes function. Most cold-adapted enzymes characterized to date are from organisms with a marine origin, and amino acid substitutions may partially reflect adaptation to halophilicity rather than psychrophilicity; particularly for secreted enzymes. Intracellular factors also exert profound effects (Fields et al. 2001). The activity and stability of EF-2 proteins from psychrophilic and thermophilic methanogens was shown to be greatly affected by interaction with cognate partner proteins (ribosomes) and intracellular solutes (Thomas et al. 2001). This example also serves to highlight that even closely related organisms can produce different types and quantities of intracellular solutes. For example the thermophile produced up to 500 mM K-glutamate whereas the psychrophile produced 100 mM K-aspartate. While these complicating factors are specifically relevant for inferring the properties of cold-active enzymes in a physiological setting, they also highlight the potential importance that interacting compounds may have in biotechnology.

3.4 Specific structural elements

Numerous good reviews describing the structural elements of cold-active enzymes have been published (Smalas et al. 2000, Feller 2003, Sheridan et al. 2000, Arpigny et al. 1994, Jaenicke & Bohm 1998, Georlette et al. 2001, Russell 2000). The following sub-sections describe important structural elements found in cold-active enzymes. Where possible these characteristics have been described in three well-

studied enzymes; an α-amylase, a xylanase and a DNA ligase from the Antarctic bacterium, *Pseudoalteramonas haloplanktis* (Table 2). Structural information about the α-amylase has also been linked to its activity and stability by examining data from mutagenesis studies (Table 3), and the activity and stability of all three enzymes (Table 4) is discussed in section 4.

3.4.1 Hydrophobic interactions

Hydrophobic interactions are defined as the gain in free energy when non-polar groups are transferred from aqueous contact in an unfolded enzyme to the hydrophobic interior of a folded molecule. This energy comprises both van der Waals interactions between non-polar groups and the removal of hydrophobic groups away from water. The interaction of surface non-polar groups with the solvent causes ordering of water molecules around the hydrophobic groups, resulting in a decreased entropy with concomitant destabilization. The major driving force for enzyme folding is the collapse of the hydrophobic core in the protein's interior. The formation of hydrophobic interactions is an endothermic reaction, therefore these interactions are generally strengthened with increasing temperature (Privalov and Gill 1988, Smalas et al. 2000).

In thermophilic enzymes stabilization is achieved by an increase in the hydrophobicity of the core and burial of surface hydrophobic residues by oligomerization. As van der Waals interactions are weak and very short range, the distance between hydrophobic groups in the enzyme interior becomes very important and determines the enthalpic contribution to hydrophobic interactions. Some studies have found that buried amino acids in cold-adapted enzymes are smaller and less hydrophobic than in their mesophilic or thermophilic counterparts (Leiros et al. 2000, Smalas et al. 2000). As a result, the less packed interior of cold-adapted enzymes may be destabilized due to reduced van der Waals forces and increased movement of internal groups.

Cold-adapted enzymes tend to have a high proportion of their hydrophobic residues exposed on the surface of the protein, thereby destabilizing the structure due to the decreased entropy of water molecules which form cage like structures around non-polar residues (Bell et al. 2002). This property has been reported for α-amylases, DNA ligases (Table 2), glyceraldehyde-3-phosphate dehydrogenases and citrate synthases (Gianese et al. 2002, Russell et al. 1998, Aghajari et al. 1998, Georlette et al. 2000) and is consistent with the genomic analysis of methanogen proteins (Saunders et al., 2003). For example, for DNA ligases, 32% of total surface area comprised of non-polar amino acids in the cold-adapted enzyme compared with 26 % from the thermophile (Georlette et al. 2003).

Cold-adaptation appears to involve a careful arrangement of the exposed and buried non-polar fraction leading to a reduced size of the hydrophobic core, and a higher proportion of non-polar residues exposed to water on the surface of the protein (Aghajari et al. 1998, Russell et al. 1998, Saunders et al. 2003).

3.4.2 Surface hydrophilicity

Most polar and charged amino acids are present on enzyme surfaces and tend to make the surfaces hydrophilic. The intramolecular, non-covalent electrostatic interactions formed are of prime importance in maintaining the secondary and tertiary structures of an enzyme (Smalas et al. 2000). The types of interactions that are formed from polar and charged amino acids are mainly comprised of salt-bridges, H-bonding and aromatic interactions. All the interactions are formed exothermically and as a result, their strength increases with decreasing temperature.

Table 2: Structural and compositional factors conferring high activity, low stability and high flexibility in cold-adapted enzymes from *Pseudoalteromonas haloplanktis*, compared with homologues from mesophiles and thermophiles

Parameter[1] \ Enzyme	α-Amylase (structure)[2]	Xylanase (structure)[3]	DNA ligase (model)[4]
Decreased number of surface charged residues	Decreased and evenly distributed	Decreased	Decreased
Increased number of acidic residues	Decreased pI	Decreased negative surface charge	Decreased negative surface charge
Increased surface neutral residues	-	-	Increased
Increased surface hydrophobicity	Increased	Increased	Increased
Decreased core hydrophobicity	Decreased number of non-polar residues in core	-	-
Decreased Arg/ Arg+Lys ratio	Decreased	Decreased	-
Decreased number of proline residues	Decreased in loops	Increased total number, increased in loops, decreased in α-helices	-
Increased number of glycine residues	Decreased in loops	Decreased	-
Increased number of methionine residues	Increased	-	-
Increased number of histidine residues	Decreased	-	-
Decreased number of disulphide bridges	Decreased	Increased	-
Decreased number of hydrogen bonds	Increased total number, decreased Arg-mediated	Increased	-
Decreased number of ion-pairs (salt-bridges)	Decreased total number, decreased Arg-mediated	Decreased	-
Increased number and longer loops	Shorter loops	-	-
Increased number of aromatic interactions	Decreased Arg-mediated	-	-
Decreased inter-domain contacts	Decreased Arg-mediated	-	-
Reduced metal binding	Lower Ca^{2+} affinity	-	-

[1] Parameter indicates what is typically associated with cold-active enzymes compared with enzymes from mesophiles or thermophiles. Descriptions in the body of the table indicate data for individual enzymes; [2] Aghajari et al. 1998, Feller 2003; [3] Petegem et al. 2003; [4] Georlette et al. 2003.

Table 3 Examples of enhanced properties of mutant proteins

Enzyme and source	Mutagenesis method	Properites of wild-type/ mutant enzymes				Remarks
		Activity (s^{-1}) [1]	T_{opt} (°C) [2]	Half-life [3]	T_m (°C) [4]	
α-Amylase *Pseudoalteromonas haloplanktis* (D'Amico et al. 2001)	Site-directed mutagenesis	697/642 (25°C)	-	5/22 (45°C)	44/46.4 (DSC)	Cold-adapted α-amylase was stabilized while retaining 92% specific activity
Xylose isomerase *Thermus thermophilus* (Lonn et al. 2002)	Directed evolution	47/412 (60°C)	80/90	95%/55% (70°C)	-	Activity and T_{opt} of thermophilic xylose isomerase was increased while half-life was decreased
3-isopropylmalate dehydrogenase *Thermus thermophilus* (Suzuki et al. 2001)	Site-directed mutagenesis	2.4/17.7 (40°C)	-	10min/10min (87°C)	87/87 (CD)	Activity of thermophilic enzyme was increased while thermostability was unchanged
Subtilisin S41 *Bacillus* sp.TA41 (Miyazaki et al. 2000)	Directed evolution	57/160 (30°C)	65/75	450min/ 1082min (60°C)	50/72 (CD)	Mutant from cold-active protease with both activity and stability simultaneously enhanced

[1] V_{max} or k_{cat} at stipulated temperature; [2] Temperature optimum for activity; [3] Half-life of inactivation ($t_{1/2}$); [4] Melting-temperature determined by differential scanning calorimetry (DSC) or circular dichroism (CD).

Table 4: Comparison of activity and stability of selected thermally adapted enzymes

Thermal class **Parameter**	**Psychrophile**	**Mesophile**	**Thermophile**
Enzyme	***α-Amylase*** (Feller et al. 1994, D'Amico et al. 2003)		
Organism	*Pseudoalteromonas haloplanktis*	Pig	*Bacillus amyloliquefaciens*
k_{cat} (min^{-1})	13980 (4 °C), 17640 (10 °C)	5820 (10 °C)	840 (10 °C)
T_{opt} (°C)	28	54	84
$\Delta H^{\#}$ (kJ mol^{-1})	35	46	70
$t_{1/2}$ (s)	14 (43 °C)	14 (60 °C)	14 (80 °C)
T_m (°C) DSC	44	66	84
Enzyme	***Xylanase*** (Collins et al. 2002, Collins et al. 2003)		
Organism	*Pseudoalteromonas haloplanktis*	*Streptomyces* sp. S38	*Caldocellum saccharolyticum*
k_{cat} (min^{-1})	30930 (10 °C)	3600 (10 °C)	5840 (70 °C)
T_{opt}	35	50	70
$\Delta H^{\#}$ (kJ mol^{-1})	21	58	41
$t_{1/2}$ (s)	2 (55 °C)	23 (55 °C)	-
T_m (°C) DSC	53	63	-
Enzyme	**DNA ligase** (Georlette et al. 2000, Georlette et al. 2003)		
Organism	*Pseudoalteromonas haloplanktis*	*Escherichial coli*	*Thermus scotoductus*
k_{cat} (min^{-1})	0.6 (4 °C), 2.02 (18 °C)	1.5 (42 °C)	0.75 (45 °C)
T_{opt}	18	42	>55
$\Delta H^{\#}$ (kJ mol^{-1})	-	-	-
$t_{1/2}$	26 s (35 °C)	124 min (35 °C)	26 min (91 °C)
T_m (°C) DSC	33	52, 54	92, 96, 101

An inherent difficulty with these studies is that interactions are effective over long distances and are therefore difficult to predict and model. Accepting this limitation, the majority of cold-adapted enzymes from bacteria and fish have been shown to have a prevalence of charged or polar amino acids on the surface of cold-adapted enzymes forming both intra-molecular interactions with each other as well as interacting with water molecules of high dielectric constant (Fields 2001, Sheridan et al. 2000). Among the charged amino acids, acidic amino acids are more prevalent on the surface of some cold-adapted

enzymes than basic amino acids. The pI of cold-adapted α-amylase (Table 2), trypsins, chymotrypsins, elastase, pepsin, citrate synthase, isocitrate dehydrogenase, malate dehydrogenase, subtilisin, β-lactamase and triosephosphate isomerase are lower than their mesophilic and thermophilic counterparts (Smalas et al. 2000). An extreme example of this is for a cold-adapted subtilisin which has 12 more acidic residues than its mesophilic homologue (Davail et al. 1994, Narinx et al. 1992). The amassing of acidic residues in defined regions of an enzyme surface may increase solvent interaction and result in charge-charge repulsions leading to an overall destabilization of cold-active enzymes. This type of clustering of negative charges is found in cold-adapted cod and salmon trypsins (Leiros et al. 1999) and β-lactamase (Feller et al. 1997).

A large number of polar amino acids on the surface of a cold-adapted enzyme will promote interactions with water thereby making the outer structure more flexible and hence prone to unfolding at moderate temperatures (Davail et al. 1994, Fields 2001). Less heat is required to melt hydrophilic interactions than hydrophobic interactions because hydration of hydrophilic groups exerts a destabilising effect while hydration of aliphatic groups stabilizes an enzyme structure (Makhatadze & Privalov 1994). A cold-active alkaline phosphatase has been shown to have an excess negative surface charge (Backer et al. 2002). However, although the majority of the surface is predominantly negative, the active site is surrounded by a positively charged area, which is likely to be important for directing the negatively charged substrate to the catalytic site. The specific distribution of charge and optimization of surface potential also appears to contribute to the function of a cold-adapted malate dehydrogenase (Kim et al. 1999), trypsin (Leiros et al. 2000) and citrate synthase (Russell et al. 1998).

In contrast to trends for an overall increase in surface charge observed for individual cold-active enzymes, the genomic analysis of Saunders et al. (2003) found that the mean fraction of accessible and buried surface composed of charged residues decreased with decreasing growth temperature (Saunders et al. 2003). The differences in this study may reflect the use of data from archaea, however this is difficult to assess as only one archaeal cold-active protein (EF-2) has been studied in any detail (Siddiqui et al., 2002, Thomas & Cavicchioli 1998, Thomas & Cavicchioli 2000, Thomas et al., 2001, Thomas & Cavicchioli 2002). It is noteworthy that a tendency towards increasing surface hydrophilicity in enzymes from both psychophilic and thermophilic bacteria has been reported (Spassov et al. 1995). While this appears to be a paradox, it may be explained by the surface charged residues in thermophiles forming networks of salt-bridges to stabilise the protein, while those in the cold-active proteins destablising the protein by increasing the number and/or strength of interactions with water molecules (Spassov et al. 1995). Perhaps one of the important lessons to be learned from these studies is that general trends may be observed by examining large genomic data sets, whereas with individual enzymes, the specific structural context of the interactions must be examined. This latter point is well illustrated by the comparisons of citrate synthases which indicated that the precise structural context of structural differences was more important than the number of changes (Bell et al. 2002).

3.4.2.1 Arginine mediated interactions

Some cold-adapted proteins have a lower ratio of Arg/(Arg + Lys) than comparative enzymes from thermophiles (Gianese et al. 2001). The guanidino groups in arginine amino acids enable more ionic and H-bond interactions to form than lysine residues, and are therefore predicted to have a more stabilising effect on proteins (Feller et al. 1997, Mrabet et al. 1992). Many cold-adapted enzymes, including α-

amylase and xylanase from *P. haloplanktis* (Table 2), have this compositional trend. However this is not the case with all cold-adapted enzymes. Uracil-DNA glycosylase from atlantic cod has three more arginine residues than its mesophilic counterpart (Leiros et al. 2003). Some of the discrepancies may be explained if arginine residues are surface exposed thereby enhancing interactions with water and imparting flexibility, or conversely if they are involved in forming specific, stabilising ionic interactions with other residues (Smalas et al. 2000).

3.4.2.2 Hydrogen bonds

Hydrogen bonds (H-bonds) are the most numerous of all non-covalent interactions. H-bonds are formed between a partially positive hydrogen atom (attached to an electronegative atom such as O or N) and an electronegative atom. The total difference in stabilization energy between a cold-adapted and thermophilic enzyme is not more than 40-50 kJ/mol. As an average of 5.4 kJ/mol is required to break a single H-bond, it is evident that the lack of only a few crucial H-bonds in cold-adapted enzymes can make a large difference to their thermolability.

Due to the close proximity (less than 3Å) and directionality (donor-acceptor angle less than 90°) required to enable H-bonds to form, they are difficult to interpret in protein structures or models (Vieille and Zeikus 2001). The number of H-bonds identified in the crystal structures of a cold-adapted α-amylase (Table 2), citrate synthase, malate dehydrogenase or uracil-DNA glycosylase is similar to their mesophilic counterparts (Aghajari et al. 1998, Russell et al. 1998, Kim et al. 1999, Leiros et al. 2003). Despite the lack of major differences in total number of H-bonds, cold-adapted enzymes may be characterised by fewer inter-domain or inter-subunit H-bonds, as is the case for the oligomeric forms of citrate synthase (Russell et al. 1998) and malate dehydrogenase (Kim et al. 1999).

3.4.2.3 Aromatic interactions

Amino acids with aromatic rings have a dipole caused by the partial negative charge of the π-electron cloud on the face of the ring, and the partial positive charge of the C-H edges of the ring. This polarity permits favorable interactions between aromatic rings at right angles to each other (aromatic-aromatic interactions) or with other groups. These interactions may contribute to enzyme thermostabilization. Aromatic interactions are predicted to be lower in a cold-adapted α-amylase (Table 2) and subtilisin (Davail et al. 1994). A comparison of β-lactamases found that 2 tryptophan based aromatic-aromatic interactions, which were predicted to be particularly important for stabilisation, were present in the mesophilic enzyme and absent in the cold-active enzyme (Feller et al. 1997). In contrast to these examples, aromatic interactions do not appear to be a feature of other cold-active enzymes.

3.4.2.4 Ion-pairs

Single ion-pairs (salt-bridges) contribute 12-21 kJ/mol of stabilisation to an enzyme. The energetic (thermodynamic) cost of ion-pair formation is very high on the surface of an enzyme due to the desolvation penalty. This is due to the fact that the free energy of desolvation is not fully compensated by the free energy released during the formation of an ion-pair in a medium of high dielectric constant (water). However, ion-pairs become more favourable with the increase in temperature as the dielectric constant of water decreases (Vieille & Zeikus 2001). As a result, ion-pairs could be important for stabilizing thermophilic enzymes. In contrast, they may be destabilizing at low temperature and contribute effectively on the surface of the protein to overall flexibility. In some circumstance they may also be strategically positioned to contribute to the stability of a cold-active protein (Bell et al. 2002).

Ion-pairs can also be formed in networks. Ion-pair networks are more stable than single ion-pairs because for each extra pair the cost of the desolvation penalty is reduced by half. As arginine residues form H-bonds and ion-pairs with carboxylic acid side chains (see Section 3.4.2.1), they are often involved in ion-pair networks. Ion-pair networks interlacing enzyme and subunit surfaces may exert a general stabilising effect (Vieille and Zeikus 2001).

There are numerous examples of cold-active enzymes having fewer salt-bridges than enzymes from thermophiles (Spassov et al. 1995). A good example is the subtilisin family of enzymes (Davail et al. 1994). Subtilisins S41 has 2 salt-bridges compared with an average of 5 and 10 in mesophilic and thermophilic enzymes, respectively. Similar findings have been found for α-amylase (Table 2) and other cold-adapted enzymes (Gianese et al. 2002). In contrast, a cold-active citrate synthase appears to have more predicted ion-pairs than four comparative enzymes from mesophiles, thermophiles or hyperthermophiles (Bell et al. 2002). This may be partially explained by inter-subunit ion-pairs counter-balancing reduced hydrophobic interactions (*i.e.* cold denaturation) that may occur at very cold temperatures. The observation of higher or lower numbers of ion-pairs in individual cold-active enzymes is mirrored in the genomic analysis of archaeal proteins, where clear trends were predicted for some proteins (*e.g.* Cpn60) although no consistent general trend was predicated (Saunders et al. 2003).

3.4.2.5 Surface loops

Enzymes are formed from well defined α-helices and β-sheets connected by flexible loops. Most loops are located on the solvent-exposed surface of an enzyme where thermal unfolding is likely to be initiated. Loops are also positioned near catalytic pockets and play an important role in the flexibility of the active site. The flexibility of the active site contributes to the high activity and high thermolability of cold-adapted enzymes (Smalas et al. 2000).

Proline and disulphide residues in loops reduce the conformational freedom of polypeptides thereby increasing the stability of the native state of the enzyme. In contrast glycine residues in loops increase the conformational freedom of the native state (Fields 2001). A cold-active lactate dehydrogenase has two extra glycine residues which are predicted to increase the rotational entropy of the peptide backbone (Fields 2001). It also has proline to alanine substitution at the N-terminal region of an α-helix. These changes are predicted to increase the flexibility of the enzyme (Fields 2001). Cold-adapted α-amylase (Table 2), β-lactamase, subtilisin, malate dehydrogenase, trypsin, b-galactosidase, DNA ligase and alanine dehydrogenase have fewer proline residues (Feller 2003, Smalas et al. 2000). Despite their potential role in stabilisation, disulphide bridges do not appear to be reduced in loops or in total number in cold-active enzymes.

Loops are also likely to contribute to flexibility by increasing enzyme solvation. Compared with mesophilic and thermophilic homologues, cold-adapted enzymes have a larger number, and longer loops connecting α-helices and β-sheets. This has been found in triosephosphate isomerase (Rentier-Delrue et al. 1993). The effect of loops regions also appear to be manifested through ionic interactions. Citrate synthases from thermophiles appear to have on average only marginally shorter loops than a cold-active citrate synthase (Bell et al. 2002). However, many of the loops appear to have diverse and specific ionic interactions with other parts of the protein.

3.4.2.6 Other features of cold-adapted enzymes

Many cold-adapted enzymes have a higher methionine content (Smalas et al. 2000). The high degrees of freedom and lack of branching, charge or dipole interactions afforded by methionine residues may enable flexibility to be maintained at low temperature (Thomas & Cavicchioli, 1998). The presence of methionine residues may also relate to adaptation to salt (Smalas et al. 2000).

The stability of secondary structural elements is dependent on their amino acid composition and location within the protein (Smalas et al. 2000). The N- and C-termini of α-helices (caps) in cold-adapted enzymes may be structurally weakened by the presence of modified charge-dipole interactions, and as a result be sites of protein unfolding (Doig & Baldwin 1995). The α-helices in a cold-active triose-phosphate isomerase tend to have positively charged N-termini and negatively charged C-termini, thereby weakening the helices (Rentier-Delrue et al. 1993). In a cold-adapted citrate synthase, flexibility of α-helices appears to be mediated by the presence of additional proline residues in the middle of two helices, which may reduce rotational degrees of freedom in the helices and compromise structural integrity (Russell et al. 1998).

4. CONTROLLING STABILITY AND ACTIVITY OF COLD-ADAPTED ENZYMES

4.1 Modified enzymes

In order for enzymes to be used succesfully for biotechnological purposes they need to possess and maintain sufficient catalytic activity. Cold-adapted enzymes tend to have good rates of catalysis but low half-lives of inactivation at moderate to high temperatures (Feller & Gerday 1997, Feller et al. 1996, Russell 2000, Marshall 1997). On the other hand, thermophilic enzymes are stable at high temperatures but lack sufficient rates of catalysis (k_{cat}) at lower temperatures due to their high activation energy of catalysis (Arpigny et al. 1994). At their respective temperature optimum for catalysis (T_{opt}), enzymes from psychrophiles, mesophiles and thermophiles tend to have similar activity (Figure 1), although this can vary depending on the representative enzymes chosen for each thermal class. As a result, more energy expenditure is required for equivalent processes at high temperature.

One approach to surmounting this problem is to mutate cold-adapted enzymes to increase their thermal stability while maintaining activity, or to enhance the activity of thermophilic enzymes without compromising their high thermal stability (Carrea & Colombo 2000). Good examples of this include the stabilisation of an α-amylase from a psychrophile while retaining most of its original activity, and the improvement in activity of a xylose isomerase and 3-isopropylmalate dehydrogenase from a thermophile while reducing or maintaining their stability (Table 3). Until recently it has been assumed that in order for an enzyme to be highly active it had to be very flexible and unstable, whereas a thermophilic enzyme had to be rigid and resist unfolding (D'Amico et al. 2002). This interdependence of high flexibility and high activity with low thermal stability of cold-adapted enzymes led to the concept of activity-stability trade-off (Svingor et al. 2001, Arnold et al. 2001). There is now evidence that enzyme stability and activity are often governed by distinct structural elements that exert different temporal effects on enzyme thermal inactivation (Lazaridis et al. 1997, Fields & Somero 1998, Merz et al. 2000). This introduces the theoretical possibility of being able to engineer an enzyme with both high activity and high stability. Importantly, this has been experimentally confirmed with a mutant of subtilisin S41 which possesses improved activity from 57 s^{-1} to 160 s^{-1}, and an increase in half-life of inactivation at 60°C from 450 min to 1082 min (Table 3).

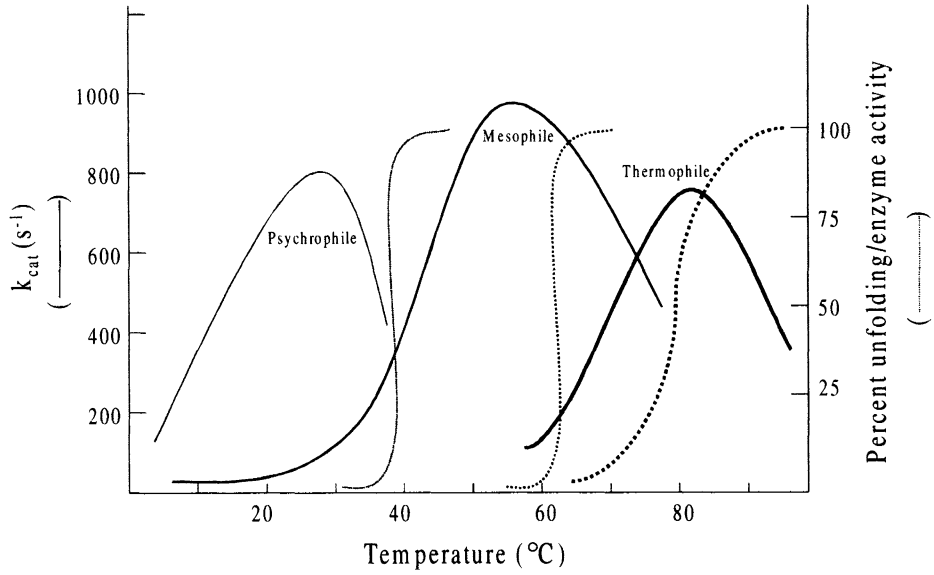

Figure 1. Effect of temperature on the activity (continuous parabolic curves) and stability (dotted sigmoidal curves) of thermally adapted enzymes. The T_{opt} for activity and the thermal stability curve for the cold-adapted enzyme (narrowest lines) are shifted towards lower temperatures compared with those from the mesophile (medium thickness lines) and thermophile (thickest lines).

4.2 Thermodynamic considerations for improving enzyme activity and stability

One of the main thermodynamic characteristic of cold-adapted enzymes is that their rates of catalysis become less temperature-dependent due to a decrease of enthalpy of activation ($\Delta H^{\#}$), in comparision with mesophilic or themophilic enzymes (Gerday et al. 1997, Lonhienne et al. 2000, Table 4). A cold-adapted α-amylase from *A. haloplanktis* has the lowest $\Delta H^{\#}$ for substrate hydrolysis and hence has highest activity at 10°C, compared with α-amylase enzymes from a mesophile or thermophile (Table 4). Conversely, the thermophilc α-amylase has highest $\Delta H^{\#}$ and the lowest activity at 10°C. The enzyme from the mesophile lies between these two extremes.

The value of Q_{10} describes how much the rate of a reaction will increase for every 10°C rise in temperature. It is calculated as: Q_{10} ($\ln k_{T1}/k_{T2} = - (E_a/R) \times \{(T_2 - T_1)/(T_1T_2)\}$ where k_{T1} is the rate of reaction at T_1, k_{T2} is the rate of reaction at T_2, E_a ($\Delta H^{\#} + RT$) is the activation energy of the reaction, and R is the Universal Gas Constant. The value of Q_{10} for substrate hydrolysis by cold, mesophilic and thermophilic α-amylases is 1.72, 2.0 and 2.84, respectively. If the respective k_{cat} of all three enzymes is extrapolated from 10°C to higher temperatures, despite the comparatively low Q_{10} of the cold-active enzyme, the k_{cat} climbs to 60,000/min at 30°C, compared with 23,500/min and 4700/min for the enzymes from the mesophile and thermophile, respectively. This concept can be extended by considering a modest increase in T_{opt} of the cold-adapted α-amylase from 28 to 40°C, which would further increase its k_{cat} to 91,000/min. Similar trends are apparent when applying these calculations to the xylanase and DNA ligase (Table 4). These example illustrate that by generating a modest increase in thermostability of a cold-active enzyme, very high reaction rates could be achieved at room temperature conditions.

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Ribozymes

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1. INTRODUCTION

Proteins have long been occupying unenviable position of biological catalysts until twenty years ago. Since then this concept needed to be relooked at with the discovery of catalysis by RNA by Thomas Cech and his colleagues. Working with the protozoan *Tetrahymena thermophila*, they found that the processing of pre-rRNA to mature rRNA involved excision of an intron some 400 nt (nucleotide) long from the 6400 nt long primary transcript in an intramolecular fashion (Krueger et al. 1982). Another instance of RNA acting as an enzyme came from the laboratory of Sidney Altman. In *Escherichia coli*, processing of pre-tRNAs is carried out by RNaseP. It is a ribonucleoprotein whose RNA component, M1RNA, is enzymatically active (Guerrier-Takada et al. 1983). Since then several distinct categories of naturally occurring catalytic RNAs have been discovered (Hutchins et al. 1986, Hampel & Tritz 1989, Sharmeen et al. 1988, Saville & Collins 1990).

Most of the naturally occurring catalytic RNAs, called ribozymes, catalyse intramolecular reactions in their natural context, exception being RNaseP. Naturally occurring ribozymes, with the sole exception of ribosome, catalyse the cleavage and ligation of RNA phosphodiester backbone. They catalyse highly sequence specific reactions determined by RNA-RNA interactions between the ribozyme and its substrate molecules. The key to the recognition and binding of substrate molecules and their subsequent cleavage reaction resides in the ribozyme. Since the topic is vast and extensive literature is available, the present article will briefly describe various types of ribozymes known to-date and also focus on basic mechanism of catalysis and applications mainly of small ribozymes such as hammerhead ribozyme.

2. CLASSIFICATION

There are seven catalytic RNA motifs that are derived from naturally occurring ribozymes. These are Group I and II introns, RNaseP, hammerhead, hairpin, hepatitis delta virus and Neurospora VS ribozymes. The first three are larger in size and may be as long as 6000 nt. Rest of the ribozymes are smaller in size.

2.1 Group I introns

As already mentioned, Group I intron was found in the precursor of *Tetrahymena thermophila* large subunit rRNA. It has also been found to interrupt a large variety of tRNAs, mRNAs, and rRNAs. All of them possess a common splicing pathway (Cech 1990). Through a series of meticulous and rigorous experiments, Cech and his colleagues demonstrated that excision of intron from 6400 nt long primary pre-rRNA was the first step in a cascade of reactions. Immediately after excision the linear intron is

converted to a 414 nt long circular intron, designated L-19 IVS that has 19 nt removed from the intron formed in the first step (Fig. 1). The presence of Mg^{+2} and guanosine are required for the splicing. It was also observed that prevention of folding of RNA resulted in the loss of activity, suggesting that the three dimensional structure of the RNA was essential for splicing.

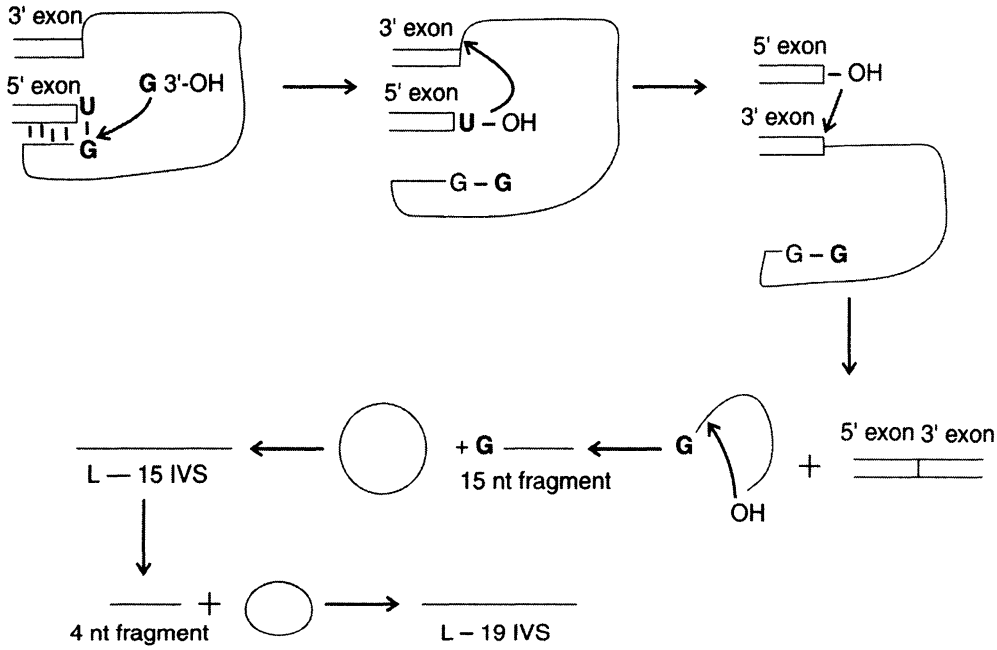

Fig. 1. Scheme showing self-slicing of Group I intron forming L-19 IVS (see text for details)

Despite considerable variability in size and sequence, Group I introns have phylogenetically conserved structures (Michel & Westhof 1990) and common reaction mechanism. The secondary structure consists of 10 paired segments (P1-P10) with P3, P4, P6 and P7 constituting the catalytic core (Fig. 2). There are four conserved sequence elements, designated P, Q, R and S. Ribozyme sequence specificity is defined by the interactions between 5' region of the intron and the two exons. There is sequence within the intron, called internal guide sequence (IGS), which constitutes P1 and P10 in the structure. The exon sequences are oriented relative to the catalytic core by base pairings with the IGS. The 3' exon splice site is further aligned by an additional pairings, called P 9.0. The 5' exon contains a highly conserved U at its 3' end. It forms a functionally important U.G pair with the IGS. There is also a conserved G at the 3' end of the intron. Finally, there is a specific binding site for the guanosine cofactor that initiates the reaction. This site involves a conserved G.C base pair in P7.

In vivo these reactions occur with the assistance of protein factors that in some cases are encoded within the intron itself (Lambowitz & Perlman 1990). Interestingly, *Neurospora* CYT-18 protein, which is known to interact with the *Neurospora* large mitochondrial rRNA and ND1 Group I introns, can substitute for an RNA domain in *Tetrahymena* Group I intron (Mohr et al. 1994). *Neurospora* introns are able to form most of the RNA secondary structures required for activity but require CYT-18 protein for *in vitro* and *in vivo* splicing activity. This convergence in function between RNA and protein factors

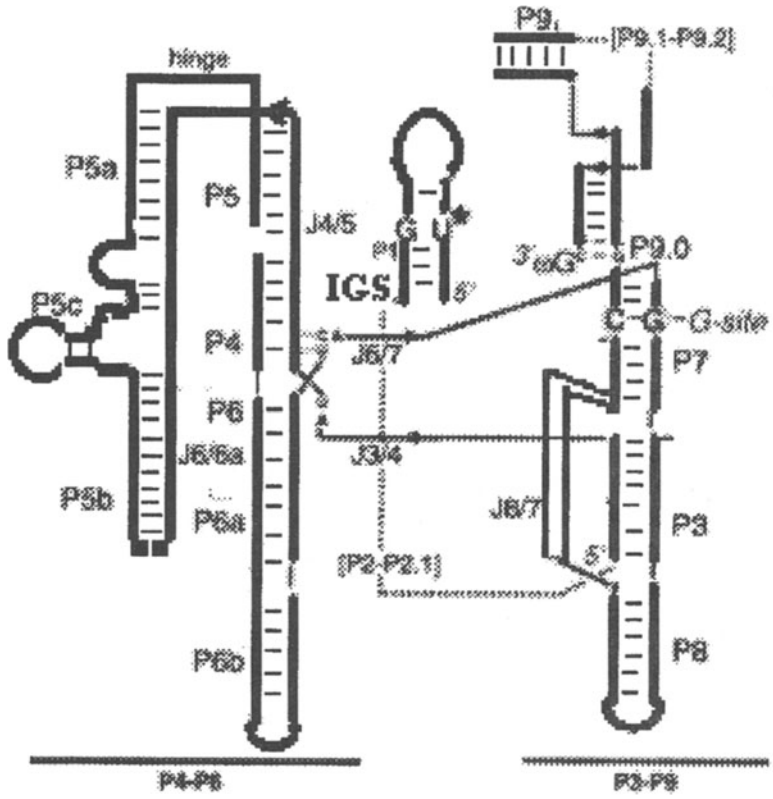

Fig. 2. Tetrahymena secondry structure of Group I intron. IGS-internal guide sequece, *site of cleavage

perhaps signals a transition from the relatively simple self splicing Group I introns to the more complex splicing pathways seen in higher eukaryotes (Weiner 1993).

Self splicing reaction proceeds via a 2-step transesterification mechanism initiated by nucleophilic attack by 3' hydroxyl group of an exogenous guanosine or guanosine nucleotide at the phosphodiester bond between the last nucleotide of exon 1 and the 5' end nucleotide of the intron (5' splice site) (Fig. 1). This results in the covalent linkage of the guanosine to the 5' end of the intron and the release of 3' OH free exon1, which remains bound to the intron by base pairing. In the second step, the free hydroxyl group of the 5' exon makes a nucleophilic attack on the 3' splice site, releasing the intron as a circular molecule and leaving a ligated exon. The reaction is completely reversible, so the intron is able to integrate between the exons (Woodson & Cech 1989).

2.2 Group II Introns

Group II introns are much less widely distributed than Group I introns and are commonly found in bacteria and other organellar genes of plants, fungi and yeast (Saldanha et al. 1993, Michel & Ferrat 1995, Bonnel & Vogel 2001). Most group II introns are present in mRNA but a few also occur in tRNA and rRNA genes. Much less is known about Group II introns. This is partly because very few of them

are required to be self splicing *in vitro*. Those that are autocatalytic require reaction conditions which are far from physiological and occur at moderately high temperature, e.g., 45°C (Jarrel et al. 1988). Extensive phylogenetic and mutational analysis led to the model for secondary structure of Group II intron (Fig. 3). It has six helical domains (I-VI) radiating as spokes from a central wheel. Domain V is believed to be the reaction centre. This and domain I are indispensable for catalysis (Jarrel et al. 1988, Koch et al. 1992). Domain VI contains highly conserved adenosine that is generally used to initiate the splicing reaction by the nucleophilic attack of its 2' OH group at the 5' splice site forming a 2'-5' phosphodiester bond at the adenosine branch site much like the splicing reaction of mRNA. This is followed by attack of 3' OH group of exon I at the 3' splice site resulting in the ligation of 2 exons and release of intron lariat (Fig. 4). In this splicing reaction recognition of 5' exon results from the interaction of intron binding sequences IBS1 and IBS2 with specific exon binding sequences EBS1 and EBS2 found in Domain I. Further interaction of ɵ and ɵ' sequences help defining 5' splice site.

2.3 RNaseP

RNaseP is involved in processing of 5' termini of tRNA precursors during their maturation. It is an essential, ubiquitous enzyme present in all cells and cellular compartments that synthesise tRNA, e.g., bacteria, eukaryotic nuclei, mitochondria and chloroplasts. All known RNaseP are ribonucleoproteins, with the exception of RNaseP of some plant protoplasts and trypanosome mitochondria (Altman & Kirsebom 1999, Salavati et al. 2001). In *E. coli*, the RNaseP consists of M1 RNA and a small basic protein, C5, of about 14kDa.The RNA component of RNaseP from bacteria is encoded by rnpB gene and varies in length between 350-450 nt (Brown 1999). There is little sequence similarity among 300 or

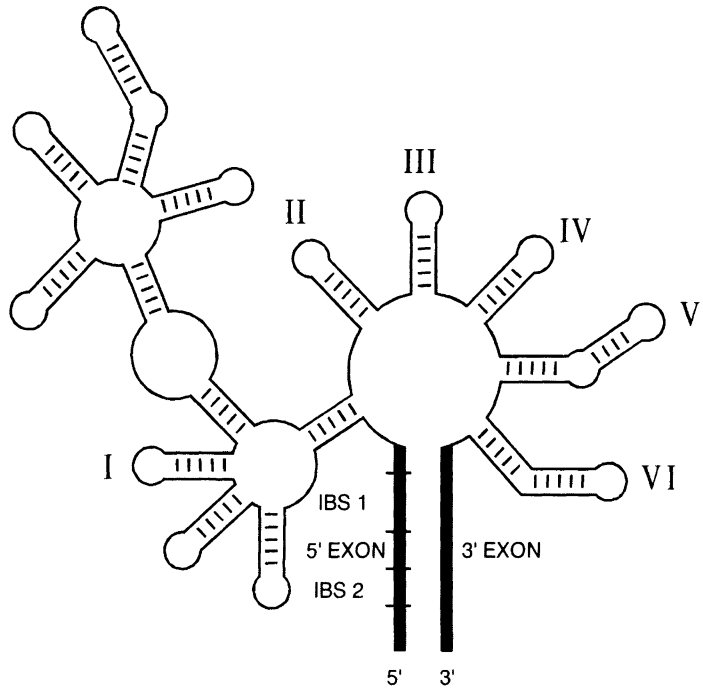

Fig. 3. Secondary structure of Group II intron. IBS –intorn binding sequence

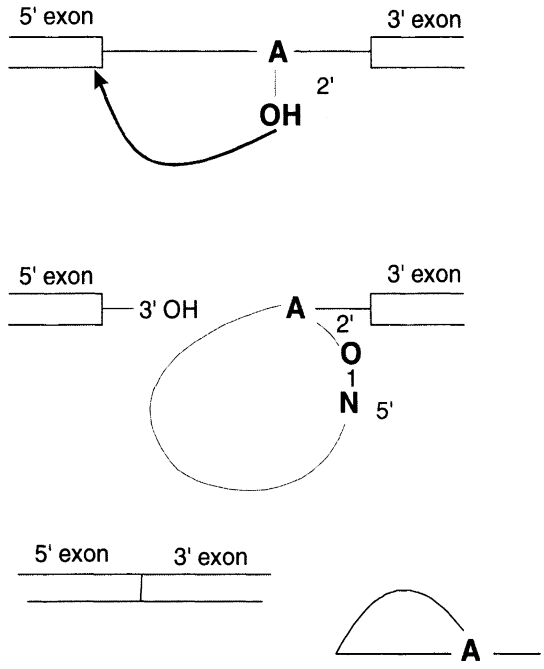

Fig. 4. Scheme showing splicing of Group II intron

so bacterial sequences except for a few short segments. Phylogenetic covariation analysis of the large data set has allowed the precise definition of the secondary structure and identification of several tertiary interactions (Brown et al. 1996, Masserie et al. 1998). RNA of RNaseP from bacteria can be divided into two distinct classes: type A and type B. The former, represented by *E. coli*, is the ancestral type found in most bacteria. Type B, represented by *Bacillus subtilis* is found in low GC content Gram positive bacteria (Haas et al. 1996). Despite differences in the secondary structural organization of type A and B RNAs, both can be modelled into a similar 3-D structure with the evolutionarily conserved nucleotide. Computer-aided modelling efforts illustrate a common phenomenon in RNA architecture for long range structural interactions that result in functionally equivalent structures. Another RNaseP, type C is found in nonsulphur bacteria (Haas & Brown 1998).

E. coli M1 RNA consists of 18 paired helices, P1-P18, whereas *B. subtilis* M1 RNA analogue is more diversified form. It folds into a similar structure but P6, P13, P14, P16 and P17 are missing and it contains helices P5.1, P10.1, P15.1 and P19. RNase P from archea can also be divided into two structural classes, type A, the most common and similar to the bacterial type A structure, and type M, a derived structure found only in two species so far (Harris et al. 2001). Similarly, eukaryotic RNaseP sequences can also be fitted into a minimal consensus secondary structure (Frank et al. 2000).

Although pre-tRNAs are the substrates for RNaseP, in *E. coli* other RNA, such as 4.5 S RNA, an analogue of eukaryotic SRP RNA, some small phage RNAs, 30S pre-rRNA are also acted upon by RNaseP. The exact mechanism by which tRNA precursor is bound is still unclear. The 3' half of the acceptor stem is thought to function as an external guide sequence (EGS), but it does not uniquely

define the cleavage site. The primary sequence does not seem to be important nor does any single element uniquely define the cleavage site. Instead, recognition could result from several redundant factors, including the distance along the coaxially stacked T stem-loop and acceptor stem, the 3'CCA sequence, the EGS alignment and a conserved guanosine 3' to the cleavage site (Altman et al. 1993). This indicates that the tertiary interaction with the substrates may constitute an important factor in the recognition by the ribozyme.

In *E. coli,* C5 is essential for activity of RNaseP *in vivo.* However, M1 RNA by itself can catalyse the reaction *in vitro.* This reaction requires high salt concentration, e.g., 1M K^+ or NH_4^+ and 10 mM $MgCl_2$ (Pace & Brown 1995), which suggests that the basic protein C5 serves as an electrostatic shield facilitating binding between RNA enzyme and the RNA substrate. RNA cleavage takes place via nucleophillic attack on the phosphodiester bond leaving 5'P and 3' OH at the cleavage site and there is no absolute requirement for divalent metal ions.

2.4 Small ribozymes

2.4.1 Hammerhead ribozyme

Some members of plant pathogens such as viroids, virusoids and satellite RNAs undergo site specific self cleavage. During infection, self-cleavage is thought to be involved in conversion of linear RNA multimers, produced by rolling circle replication, into unit sized progeny. Self-cleavage of plant infectious RNAs was discovered by Bruenning and his colleagues working on satellite RNA of tobacco ringspot virus, sTobRV (Buzayan et al. 1986). Symmons and coworkers (Forster & Symmons 1987a, b) provided structural basis by identifying conserved sequences. The secondary structure that has a hammerhead shape was common to a large class of self-cleaving RNAs. It was quite remarkable that the hammerhead, which has a small size of 55nt RNA could undergo self-cleavage.

Uhlenbeck (1987) reformed an active self-cleaving RNA by combining the top and bottom strands of the hammerhead. In the process, not only the size was reduced to 43 nt but the lower strand could carry out multiple cleavage reactions on the upper strand, thus behaving like a true enzyme (Fig. 5). One

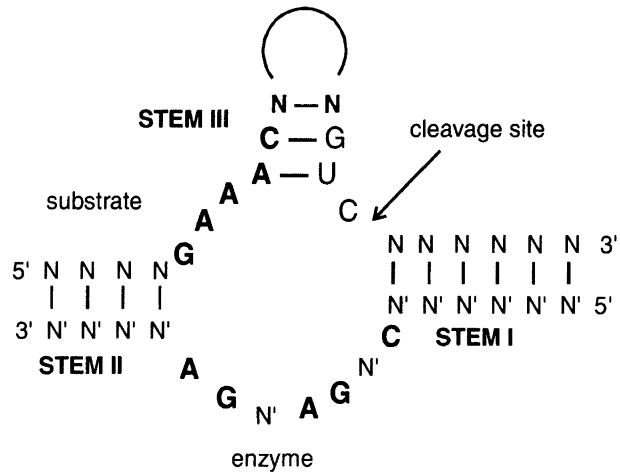

Fig. 5. Hammerhead ribozyme substrate complex based on Uhlebeck (1987)

constraint still remained in the trans cleavage reaction of Uhlenbeck's strategy. In this, there are several bases that are conserved in the target sequence, thus restricting the choice of target in trans cleavage. This was got around by Haseloff and Gerlach (1988) who included most of the conserved bases in the enzyme strand leaving very little sequence constraint on the substrate (Fig. 6). This ensured flexibility in the design of ribozyme for diverse targets.

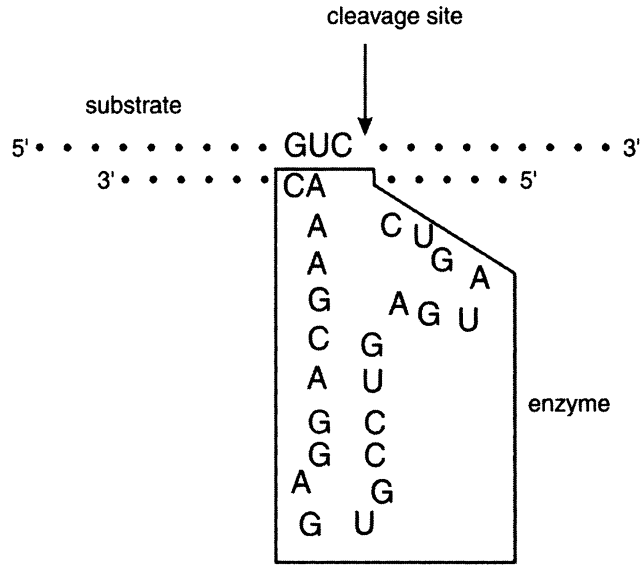

Fig. 6. Hammerheaded ribozyme-substrate complex based on Haseloff and Gerlach (1988); Boxed region represents the ribozymes

Hammerhead ribozyme has three basic components: I) a 22 nt catalytic domain, ii) base pairing sequence flanking the susceptible 3'-5'-phosphodiester bond, and iii) a recognition sequence on the target RNA such as GUC. The cleavage reaction occurs 3' to the recognition sequence with formation of a terminus containing 2', 3'-cyclic phosphodiester and a 5' OH terminus on the 3' fragment. Two crystal structures of noncleavable variants of the hammerhead ribozyme have been solved, one with noncleavable DNA as substrate strand and the other with an RNA substrate containing a 2'-O-methyl group at the cleavage site (Doherty & Doudna 2000). Based on fluorescence and native gel electrophoresis data, a Y shaped structure formed by three helices, Stems I, II and III, was proposed (Fig. 7). Stem I forms a sharp angle with stem II, which is coaxial to stem III.

2.4.2 Hairpin ribozyme

The first naturally occurring hairpin motif was identified in the negative strand of the satellite RNA of tobacco ringspot virus (sTobRV). The other hairpin ribozymes are found in the satellite viruses of arabis mosaic virus (sARMV) and chicory yellow mottle virus (sCYMV). The hairpin ribozyme from sTobRV consists of four stem regions that when lined up coaxially, somewhat resemble a hairpin (Fig. 8). The catalytic centre consists of two minimal sequences of sRNA: a catalytic RNA with 50 satellite bases and a substrate RNA with 14 satellite bases, which cleaved to form the corresponding 5' and 3' fragments

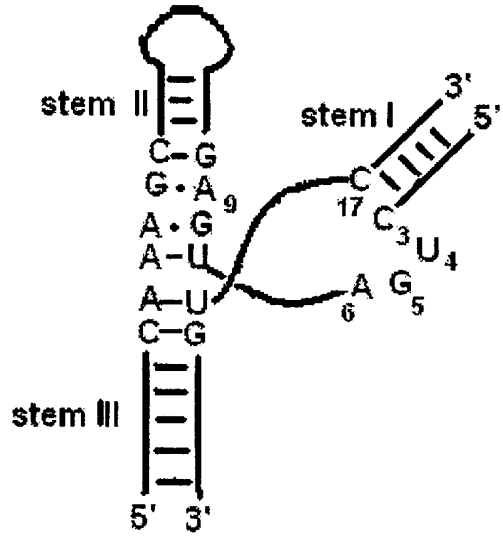

Fig. 7. Y-shaped model of Hammerhead ribozyme (adapted from Dohery & Duodna 2000)

(Hampel et al. 1990). The proposed model suggests the existence of four helical regions, which are the structural elements that can largely be changed, as long as the integrity of the helices is maintained. Helices 3 and 4 are within the ribozyme itself and helix 1 (6 bp) and helix 2 (4 bp) separated by an NUGC loop in the substrate strand. The cleavage takes place 5' to the G of GUC. This G is essential but altering the other conserved position can dramatically reduce the activity as well. The hairpin ribozyme-substrate

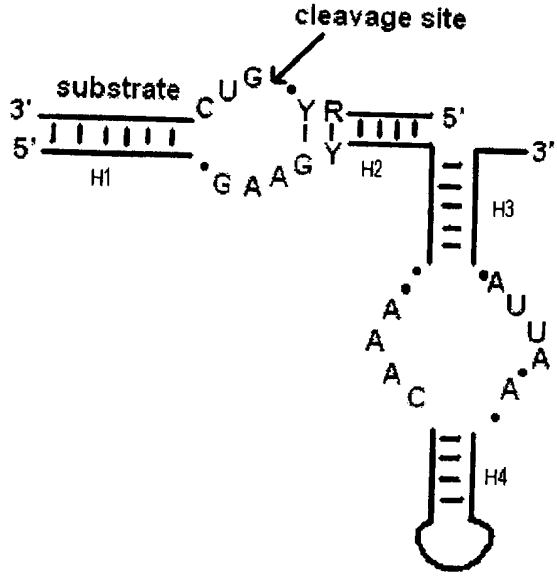

Fig. 8. Hairpin ribozyme -substrate complex

complex forms two domains viz., ribozyme and the substrate and an internal loop. Domain B is entirely composed of ribozyme. After formation of the ribozyme-substrate complex, an essential conformational change must occur. Domain A folds upon domain B and the nucleotides of both the loops interact creating a buried active site. This process, known as docking, is essential for the activity of the ribozyme (Walter et al. 1998). Like hammerhead ribozyme, hairpin ribozyme also produces products terminating in 2', 3'- cyclic phosphodiester and 5' OH.

2.4.3 Hepatitis Delta Virus

Hepatitis Delta Virus (HDV) is an infectious agent that exists as a satellite RNA of hepatitis B virus (Taylor 1992). It is widespread and causes severe fulmination in hepatitis infected patients. It has a circular RNA genome, about 1700 nt long and encodes a single protein that is expressed in two forms due to RNA editing event. The RNA is believed to replicate by the double rolling circle mechanism which requires self-cleavage by closely related versions of a catalytic domain contained within the genomic and antigenomic RNAs (Wu et al. 1989). The 2 ribozymes are similar in sequence and structure, though the proposed common secondary structure differs from those of other small catalytic RNAs. The minimal domain containing self cleaving activity has one nucleotide 5' and 84 nucleotide 3' to the cleavage site for both domains. Based on the crystal structure of the genomic cleavage product, HDV ribozyme is folded into a double pseudoknot containing four helical stems (P1-P4). One additional small stem P1.1 is coaxially stacked upon P4, while P2 is stacked on P3 (Ferre D'Amre et al. 1998, Fig. 9). There are three joining regions between the helices (Tanner et al. 1994). Like hairpin and hammerhead ribozymes, the catalytic activity of HDV is more efficient in the divalent cations. However, the crystal structure shows a deep active site devoid of well ordered divalent metal ions, suggesting that divalent cations are not required to stabilize the structure of the cleavage product.

2.4.4 VS ribozyme

The VS ribozyme was isolated from the mitochondria of natural Varkud-1 strain of *Neurospora* (Saville & Collins 1990). The Varkud plasmid is a retroplasmid, which encodes a reverse transcriptase, and a

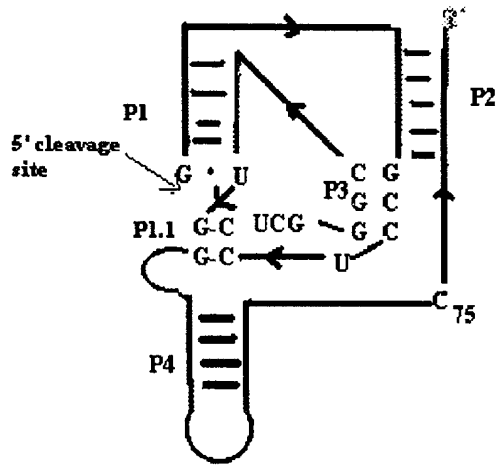

Fig. 9. Two dimension structure of genomic hepatitis delta virus (adapted form Ferre-D' Amare et al. 1998)

small, unrelated RNA (VS RNA). The VS RNA is transcribed from circular or multimeric VS plasmid by a mitochondrial RNA polymerase. The resulting transcripts are subsequently site specifically cleaved and ligated to form circular 881 nt long, RNA monomers, which are reverse transcribed and made double stranded to form the mature VS plasmid (Kennell et al. 1995). The catalytic activity has been restricted to a 154 nt long fragment, retaining 1 nt 5' of the cleavage site and 153 nt at its 3' site. This structure can further be reduced to 121-126 nt (Rastogi & Collins 1998). The catalytic domain of VS RNA is converted to a trans cleaving ribozyme by using a 144 nt fragment of the VS RNA. The minimal substrate consists of 1 nt 5' and 19 nt 5' to the cleavage site, and it forms a short stem-loop structure (Perrotta & Been 1990).

3. CATALYSIS

All the ribozymes known to-date catalyse cleavage and ligation of RNA backbone. The cleavage reaction carried out by all of them is phosphoryl transfer of different forms. In the case of small ribozymes, the cleavage is brought about by the attack by adjacent 2' oxygen nucleophile of the phosphate in the RNA backbone, resulting in the products with 2', 3' cyclic phosphate and 5' OH termini. In contrast, such attack is by remote 2' oxygen in Group II intron and by 3' oxygen of exogenous guanosine in group I intron (Fig. 10).

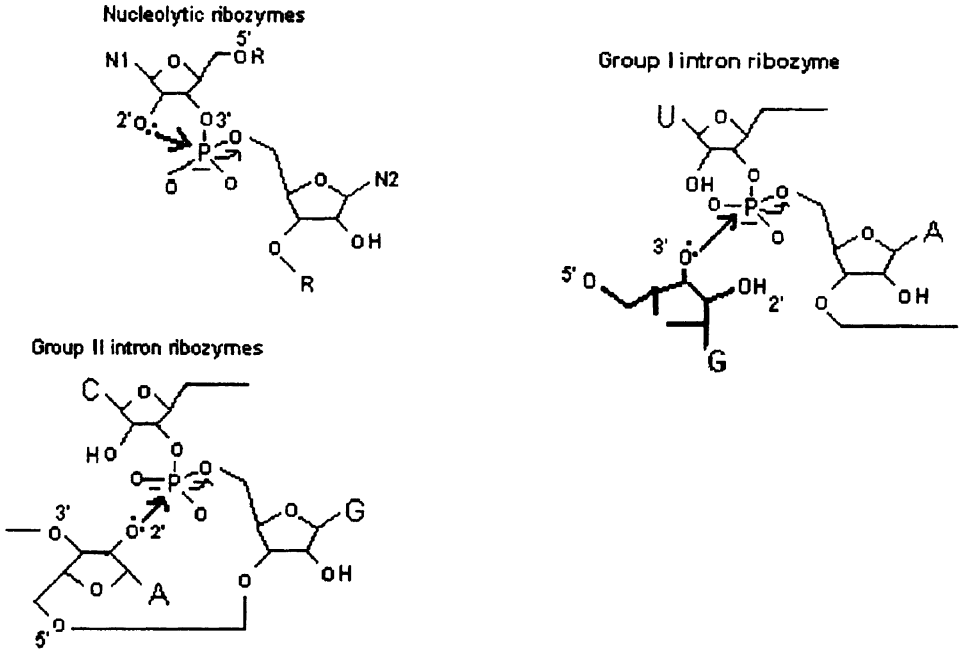

Fig. 10. Chemical mechanism showing cleavage of phosphodiester bonds in a) nuleolytic ribozymes b) Group I, c) Group II intron ribozymes

The nucleophilic attack mentioned above follows, in the case of small ribozymes an S_N2 mechanism in which the reaction is accompanied by an inversion of configuration at the phosphate as revealed by replacing one of the nonbridging oxygen atoms by sulphur (Van Tol et al. 1990). The transition state is an oxyphorane intermediate with a trigonal bipyramidal structure containing the attacking 2'-O and departing 5'-O atoms at apical positions (Fig. 11).

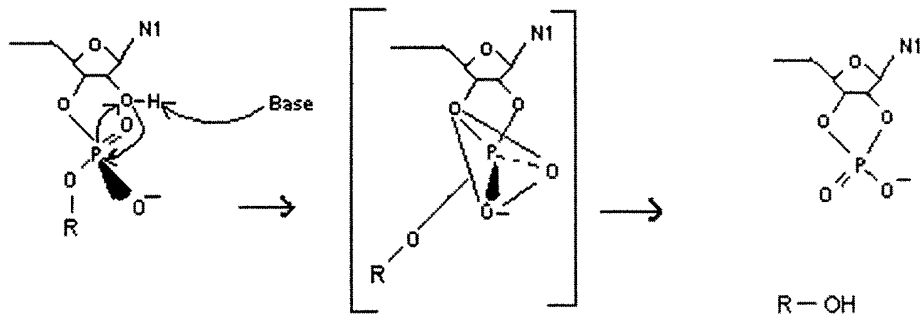

Fig. 11. Catalytic processes showing in line attack leading to the formation of 2'-3' cyclic phosphate and 5' OH products in Hammerhead ribozyme

A similar reaction carried out by a protein enzyme, RNaseA, occurs via acid base catalysis, in which one histidine imidazole group (His 12) acts as a general base to deprotonate the 2' OH, while the other (His 119) protonates the departing 5' oxyanion as a general acid. The pentacoordinated phosphate of the transition state is thought to be stabilized by the positive charge of a nearby lysine side chain (Richards & Wyckoff 1971). It is the precise positioning of these groups and the ability of the protein to modulate pK_as of the reactive histidines that are responsible for the rate acceleration by RNase A. Since metal ions, such as Mg^{+2} and other divalent cations, play a crucial role in the folding of RNA molecules, thus creating the active geometry of ribozymes and RNA structures often contain specific binding sites for divalent ions, ribozymes may carry out metal ion associated catalysis. Divalent metal ions, particularly Mg^{+2}, could play different roles in ribozyme catalysis. A metal ion coordinated to a hydroxide might activate a –OH or water nucleophile by deprotonation or a divalent ion might directly coordinate the nucleophile oxygen, making oxygen more susceptible to deprotonation by OH^-. Metal ions also might stabilize the transition state by direct inner-sphere coordination to the pentavalent scissile phosphate group and might stabilize the leaving group by protonating or directly coordinating the leaving oxygen atom. Metal ions might also stabilize the transition state structure by donating positive charge.

Recent results suggest that the mechanisms used by various ribozymes differ than previously thought. *Tetrahymena* ribozyme requires divalent metal ions in order to attain three dimensional structure. This can also be met by a variety of divalent cations and partially met by high concentration of monovalent cations (Downs & Cech 1996). Moreover, there is a requirement for Mg^{+2}, not met by Ca^{+2}, in correct positioning of the 5' splice site helix within its active site. Since metal ions are required to fold the ribozyme, it has been difficult to identify additional metal ions involved directly in active-site chemistry, especially since they are already bound at the 2mM Mg^{+2} required for ribozyme folding. This was addressed by experiments in which key oxygens were substituted by S or N, which provided strong evidence that three directly coordinated Mg^{+2} ions participate in the catalytic mechanism to activate nucleophile, in this case the 3' OH of guanosine and stabilize the leaving group (Fig. 12).

In manner similar to the above, sulphur substitution of nonbridging pro-Rp oxygen of scissile phosphate of hammerhead ribozyme resulted in the reduced activity which could be retrieved by the addition of

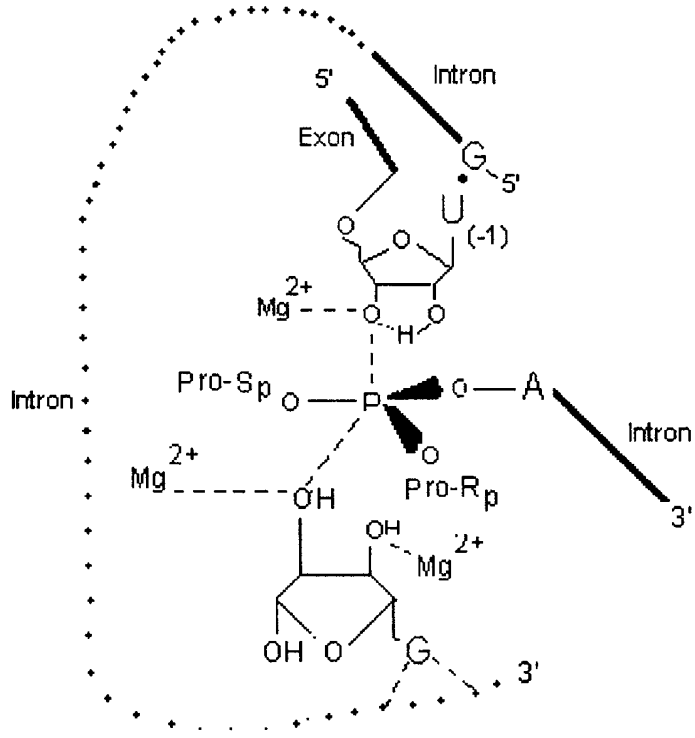

Fig. 12. Transition state intermediate of Group I intron - Mg^{+2}

thiophilic metal ion, suggesting that a metal ion directly coordinates this oxygen in the transition state. The reaction rate increases linearly with pH, indicating that the nucleophile is activated by a hydroxide ion. Either a metal hydroxide deprotonates the 2' OH directly or a metal ion coordinated to the 2' OH increases the acidity of 2' oxygen, rendering it susceptible to attack by a OH^- from solution. The ion that is coordinated to the pro-Rp oxygen could perform either function. In the case of two metal ion mechanism for self cleavage, additional directly coordinated ion should stabilize the leaving group oxygen, for which there is no evidence available. These results raise doubt on the number of divalent metal ions directly involved in catalysis. The ability of divalent cations at extremely high ionic strengths may suggest that divalent cations are not essential cofactors in the reaction. However, at least one directly coordinated Mg^{+2} may be involved in catalysis *in vitro*. In the case of hairpin ribozyme, changes in pH and substitution of sulphur as described above for hammerhead ribozyme have minimal effect on the rate of reaction under a wide variety of conditions. Besides, cobalt hexamine, a metal complex inert to changes in its coordination sphere, supports full hairpin ribozyme cleavage, providing evidence against the importance of direct coordination of metal to the RNA (Nesbitt et al. 1997).

4. RIBOZYME KINETICS

Extensive studies have been conducted on the kinetics of ribozyme catalysis. In a multiple turnover event several factors contribute to the overall reaction rate, viz., substrate recognition binding and

release, bond cleavage and ligation, product release and rebinding. A Michaelis-Menten mechanism of hammerhead ribozyme has been established by using multiple and single turnover conditions for the formation of the ribozyme-substrate complex and its subsequent conversion to products (Fedor & Uhlenbeck 1990, Perreault 1990). Under multiple turn over conditions, the catalytic rate constant, k_{cat}, is a measure of rate limiting step, which can be cleavage, conformational transitions of the ribozyme-substarte complex or product release. Complete description of minimal kinetic scheme was first accomplished for the hammerhead ribozyme, using a construct with stem II connected with a loop and duplexes of eight base pairs in stems I and III. With this system it was found that to a good approximation, binding and release of substrate and product follow the kinetics expected for formation and dissociation of standard Watson-Crick duplexes in the stem regions. The rate of chemical step, i.e., bond cleavage, was found to be essentially same for different hammerheads under a given set of conditions. Extension of the stems generally results in an increased stability and markedly decreased k_{cat}.

It is notable that the cleavage rates for representatives of all four small ribozymes are of the same magnitude, ~1 min^{-1}. It is equally notable that the equilibrium between product and substrate for the hairpin ribozyme is inverted relative to that of the hammerhead, with ligation being favoured over cleavage (Nesbitt et al. 1997).

5. APPLICATIONS

Ribozyme has the ability to inhibit the expression of a specific gene and this property has three major applications: i) as a tool for molecular biology, e.g., the effect of a specific gene on a cell could be found by inhibiting its expression using ribozyme, ii) for generating genetically altered organisms such as virus resistant plants, animals, novel strains, plants or animals which no longer possess a certain undesirable trait by selectively blocking specific gene expression and iii) in gene therapy. However, by and large the applied research with ribozymes is directed in evaluating its efficacy as therapeutic agents.

5.1 Design of ribozymes

Ribozymes are promising tools as therapeutic agents. Although mutational, chemical and physical investigations have contributed to our understanding of how ribozymes act *in vitro*. For both *in vitro* and *in vivo* use of ribozyme, the design of optimal ribozyme to a target RNA has to be experimentally determined. Some of the basic rules for such design will be illustrated using hammerhead ribozyme as an example. Although the target site for cleavage was initially 5' GUC 3', subsequent studies showed that this sequence requirement can be modified to 5' NUH 3' of target RNA, where N is any nucleotide and H can be U, C or A. This cleavage rule has been further extended from the work of several groups (Perimann et al. 1992, Shimayama et al. 1995, Zoumadakis & Tabler 1995, Kore et al.1998) reported that the NUH rule can be changed to NHH rule, where H is any nucleotide but G. However, this can be further complicated when target RNA structure in cellular environment is taken into consideration.

The length of the 3' arm is apparently more critical for specificity than that of the 5' arm (Hertel 1996). For a long substrate RNA, intramolecular structures of the target RNA could interfere with the binding of a ribozyme, for *in vivo* application, cellular proteins could also interfere with ribozyme activity. Results from variations in arm length of ribozymes from 5-18 nt have indicated that the ribozyme activity is closely related to the arm length both in symmetric and asymmetric models, and this depended somewhat on the sequence context (Sioud 1997).

Ribozymes can be produced chemically, biochemically or biologically. During chemical synthesis, ribozymes can be modified by incorporating deoxynucleotides, phosphoramidates to increase stability. The arms of ribozyme which hybridizes with target are responsible for site specificity. In addition to site specific cleavage, ribozymes can perform site specific ligation also which can be exploited in repair of mRNA. Significant progress has been achieved in engineering, but still a lot has to be known about it in order to use this in intracellular applications. The intracellular efficiency of ribozyme depends on five major factors.

1. The delivery of ribozymes to appropriate cells
2. The efficient expression of ribozymes in these cells
3. The co-localization of ribozymes in the same intracellular compartment as the target substrate RNA
4. The specificity of ribozyme to recognize and cleave only its target substrate RNA
5. Turn over

5.2 Delivery

The efficiency of ribozyme as therapeutic agents depends on the methods for delivery of ribozyme for ribozyme expression to the appropriate target cells. The delivery of gene constructs using retroviral vectors has been most exploited approach for gene transfer protocols. These vectors can be engineered to deliver either RNA polymerase II or pol III transcriptional units for ribozyme expression.

Delivering ribozyme into cellular environment includes two methods.

5.2.1 Exogenous delivery

In this preformed ribozymes are delivered into cells using different techniques. Exogenously delivered ribozymes are subjected to short circulation lifetime because of endogenous nucleases and less cellular uptake. Exogenously administered molecules enter cells *in vitro* by a combination of fluid-phase (pinocytosis), adsorptive and receptor mediated endocytosis. The cellular uptake can be improved by liposome mediated delivery. Chemically modified c-myb ribozymes were targeted to smooth muscle cells resulted in the reduction of c-myb expression and decreased cell proliferation (Javis et al. 1996). In another study two chemically modified hammerhead ribozymes were targeted to N-ras oncogene using an N-ras luciferase gene fusion as a reporter system and found 55% reduction of liciferase activity in transfected cells (Scherr et al. 1997). In another study liposome entrapped ribozymes targeted against tumor necrosis factor (TNF) were introduced into mice by peritoneal infection and 60% cells received ribozymes. Ribozymes were still active two days after infection and reduced lipopolysaccharide induced TNF protein level by 70%. Cationic liposomes are widely used as they can easily form lipid-nucleic acid complexes (lipoplexes) because of opposing electrostatic charges. By virtue of positive charge these cationic liposomes have high affinity to cell membranes, which are negatively charged, and gains easy entry into cells by adsorptive endocytosis. Kisich and his coworkers also achieved reduction in the expression of leukocyte type 12-lipoxygenase expression in vascular smooth muscle cells by delivering hammerhead ribozymes using cationic liposomes (Kisich et al. 1999). Cationic liposomes are formed by incorporating cationic lipids such as DOTAP, lipofectin into liposomes. One major problem with liposome mediated delivery is that ribozymes becomes trapped in the endosome and must be released into intracellular space to reach its target. To facilitate subsequent release from endosomal or

lysosomal compartments, commercially available lipoplex transfection agents (also called transfectins or cytofectins) are used, which contain a helper lipid such as phosphotidyl ethanolamine (DOPE). DOPE is an inverted cone shaped lipid that aids cytosolic release by fusing to and /or disrupting the endosomal membrane possibly by the formation of lipid structures (Zelphati et al. 1996). Optimising the amount of cationic lipid used is also essential to minimize the potential toxicity to cultured cells.

Another development to enhance release from endosomal compartments is the use of pH sensitive fusogenic liposomes. These consist of a bilayer forming lipid such as DOPE and a titratable acidic amphiphile such as oleic acid (OA) or cholesterylhemisuccinate (CHEMS) (Monica Cristina et al. 1998). At pH 7 amphiphile maintains lipid mix in bilayer structure and as the complex moves through the endosome pH decreases, amphiphile gets protonated, resulting in the collapse of liposome. The liposome fuses with endosomal membrane releasing the contents into cytoplasm. The anionic nature of pH sensitive liposomes leads to poor encapsulation of oligodeoxynucleotides and ribozymes. To overcome this problem cationic amphiphiles were developed. They consist of hydrophobic cholic acid group covalently linked to spermine or spermidine groups, which enable association with nucleic acids. Delong and his coworkers obtained 250 fold increase in cellular uptake by using cationic amphiphiles compare with naked oligonucleotides (ODNs) (Delong et al. 1999). The rapid degradation and pharmacokinetic elimination of nucleic acids *in vitro* and *in vivo* suggests that the pharmacological effects of ribozymes are likely to be short lived. Repeated administration is required to increase the duration of ribozymes for good efficacy. Biodegradable polymers as delivery systems provide sustained release of active compounds, which can improve pharmacokinetics and pharmacodynamics of ribozymes. The most widely used polymers are polylactides (PLA) and copolymers of lactic acid and glycolic acid P (LA-GA). These polymers have been evaluated for their potential as delivery agents for antisense ODNs and ribozymes. These can be delivered locally or parenterally or administered systemically. They are used routinely in resorbable surgical sutures and in commercially available as sustained release preparations (Ex Zoladex TM, AstraZeneca, Alderley Edge, UK).

Another method of delivery of ribozymes to cells includes receptor mediated endocytosis. Hudson and coworkers demonstrated three fold increase in cellular uptake of ribozymes conjugated to a transferring receptor antibody (TFA) compared with free ribozymes. This uptake was temperature dependent, indicative of a receptor mediated process and inhibited by excess free TFA.

5.2.2 Endogenous delivery

This delivery technology is most widely used in gene therapy and requires the cloning of ribozymes encoding gene construct into an expression vector. Then the cloned construct has to be introduced into target cells by transfection. Two types of vectors are in use. They are non viral and viral vectors. Viral vectors are widely used because of the inherent property of viruses to deliver DNA into cells. Among these the retro viral, adeno viral and lenti viral vectors are commonly used. All these vectors have some advantages as well as some limitations. Retro viral vectors have the advantage of stable integration with host cell genome and the absence of expression of viral proteins leads to less immune response (Weltch et al. 1998). The limitations are low efficiency of transduction into primary cells, inability to transduce non dividing cells, random integration into host genome and trasnscriptional silencing of encoded genes. Adeno viral vectors do not integrate with host genome instead remains as extrachromosomal element and replicates in synchrony with cell divison, which results in high viral titers (Czupayku et al. 1997).

They provide very efficient transduction of both dividing and non dividing cells (Lieber et al. 1996). Expression of trans genes is only transient in actively dividing cells and adeno viral vectors express some viral proteins at low level, which results in host immune response against the transduced cells. Most recently 'gut less' adeno viral vectors have been developed that lack all viral coding sequences thus making these vectors ideal for delivering ribozymes (Parks et al. 1996).

Adeno associated virus is attractive as it is a small non-pathogenic virus that can stably integrate a ribozyme expression cassette with host genome without the expression of any viral genes. An alpha virus provided high cytoplasmic expression of ribozymes and transduction to mammalian cells. Lenti virus also can be used for ribozymes expression due to their ability to integrate in nondividing cells. Different retro viral vectors have been used for ribozymes delivery in the field of cancer or viral diseases such as AIDS. T cells have been transduced with hairpin or hammerhead ribozymes to HIV by using retro viral vectors (Kronenwetter et al. 1996). Adeno viral vector was used to investigate the therapeutic use of ribozymes for the treatment of hepatitis and genetic disorders *in vivo* (Huang et al. 1997). The level of human growth hormone mRNA expression in transgenic mice was reduced by ~96% after delivering ribozymes by adeno viral vector. In another example adeno virus encoded ribozymes were used for treating Epstein-Barr virus infections. The above examples illustrate that viral vectors act as efficient vehicles to take ribozymes to their destination.

5.3 Expression strategies

The success of ribozyme mediated gene inactivation depends on the choice of expression system. Expression system includes promoter to regulate the level of ribozyme expression, *cis* appending elements such as capping, polyadenylation signals, which offer stability to ribozymes and localization signal, which target ribozymes to specific location.

In nature polIII promoter directs a high level of expression of small, stable RNAs such as tRNA, 5SRNA and U6 small nuclear (sn) RNA. A mouse U6 sn RNA- hammerhead chimeric ribozyme directed against chloramphenicol acetyltransferase (CAT) RNA was found to be effective in HeLa cell culture (Dahlberg et al. 1992). A human tRNAMet and a U6-snRNA polIII promoter have been used to express ribozymes directed to HIV (Good et al. 1997). In polIII transcriptional systems promoter elements are located upstream of the coding sequence. An important feature of these systems is that a minimum amount of cis appended sequences are required for proper initiation and termination of transcripts. For example U6 system requires atleast 30 nucleotides of mature U6 coding region to cap transcripts with g- methyl phosphate (Noonberg et al. 1994). In polIII system the termination signal, a stretch of five uridines follows the coding region of transcripts. Non ribozymic flanking sequences arising from expression cassettes some times interfere with proper folding and activity of ribozymes. To over come this problem these sequences are sequestered in a secondary structure making them unavailable for interaction with ribozymes (Thompson et al. 1995).

Viral promoters also can be used for ribozymes expression. Moloney murine leukaemia LTR promoter is more efficient than cytomegalo virus and tRNA Met promoters (Zhou et al. 1996).

PolII snRNA promoter has also been used for ribozyme expression and these target ribozymes to cytoplasmic compartments that provides colocalization with the target. A U1 snRNA chimeric ribozymes directed against HIV-Rev pre mRNA was found to accumulate in large amounts in Xenopus laevis oocytes and to reduce the level of target RNA. The expression of ribozymes by the human U6 in which

capping signal was appended to the ribozymes resulted in inhibition of HIV-I replication in cotransfection assay. U6 promoter provides capping and nuclear localization. Capping is believed to increase the stability of transcript against endogenous nucleases.

Additional control over ribozymes expression is required while expressing ribozymes in transgenic models. In this regard tissue specificity and control over expression levels are the major concerns. Tissue specificity can be obtained by using tissue specific promoters. This approach has been successfully used with tyrosinase promoter, which exclusively expressed ribozymes in melanocytes (Ohta et al. 1996). Another problem, controlling the level of expression can be solved by using inducible promoters like tetracycline. Juhl and his coworkers are able to turn on and off of the ribozyme expression by tetracyclineinducible promoter in both cell culture and in animals (Juhl et al. 1997)

5.4 Identification of accessible sites

RNA adopts a complex secondary structure consisting of predominantly single stranded and double stranded regions. Traditionally, site selection is based on the computational secondary structure prediction of target RNA. However it is not clear how predictive such computations are. Experimental approaches rely on RNase II cleavage of RNA in an RNA-DNA hybrid. In one example, an algorithm that takes into account the secondary structure of target as well as ribozymes was used to select 26 potential cleavage sites *in vivo* on a C-myb RNA transcript, to which complementary nucleotides were annealed and complexes treated with RNase II (Christoffersen et al. 1994). Eighteen were then synthesized that were targeted to the sites and had given reasonable degree of cleavage.

5.5 Stability

Stability of the ribozymes is the major concern. If ribozymes are expressed *in vivo* its stability can be increased by using polyadenylation signals and U6 promoter that provides cap (Rossi 1995). Producing circular ribozymes *in vivo* using permuted intron exon sequence is another technique employed to increase stability. The circular ribozymes have enhanced resistance to nuclease degradation relative to the linear form of ribozyme, suggesting circularization *in vivo* as a viable alternative to chemical modification *in vitro* to protect from nucleases. A circularly trans-acting hepatitis delta virus ribozyme was successfully used on synthetic substrates in Hela cell and nuclear extracts (Puttaraju et al. 1993). Circular RNAs generated by splicing are devoid of flanking sequences that could potentially interfere with function. As circular RNAs are not the primary substrates for exonucleases they may have increased half lives relative to linear molecules with similar sequence (Puttaraju & Been 1996). Circular ribozymes can be synthesized *in vitro* by using T4 RNA ligase. The efficiency of circularization can be increased by using ODN as template (Wang & Raffner 1998). The template should be designed to prevent the precursor from folding into an unsuitable structure. In order to increase stability of ribozymes for *in vitro* applications nuclease resistant ribozymes have been used. Sioud and Sorensen have used a synthetic protein kinase Cα ribozyme with complete 2-aminopyramidine substitution for pyramidine. They have observed retention of cleavage activity and 14,000 fold increased half life in serum compared to unmodified ribozyme (Sioud & Sorensen 1998).

5.6 Colocalization

Colocalization is an important issue to be considered for the effective inhibition of gene expression by ribozyme. Localization provides easy accessibility of target to ribozyme, which in turn leads to high

catalytic activity. To demonstrate this Sullenger and Cech have taken retroviral dimerization domains forcing encapsulation (co-packaging) of transcript encoding ribozyme with the retroviral RNA encoding the target lacZ. The ribozyme was only effective when the RNAs were copackaged. This indicates that ribozymes when copackaged along with the virus carrying LacZ gene decreased its expression level by cleaving it, but failed to inhibit LacZ when packaged separately (Sullenger & Cech 1993). This result indicates the importance of colocalization for ribozyme's efficiency.

5.7 Specificity and turnover

The specificity of ribozyme is an important factor to be considered while designing it. Ribozyme should recognize its substrate specifically from the pool of other RNA molecules; otherwise it would lead to deleterious effect on the host. Specificity of ribozyme depends on its arms, which hybridize with the substrate. In *in vivo* applications ribozymes with longer arm length have given good results. The cleavage efficiency of ribozyme with 7 nucleotides in each arm is more against 1000 nt long HIV-1 transcript than that of a ribozyme having 5 nucleotide in each arm (Heidenrich & Eckstein 1992). The reason might be that the longer arms bind to the substrate by interaction with single stranded regions and make the cleavage site available for ribozyme. However, longer arms are not always beneficial. A ribozyme with 7-8 nt in each arm is more efficient than the one with 10-13nt arms in cell culture conditions (Lieber & Strauss 1995). The above examples show that optimum arm length differs in different ribozyme-substrate combination and has to be determined experimentally. Sometimes, effectiveness of a ribozyme designed to a single target sequence in the substrate RNA may not give optimum result due to complex local secondary structure. Designing a ribozyme targeted to multiple sites elicits better response (Chen et al. 1992).

5.8 *In vitro* evolution

In nature evolution is a continuous and slow process that occurs through millions of years. It is not possible for a human observer to visualize it. Hence researchers started with *in vitro* evolution of enzymes and ribozymes to obtain better catalytic properties. The principle of *in vitro* evolution is based on Darwin's 'survival of the fittest' hypothesis. In this also molecules with desirable qualities are selected and undesirable molecules are rejected.

In vitro evolution brings about genotypic change to achieve desired phenotype. Sol Spiegelman exploited this for the first time in 1960 with RNA bacteriophage QB and obtained RNA whose replication time was shortened. The ligand that emerges from *in vitro* selection is called "Aptamer". The first RNA aptamer targeted to a small biomolecule was directed at ATP, one of the most important cofactor in contemporary metabolism (Sassanfar & Szostack 1993). This aptamer was isolated by passing a random RNA pool over a column of immobilized ATP, washing away unbound sequences and then eluting with free ATP. After six cycles of selection and amplification ATP binding sequences dominated the pool. Cloning and sequencing of these RNA revealed a common 11- nucleotide motif that always occurred within the same secondary structural context. Not every aptamer selection leads to the molecules with same secondary structure for example heptamers of arginine or guanosine binders showed different structures. The isolation of reverse transcriptase and invention of Polymerase Chain Reaction made it easy to evolve any nucleic acid *in vitro* (Connel & Yarus 1994). A variation on the aptamer selection can be used to evolve catalytic nucleic acids. Such approaches have been used to impart new function to known ribozymes, to create new ribozymes and to understand ribozyme structure, catalytic mechanism and folding pathways

(Michel et al. 1990, Beaudry & Joyce 1992). RNA world hypothesis has stimulated many experiments to determine the range of reactions that RNA can catalyse.

Robertson and Joyce made a pool of six variants of *Tetrahymena* group I intron that could "tag" their 3' ends by performing transesterification reaction with a separate RNA fragment (Robertson & Joyce 1990). This mixture was subjected to new selective pressure in which RNA substrate was replaced by DNA substrate. Repeated cycles of *in vitro* selection and *in vitro* amplification have been widely used to isolate rare nucleic acid sequences with specified biochemical properties from a large pool of random or degenerate sequences. A similar strategy was used to fish out a ribozyme, which can ligate a substrate oligo nucleotide to its own 5' end. The substrate sequence was then used as a tag to separate rare ligated molecules from inactive molecules. To generate random pool of RNA (RNA 0 Pool), the template DNA was constructed by joining pools of N72, N76 (Bartel and Szostack 1993), after digestion to get compatible sites for directional ligation in such a way that N72 flanks N76 at both ends. This has yielded 540 bp full length DNA that contains 220 random base pairs. The central segment is flanked by two conserved segments, at one end a 42bp segment containing T7 promoter and coding 5' constant region that binds to the substrate and participates in the ligation reaction and the other end a 20bp segment coding for 3' constant region. This DNA pool was *in vitro* transcribed to get RNA 0 pool. To minimize the problem of RNA aggregation the pool of RNA molecules was immobilized on agarose beads before the addition of Mg^{2+}. A biotinylated oligo nucleotide was annealed to 3' constant region of pool RNA and the mixture was then incubated with avidin agarose beads. Once attached to agarose, the pool molecules could not diffuse to form intermolecular interactions. During incubation the temperature was cycled between 25°C and 37°C to encourage RNA molecules to form alternate conformations. After ligation incubation, the pool RNA was eluted from agarose and passed through oligo nucleotide affinity column to selectively bind molecules containing tagged sequence of substarte. RNA bound was eluted and reverse transcribed to get DNA, which was further amplified by using specific primers. Again *in vitro* transcription, selection and amplification steps were repeated several times. Nearly 3% of pool 4 RNA became ligated to the substrate during first hour of incubation and large portion of it ligated at 16 hours. To generate a broad-spectrum variants pool 4 DNA was subjected to error prone PCR. Optimum Mg^{2+} concentration required for ligation is less for the pool after PCR mutagenesis compared to its previous pool.

6. CONCLUSIONS AND PERSPECTIVES

Since catalytic RNA was discovered, great strides have been made in the development in this field. Ribozymes have found applications in research, medicine and agriculture. Recently, there has been a spurt in using ribozyme in gene inactivation in human pathogenic conditions and clinical trials are underway. In this, attempts are being made to analyse the efficacy and validity of ribozymes as therapeutic agents. Being catalytic is an added advantage of this class of compounds. Further, *in vitro* selection techniques have opened up the possibility of making ribozymes with improved and novel properties. This may lead to the isolation of completely new RNA inhibitors. With *in vitro* evolution coupled with rational design it may be possible to synthesize oligonucleotide tagged proteins which could be useful as affinity tags or could function as amplifiable identifying markers. Ribozyme technology will also have great potential in post genomic era allowing rapid functional determination of RNA sequences.

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Hybrid Enzymes

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1. INTRODUCTION

One of the large avenues of modern biotechnology is the improvement of bioprocess by changing properties of biomolecules. In particular, enhancement of catalytic properties of enzymes by protein engineering is undergoing the most exciting development in its history. It promises unprecedented expansion in the scope, and applications of modified or improved enzymes with desired physical and catalytic properties. Enzyme design with special capabilities involves manipulation of DNA to construct hybrid biocatalysts. Hybrid or chimeric enzymes can be defined as a biocatalyst with altered DNA sequence originated from one or more different parent/s.

There are two complementary strategies currently available for generation of hybrid enzymes: rational redesign, and directed evolution. In both cases, the enzyme gene encoding, a suitable expression system, and a sensitive detection system are a prerequisites. Although both approaches have been successfully applied, each has limitations (Chen 2001).

Rational protein design by site-directed mutagenesis is still a very effective way to elaborate improved enzymes. However, a serious limitation of this strategy is the knowledge of enzyme structure as well as the relationship between sequence, structure and mechanism-function, which is very sensitive information and is not always available. On the other hand, rapid progress in solving protein structure by NMR spectroscopy and the increasing number of sequences stored in public data bases have significantly eased access to data and structure. Using molecular modelling, it has been possible to predict how to increase the enzyme selectivity, activity, and stability, even if there are no structural data available and the structure of the homologous enzyme is used as a model (Kazlaukas 2000; Guieysse et al. 2003). It is also important to mention that such model place an emphasis on enthalpic interaction but omit entropic contributions. Considering that the entropic factor in short-term range is generally associated to hydrophobic interactions between macromolecules and molecule residues, exclusion of entropy contributions might be one reason why rational design sometimes does not live up to expectations (Ottosson 2001). Depending on the mutagenesis purpose, amino acid substitutions are often selected by sequence comparison with homologous sequences. Minor sequence changes by a single mutation-point may cause significant structural disturbance. Thus, comparison of the three-dimensional structures of mutant and wild-type enzymes are necessary to ensure that a single mutation is really site directed.

Directed evolution is now used routinely to improve enzyme fitness for a given application. Molecular diversity is typically created by random mutagenesis and/or recombination of a target gene or a family of

related genes. Upon inserting the library of mutant genes in an appropriate microorganism, mutant enzyme are expressed and screened for the reaction activity of interest. The gene(s) encoding those improved enzymes are used as parents for the next round of evolution. This creates an evolutionary pressure, leading to the formation and identification of an even better enzyme. Since the process can be repeated as often as needed, a type of "Darwinian" principle holds, without the knowledge of enzyme structure and/or mechanism.

Rational redesign and directed evolution both have their distinct advantages and yet the technologies are complementary. It has been clearly demonstrated that directed evolution can effectively augment rational redesign even though a great deal of knowledge about structure and function is available. Using rational design it is possible to introduce key residues or structural elements that are no usually attainable via a random process. Random mutagenesis was then employed to generate subtle changes that would fine-tune protein packing and function. Conversely, random mutagenesis can also provide critical information for implementing a more rational protein engineering strategy. The information can be used to minimize the sequence space that must be searched for future random mutagenesis experiments or to suggest target for rational site directed mutagenesis (Chen 2001).

As pointed out by Nixon et al. (1998), hybrid enzymes can be generated in a number of ways. An existing enzyme can be altered by a single mutation point (or series of mutation points) based on structures existing in other enzymes. Similarly, secondary-structural elements or whole domains of enzymes, or monomeric units of multimeric enzymes, can be exchanged. Fusions between two enzymes that have separated and distinct activities are also considered hybrid enzymes.

Hybrid enzyme approaches seek to recruit established functions and properties from existing enzymes and incorporate them into the engineering enzyme. These techniques have been shown to be useful in the alteration of non-enzymatic as well as enzymatic properties and also as tools for understanding structure-function relationships. In addition, the creation of hybrid enzymes can expand the potential uses of natural enzymes. Enzymes, or fragments of enzymes, could potentially serve as building blocks for proteins capable of catalyzing reactions not observed in nature.

In this chapter, the state of the art and trends of hybrid enzymes technology is analyzed and discussed.

2. METHODS FOR GENERATION OF HYBRID ENZYMES

2.1 Rational protein design: Combining theory and experiment

The rational design of protein structure and function is rapidly emerging as a powerful approach to test general theories in protein chemistry. *De novo* creation of a protein or an active site requires that all the necessary interactions be provided. The design approach is, therefore, a way to test the limits of completeness of understanding experimentally. Furthermore, if the experiments are devised in a progressive fashion, such that the simplest possible designs are tried first, followed by iterative additions of more complex interactions until the desired results is achieved, it may be possible to identify a minimally sufficient set of components. At the center of the design approach is the "design cycle", in which theory and experiment alternate. The starting point is a development of a molecular model, based on rules of protein structure and function, combined with an algorithm for applying these. This is followed by experimental construction and analysis of the properties of the designed protein. If the experimental outcome is failure or partial success, the next interaction of the design cycle is started in which additional

complexity is introduced, rules and parameters are refined, or the algorithms for applying them are modified (Hellinga 1997)

2.1.1 Site-directed mutagenesis using the megaprimer PCR method

Oligonucleotide-directed site-specific mutagenesis is used routinely to introduce desired mutations into target DNA sequences. A variety of protocols have been established to achieve efficient mutagenesis, including several that use the polymerase chain reaction. Polymerase chain reaction (PCR) is a DNA amplification technique in which an initial small amount of DNA is replicate in consecutive cycles increasing its concentration exponentially. The first step of PCR is the DNA denaturalization into single strands. The second step is the annealing of a primer to the DNA single strands. Primers consist of two DNA oligonucleotides with lengths of 15-30 base pair complementary to the ends of the amplified region. The third step is primer extension by a polymerase. Complementary nucleotides to the single strand template are added by using the original sequence as a template, extending the complementary strands until normal DNA double strands are recovered.

Among the PCR-based protocols, the megaprimer method introduced by Kammann et al. (1989) appears to be particularly simple and cost-effective. This method involves two round of PCR that utilize two flanking primers and one internal mutagenic primer containing the desired base substitution (s). The first PCR is performed using the mutagenic internal primer and the first flanking primer. The product of this first PCR, the megaprimer, is purified and used, along with the second flanking primer, as a primer for a second PCR. The final PCR product contains the desired mutation in a particular DNA sequence. Most mutagenic strategy based on this two-step PCR scheme required an intermediate purification step of the first PCR reaction products to prevent leftover primers from the first PCR from interfering with the second PCR step. This purification step, usually accomplished by agarose gel electrophoresis and subsequent elution of the DNA of interest, is time-consuming and labor intensive. Ke & Madison (1997) described a useful approach, which provides rapid and highly efficient site-directed mutagenic in a single tube without any intermediate purification steps or additional manipulation or treatment of PCR products. This mutagenic strategy utilizes primers with significantly different melting temperatures (Tm) to initiate the two PCR steps. This experimental design allows the use of an elevated annealing temperature in the second reaction to ensure selective synthesis utilizing two of the primers present in the reaction mixture. The two flanking primers are different in length. The short, reverse flanking primer is usually 15-16 bases long and typically has a calculated tm of 42-46 °C. The long, forward flanking primer is designed to be 25-30 base long and has a calculate Tm between 72 and 85°C. The first PCR reaction is carried out using the short flanking primer, the internal mutagenic primer and a low annealing temperature. Without purification step, the second, high Tm flanking primer is added directly to the reaction tube. The second step PCR is performed using a high annealing temperature, usually 72°C. The high annealing temperature assures that the final PCR product will be generated through selective priming by the megaprimer product of the first PCR and the high Tm flanking primer. The short flanking primer that remains from the first PCR does not interfere with the second PCR synthesis because of its poor efficiency of annealing to the template at 72°C. The authors report an average mutagenesis efficiency of 82%.

2.2 Directed evolution

A key step in the directed evolution experiments cycle is the introduction of new genetic diversity to the library. There are two basic ways for diversity introduction: error-prone PCR (ep-PCR) and DNA recombination. Unlike ep-PCR where no exchange of genetic material occurs between parent sequences, DNA recombination methods rely on the mixing and concatenation of genetic material from a number of parent sequences. Recombination protocols include DNA shuffling (sexual PCR), staggered extension process, random priming recombination, DNA family shuffling, heteroduplex recombination, random chimeragenesis on transient templates, chimeric gene construction without reference to restriction sites, incremental truncation for the creation of hybrid enzyme, creation of multiple-crossover DNA libraries independent of sequence identity, sequence homology-independent protein recombination, random multi-recombinant PCR, structure-based combinatorial protein engineering, general method for sequence-independent site-directed chimeragenesis, etc.

2.2.1 Error-prone PCR

Error-prone PCR protocols were used in early-directed evolution experiments (Arnold, 1996). Random point mutagenesis by Ep-PCR replication process (Cadwell & Joyce 1992) intentionally introduces copying errors by imposing mutagenic reactions conditions. Thus, it involves a modified PCR protocol that uses variations in $MgCl_2$ or $MnCl_2$ concentrations to achieve an average mutation level of 2-5 base substitutions per gene, corresponding to an average exchange of one amino acid per mutated protein. Researchers should be aware that because of the inherent mutation bias of ep-PCR and the restrictions imposed by the genetic code, this method is not suitable for introducing all 20 amino acid residues at each position of the protein. In fact, an average of 5.7 amino acid substitutions are accessible for any given amino acid residues using this method (Miyazaki & Arnold 1999).

2.2.2 Homologous DNA recombination

2.2.2.1 DNA shuffling

DNA shuffling, pioneered by Stemmer (1994a and b), mimics the process of natural recombination. This technique involves recombinative exchange of small DNA fragments between two or more closely related genes, thereby leading to the creation of new genes. First, an initial set of parent sequences sharing a number of desired traits are selected for recombination. Next, the selected sequence undergo random fragmentation typically using DNAase I. Double-stranded fragments within a certain size rang (e.g. 100-200 bp) are retained. The retained fragments are then reassembled by thermocycling with a DNA polymerase (PCR without primers). As in regular PCR, this involves first the denaturalization of the double-stranded fragments into single stranded ones. Denaturing step is followed by annealing process in where single-stranded fragments anneal to other fragments overlapping by a sufficiently large number of complementary bases to form $3' \rightarrow 5'$ overhangs. The third step is polymerase extension (Figure 1). Note that the 3' overhangs are not changed because DNA polymerase only possesses $5' \rightarrow 3'$ activity. These three steps are repeated and the average fragment length increases after each cycle. After a number of cycles, DNA sequences of the original length are obtaining. Finally, regular PCR with primers is utilized to amplify the reassembled strands. The key advantage of DNA shuffling over ep-PCR is that it can recombine a large number of mutations within a few selection cycles quickly yielding functional blocks with combination of beneficial mutations (Moore & Maranas 2000).

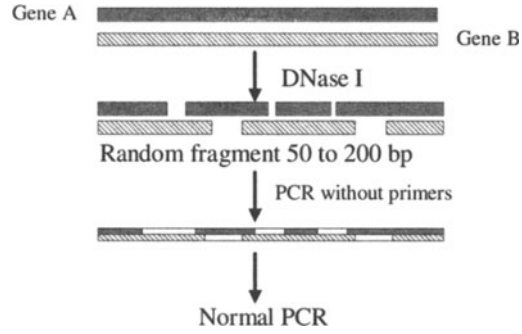

Figure 1. DNA Schuffling

2.2.2.2 Staggered extension process and random priming recombination

Staggered extension process (StEP) and random priming recombination (RPR) are two variations of recombinative DNA technology developed by Arnold's laboratory. StEP involves PCR amplification of a gene using a set of primers and short time intervals (e.g. 5 s) for annealing and for polymerization. Truncated oligomers are dissociated from the template and allowed to anneal randomly to different templates, thereby accomplishing recombination. Repetition of this process results in a staggered extension of the DNA template during PCR (Zhao et al. 1998). RPR method uses random hexamer oligonucleotide primers for PCR that yield a collection of random oligomers containing additional mutation points introduced by the polymerase reaction. After removal of the full-length template, oligomers can reassemble with other oligomers containing mutations and are finally amplified to yield complete genes, which are shuffled by a recombination-like process (Shao et al. 1998).

2.2.2.3 Enzyme evolution by DNA family shuffling

Family shuffling represents a potentially powerful approach to generating novel sequences that encode functionally interesting proteins. Even when the homologous parent proteins differ at a large number of amino acid residues (as much as 30 or 40%), a significant fraction of the resulting chimeric proteins retain some level of function. Kikuchi et al. (1999) reported a method in which they replaced the DNAase I fragmentation step by DNA digestion with restriction enzymes in an effort to reduce the background from parental sequences. Using restriction enzymes dramatically increased the frequency of chimera formation between two genes that share 84% identity at the DNA sequence level (from 1% to almost 100%). Digestion of each of parental genes with several sets of restriction enzymes followed by digest reassembly in two steps results in the formation of more diverse chimeric structures.

Joern et al. (2002) reported a useful analysis of shuffling gene library using two tools developed for this purpose. The first is a probe hybridization method in which a set of labeled probes that anneal to specific parental gene position is used to determine where sequences corresponding to the different parents appear in the chimeric genes. Due to crossovers are favored in regions of high sequence identity, if the parent pool contains parents with a low level of sequence identity to others, few recombination sites will be available among the low-identity parents. Thus, clones containing sequences information from the low-identity parent are relatively less diverse than the library as a whole. Fragments from a low-identity

parent tend strongly to reassemble into full-length wild-type genes, which further reduce diversity. Thus, one useful strategy for avoiding reassembly of wild-type genes of a low-identity parent is to use only parts of this parent rather than a complete gene in the shuffling reaction. The second tool is a sequence-based hybridization preference model that can be used for identifying preferred crossover sites and estimating relative frequencies of crossovers for particular regions.

2.2.2.4 Heteroduplex recombination

Volkov et al. (1999) described a simple method, termed heteroduplex recombination, which creates libraries of chimeric DNA sequences derived from two homologous parental sequences. A heteroduplex formed *in vitro* is used to transform bacterial cells where repair of regions of non-identity in the heteroduplex creates a library of new sequences composed of elements from each parent. Heteroduplex recombination generates multiple crossovers and should be particularly useful for recombining large genes or entire operons.

2.2.2.5 Two-step overlapping polymerase chain reaction

Construction of a fusion gene by combining two separate PCR products with overlapping sequence into one larger product is another useful strategy (Xu et al. 2001). Two separate DNA fragments are each amplified in a first PCR using primers 1, 2 and 3,4 respectively. The two PCR products then were mixed at an equal molar ratio and amplified by using primers 1 and 4 (Figure 2).

2.2.2.6 Random chimeragenesis on transient templates (RACHITT)

Coco et al. (2001) introduced a conceptually distinct and improved alternative to sexual PCR for gene family shuffling. Random chimeragenesis on transient templates (RACHITT) employs no thermocycling,

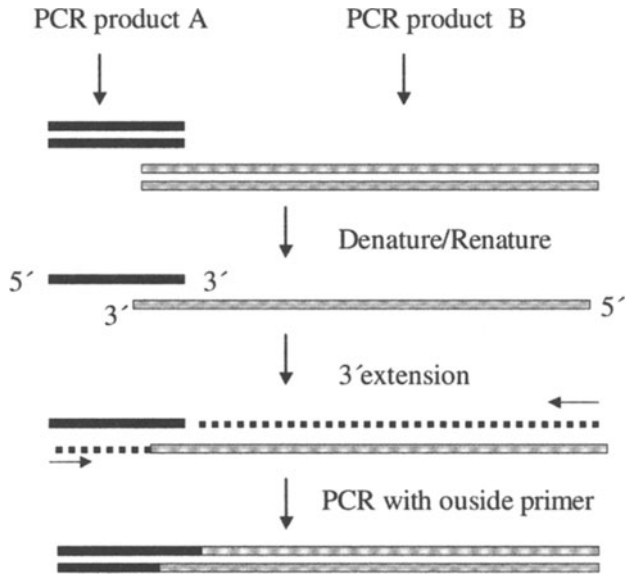

Figure 2. Two-step overlapping PCR

strand switching or staggered extension but rather the trimming, gap filling and ligation of parental gene fragments hybridized on a transient DNA template (Figure 3). This method generated chimeric libraries averaging 14 crossovers per gene. The authors also observed an unprecedented four crossovers per gene in region of 10 or fewer bases of sequence identity, no unshuffled parental clones or duplicated "sibling" chimeras and relatively few inactive clones.

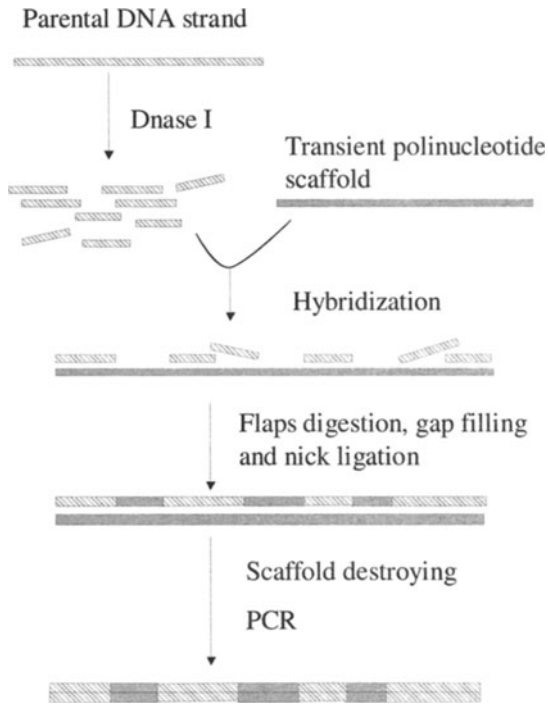

Figure 3. Random Chimeragenesis on Transient Templates (RACHITT)

2.2.2.7 Chimeric gene construction without reference to restriction sites

Sequence databases now abound with open reading frames related to gene products of known function. Functional analysis of numerous homologous undoubtedly will involve various chimeric constructs between the characterized gene product and the unknown open reading frame. Traditionally, the exchange has been performed by cutting out a segment from the donor DNA, using restriction sites common to the two genes, and ligating it into the recipient DNA. However, more often that not, such convenient restriction sites are not available, and chimeras are some time constructed with great ingenuity (Ba-thein et al. 2001). One way to overcome this challenge is to engineer a restriction site by site-directed mutagenesis, but because convenient sequences must be found for the alterations, the precise manipulation of DNA is not always possible. Shigaki & Hirschi (2002) presented an universal method to exchange practically any segment of two homologous sequences without regard to restriction sites. Toward that end, the authors take full advantage of the unique properties of Class IIS restriction enzymes. By

employing this method, only two to four nucleotide homologies are necessary to exchange segments between genes, with no requirement for common restriction sites. Class IIS restriction enzymes recognize non-palindromic sequences, and cleave at sites that are separate from their recognition sequences (Shigaki et al. 2001). Class IIS enzymes have two useful properties. First, when a Class IIS enzyme recognition site is engineered at the end of a primer, the site is cleaved off when digested. Second, overhangs created by Class IIS enzymes are template-derived and thus unique. This is in clear contrast to regular class II restriction enzymes such as *Eco*RI, which creates an enzyme-defined overhang that ligates to any *Eco*RI-digested end. The unique overhangs produced by Class IIS enzymes can be ligated only to their original partner.

This technique not only overcomes the difficulties inherent in segment exchange but also enables the simultaneous creation of numerous chimeras, thus facilitating medium-throughput screening for protein domains of interest.

2.2.3 Non homologous DNA recombination

The increasing numbers of protein structures available and the study of the enzyme structural families has shown that many enzymes with little or no DNA homology can have high protein structural homology. Constructing hybrid of such structural homologues is also an important empirical strategy for engineering of novel activity.

2.2.3.1 Incremental truncation for the creation of hybrid enzyme (ITCHY)

Incremental DNA truncation is an alternatively new approach for combinatorial protein engineering (Ostermeier et al. 1999). For domain swapping, it can be difficult to predict exactly which fusion points will produce an active hybrid enzyme. The use of incremental truncation in the creation of hybrid enzyme libraries solves this problem by a stochastic method. ITCHY was developed to produce functional interspecies hybrids in a manner independent of DNA homology. Whereas crossovers points between genes in traditional DNA shuffling are defined and confined by the regions of identity, shuffled ITCHY library crossover points are defined by the fusion-point. Incremental truncation of DNA allows the construction of libraries containing many or all-possible truncations of a gene, gene fragment or DNA library in a single experiment. This method is performed on a linear piece of DNA containing a gene fragment or DNA library that has both one end protected (RE3', that produces a 3'overhang) and the other end susceptible (RE5', that produces a 5' overhang) to Exo III digestion. This reaction is performed by DNA digestion, which is performed slow, directional and controlled. Aliquots are frequently removed and the digestion quenched. Thus, by taking multiple samples over a given time period the authors create a library of all possible single base-pair deletions of a given piece of DNA. The end of DNA can be blunted by treatment with S1 nuclease and klenow so that unimolecular ligation results in the desired incremental truncation library (ITL). For some applications, additional DNA manipulations are performed before recircularizing the vector. An example of ITCHY is summarized in Figure 4.

ITL can also be created through the incorporation of α-phosphothioate dNTPs by PCR (THIO-PCR) or by primer extension truncation (THIO-extension truncation). In THIO-PCR truncation the entire plasmid, containing the gene to be truncated, is amplified by PCR using a mixture of dNTPs and a small amount of αS-dNTPs, which are randomly incorporate into DNA. ExoIII cannot remove αS-dNMPs. Thus, a distribution of truncation lengths, defined by the sites of incorporation of the αS-dNTPs, is created

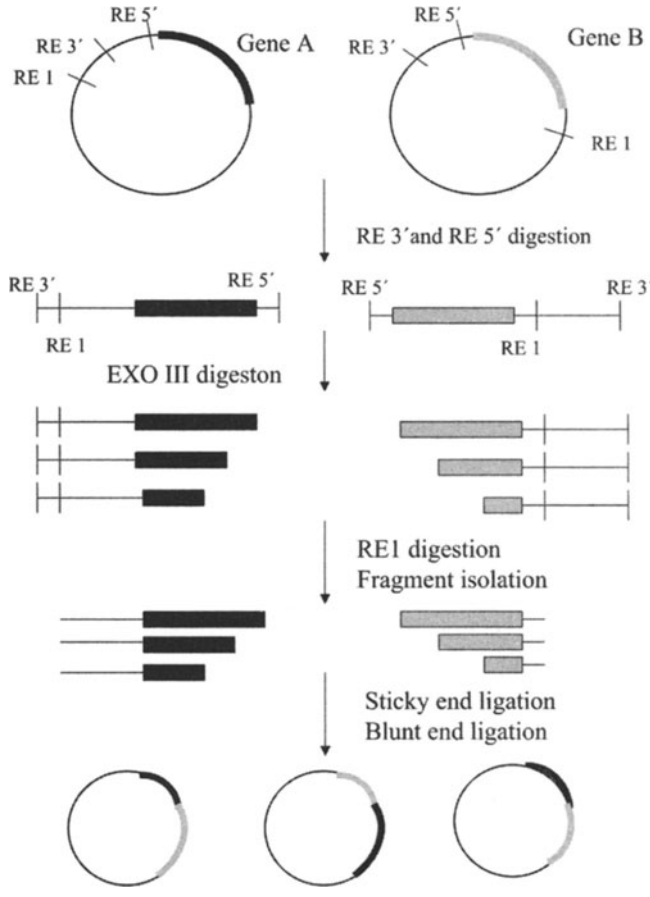

Figure 4. Incremental truncation for the creation of hybrid enzyme

upon ExoIII digestion. In THIO-extension truncation, αS-dNTPs can only be incorporate in the region exposed by the first ExoIII digestion. Thus, the range of incorporation of αS-dNTPs is limited by an initial truncation of average length. Subsequent steps are the same as in time-dependent truncation (Lutz et al. 2001a, Ostermeier 2003).

Protein splicing using *trans*-inteins technology is another interesting approach. Inteins are intervening sequences that are excised from precursor proteins by a self-catalytic mechanism. The regions flanking an intein are called exteins. Intein technologies involve protein splicing, autoproteolysis and biosynthesis of polypeptide building blocks (Perler & Adam 2000). The ability of trans-intein to fuse potentially any two polypeptides is well suited for the creation of hybrid enzyme libraries (Figure 5). In this application, incremental truncation resulting in a fusion protein of an ITL of A (gen, gene fragment or gene library) to one half of the trans-intein (N-intein) and an ITL of B (gen, gene fragment or gene library) to the other half of the Trans-intein (C-intein). The interaction of heterodimer (I_N:I_C) directs the splicing reaction resulting in the join of A to B with a native peptide bond and the release of I_N:I_C (Ostermeier et al. 1999).

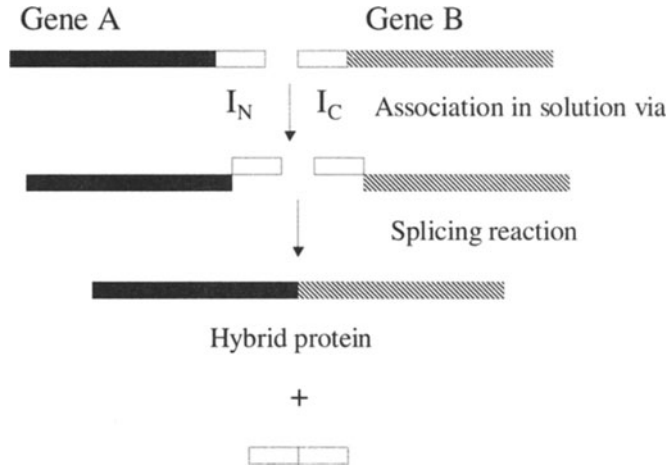

Figure 5. Protein splicing with trans-intein gene fragment

2.2.3.2 Creation of multiple-crossover DNA libraries independent of sequence identity

Diversification of parental sequence (s) is a critical component in the directed evolution of enzymes and other proteins. SCRATCHY (Lutz et al., 2001b) is an alternative method that enables combinatorial engineering of target proteins independent of sequence identity. SCRATCHY consists of a combination of two distinct methods for chimeric gene libraries construction: Incremental truncation for the creation of hybrid enzymes and DNA shuffling. ITCHY is used to create a comprehensive set of fusions between fragments of genes in a DNA homology-independent fashion. This artificially family is then subjected to a DNA-shuffling step to augment the number of crossovers. In parallel, Lutz et al. (2001) developed a modeling framework named eSCRATCHY to address *in silico* questions concerning the application of the SCRATCHY protocols. Crossovers prepositioned in the ITCHY step were shown to be preserved in the reassembled sequences, and the formation of multiple-crossover hybrids was correctly predicted. However, when attempting chimeragenesis between two distantly related parents, low sequence identity generally prevents the formation of homology-dependent crossovers during the shuffling step. This is because of the absence of homologous recombination and the fact that allocation of non-homologous crossovers derived of ITCHY clones, which contain one crossover per gene, essentially follows a Poisson distribution centered on this crossover number. In order to improve sequence diversity in SCRATCHY libraries, Kawarasaki et al. (2003) developed a simple and efficient method for library creation termed enhanced crossover SCRATCHY, that significantly increases the number of clones containing multiple crossovers. Complementary chimeric gene libraries generated by ITCHY of two distinct parental sequences are created, and are then divided into arbitrarily defined sections. The respective sections are amplified by skewed sets of primers (i.e. a combination of gene A specific forward primer and gene B specific reverse primer, etc.) allowing DNA fragments containing non-homologous crossover points to be amplified. The amplified chimeric sections are then subjected to a DNA shuffling process generating an enhanced crossover SCRATCHY library. Alternatively, the pooled chimeric fragments are allowed to assemble by overlapping extension to construct a library of chimeric gene with the defined number of crossovers.

2.2.3.3 Sequence homology-independent protein recombination

Sieber et al. (2001) reported a method for sequence homology-independent protein recombination (SHIPREC) that produce libraries of hybrid sequence in which the crossovers retain proper sequence alignment and therefore occur predominantly at structurally related sites. The goal is to create libraries of hybrid genes from distantly related sequences. To maximize the fraction of hybrid genes encoding functional proteins, the crossovers should occur at positions that are in similar structural environments. The authors therefore developed a method that uses sequence length rather than sequence similarity to achieve crossovers at position likely to be structurally related. A gene dimmer that consist of (from 5′ to 3′) the gene protein 1, a linker sequence containing useful restriction sites, and the gene of protein 2 is constructed. This dimmer is fragmented (e.g., by digestion with DNase I in the presence of Mn^{+2}) and treated to produce blunt ends (e.g., using S1 nuclease or T4 polymerase). Fragments of the length of a single gene (plus the length of the linker sequence) are separated from the pool (e.g., by agarose gel electrophoresis). Single-gene length fragments are circularized by intramolecular blunt-end ligation. Linearization of the circular DNA pieces by restriction digestion in the linker region creates a library of chimeric genes with members having an N-terminal part from protein 2 and a C-terminal part from protein 1, the crossovers being distributed over the entire length of the gene. The chimeric genes are cloned into an expression vector directly or after amplification by PCR using one terminal primer from each of a two parents. The fragment size selection ensures that the two amino acids that meet at the crossover are in similar positions in the parent structures.

2.2.3.4 Random multi-recombinant PCR

Development of a new methodology to create protein libraries, which enable the exploration of global protein space, is an exciting challenge. Tsuji et al. (2001) developed a method termed random multi-recombinant PCR (RM-PCR), which permits the shuffling of plural DNA fragments without homologous sequences in a single PCR. Thus, could be a useful strategy for protein engineering to create new proteins and as a tool in protein science to understand relationships between folding, function and evolution of globular protein. In order to evaluate this methodology, the authors applied it to create two different combinatorial DNA libraries. For the construction of a random shuffling library, RM-PCR was used to shuffle six DNA fragments each encoding 25 amino acids. This affords many different fragments sequences whose every position has an equal probability to encode any of the six blocks. For the construction of the alternative splicing libraries, RM-PCR was used to perform different alternative splicing at the DNA level, which also yields different block sequences. DNA sequencing of the RM-PCR products in both libraries revealed that most of the sequences were quite different, and had a long open reading frame without a frame shift or stop codon. Furthermore, no distinct bias among block was observed.

Evolution of eukaryotes is mediated by sexual recombination of parental genomes. Crossover occurs in random, but homologous, positions at a frequency that depends on DNA length. As exons occupy only 1% of the human genome and intron about 24%, by far most of the crossovers occur between exons, rather than inside. The natural process of creating new combinations of exon by intronic recombination is called exon shuffling. Another use of RM-PCR is to construct "*in vitro* exon shuffling" libraries, as described by Kolkman & Stemmer (2001). They proposed an alternative method for combinatorial reassembly of gene fragments without sequence homology. They used chimeric oligonucleotides encoding parts of two domains as primers to amplify targeted sequences, and the amplified sequences (termed

pre-made PCR fragments) were then mixed and used as both primers and templates in a PCR-like reaction without the primers that reassemble the full length genes. For example, exon shuffling of human pharmaceutical proteins can generate libraries in which all of the sequences are fully human, without the point mutations that raise concerns about immunogenicity.

2.2.3.5 Structure-based combinatorial protein engineering

Structure-based combinatorial protein engineering (SCOPE) is a semi-rational protein engineering approach that uses information from protein structure coupled with established DNA manipulation techniques to design and create multiple crossover libraries from non-homologous gene (Maille et al. 2002). On the basis of the concept of exon shuffling, SCOPE utilizes structural information from proteins to design coding elements of genes that correspond to structural elements present in both proteins. In a series of PCR reactions, hybrid oligonucleotides (primer composed of sequence from both parents that code for variable connections between structural elements) act as surrogate introns to direct the assembly of coding segments to create hybrid genes. Iteration of the process enables the synthesis of all desired combinations of structural elements.

In light of accumulating data from structural biology efforts and the desired to engineer the activities of proteins for commercial, therapeutic and fundamental scientific pursuits, the SCOPE approach will likely become of increasing utility. Functional proteins are estimated to be sufficiently abundant in sequence space, (occurring ~1 in 10^{11}) (Keefe & Szostak 2001). Searching large regions of sequence space using a block-wise or exon shuffling approach with distantly related proteins and an intelligent bias from structural information narrows the search considerably. Current model of evolution predict that the rate-limiting event in the acquisition of new protein folds and functions is the exchange of low energy secondary structure providing a theoretical underpinning (Bogarad & Deem 1999). One caveat is that proteins made by building block approaches may require further optimization by exploration of local sequence space by random mutagenesis and DNA shuffling (Tsuji et al. 1999). Thus, the controlled construction of chimera by SCOPE provides the means of exploring global protein space with a knowable probability of searching it.

2.2.3.6 General method for sequence-independent site-directed chimeragenesis

Sequence-independent site-directed chimeragenesis (SISDC) is a simple, cost effective and general method for site-directed recombination, which allows crossovers at multiple sites and is independent of DNA sequence identity shared by the parent genes (Hiraga & Arnold 2003). In this method, ligation of building blocks will easily regenerate a full-length and high-recombined library through the combinatorial assembly of DNA fragments from different parents. All fragments can be connected in the right order, without any insertions at crossover point. This strategy can be used for recombination of multiple parent sequences, or elements from multiple parents. It consist of four steps of simple genetic techniques:

1) Amplification of building blocks with marker tag by PCR.
2) Restriction digestion of tag regions.
3) A single ligation between elements.
4) Library amplification by PCR.

The nucleotide sequences of the parent genes (A and B) are aligned, and consensus sequences at each targeted site are determined. The marker tags contain consensus 5bp sequence, a type IIb endonuclease (*Bae*I) recognition sequence, two variable regions, a constant region and a *Sma*I recognition site. Inserted tag regions are removed by treatment with endonuclease and specific sticky ends are produces. Mixed fragments can identify their neighbors, and the fragments from different parents ligate with each other in the right order. After this step, digestion *Sma*I can eliminate any untreated tag remaining in the final, chimeric library.

A mayor advantage of the SISDC method is that the number of primers for inserting the tag sequence into the parental genes scales linearly with the number of parental genes. In addition, SISDC is expected to be less mutagenic than PCR mediated methods, because the random error introduced during interactive PCR steps are avoided.

2.2.3.7 Three-step overlapping polymerase chain reaction

Some times scuffling sites were selected for the construction of chimeric enzymes. This construction was carried out using a three-step polymerase PCR reaction. In the first step, both full-length gene and the gene fragment of interest plus a linker were amplified using primer 1,2 and 3,4 respectively. Primers 1 and 4 have proper restriction sites for cloning into a vector, whereas primers 2 and 3 have 20 bp overlapping regions, which are used as templates for self-priming in the second step of the overlapping PCR. In the last step, primers 1 and 4 are used to amplify the full-length chimeric gene (Figure 6) (Kittur et al. 2003).

3. METHOD TO PROTECT A TARGETED AMINO ACID RESIDUE DURING RANDOM MUTAGENESIS

PCR mutagenesis is widely used to generate libraries of mutant sequences that are used to search for improved and/or novel functions of proteins. The mutation rate is usually set relatively low, to discover

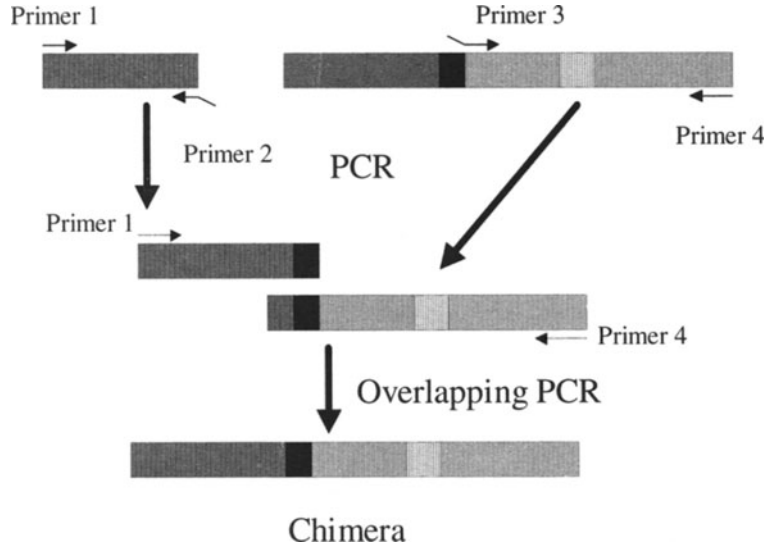

Figure 6. Three-step overlaping PCR

single amino acid substitutions that confer the desired phenotype. The ability to discover those mutations can be substantially decreased by experimental factors such as mutation bias in the PCR. PCR-based mutagenesis generates certain types of mutations more frequently than others. Beneficial, but rare mutations are, therefore, less likely to be discovered especially when a limited number of clones can be screened. When more clones are screened, variants with rare mutations are difficult to identify because they are overshadowed by other, more frequent mutations. To generate a random mutant library that is free from mutation at a particular amino acid residue, Udemo et al. (2003) replace the codon of interest with a detachable, short DNA sequence containing a BsaXI recognition site. After PCR mutagenesis, this sequence is removed and intramolecular ligation of the sequences flanking the insert generates the gene. The three-base cohesive ends for ligation correspond to the codon for the targeted residue and any sequences with mutations at this site will fail to ligate. As a result, only the variants that are free from mutation at this site are in the proper reading frame.

4. HYBRID PROTEIN SELECTION METHOD

4.1 DNA display for *in vitro* selection of diverse peptide libraries

Formation of DNA-peptide conjugate is an essential step in DNA display. Yonezawa et al. (2003) described the use of a DNA display system for *in vitro* selection of peptide ligands from a large library of peptides displayed on their encoding DNAs. The method permits completely *in vitro* construction of a DNA-tagged peptide library by using a wheat germ in vitro transcription/translation system compartmentalized in water-in-oil emulsion. Starting with a library of 10^9-10^{10} random decapeptides, 21 different peptide ligands were isolated for monoclonal antibody anti-FLAG M2. A streptavidin-biotin complex was used as a connector for the peptide DNA linkage, and the method was named STABLE to indicate "streptavidin-biotin linkage in emulsions" (Doi 1999). Thus, a DNA library encoding streptavidin-fused peptides is labeled with biotin and compartmentalized in water-in-oil emulsions containing and *in vitro* transcription/translation system. In each compartment, stravidin-fused peptides are synthesized and attached to the template DNA via biotin labels. DNA-peptide conjugates are recovered from the emulsion and subjected to affinity selection on immobilized bait. After washing and elution, the DNA portion of the bound molecules is amplified to the next round of selection or identified by sequencing.

4.2 A universal, vector-based system for nucleic acid reading-frame selection

One of the most relevant aspects to obtain desired reading frames from nonsense sequences is the separation of libraries of combinatorial homology-independent series using protein-engineering methods. Lutz et al. (2002) constructed a vector, pSALect, which utilizes a dual selection system (head + tail) to identified nucleic acid sequences with desired reading frame. The fundamental idea of the approach is to apply two functionally distinct, selection criteria on the gene of interest or more precisely on its reading frame. Only a fusion protein with an intact head section will be transported to the periplasm whereas N-terminally truncated translation products, originated for example by internal ribosomal binding sites, are trapped in the cytoplasm. The dual selection scheme fuses the gene of interest between a head and tail selection marker. Each is based on a different selection criterion, yet the two are functionally linked. The head section encodes for a peptide signal sequence, directing periplasmic transport of the C-terminal fusion construct. The tail market is a reporter that only functions in the periplasmic environment (e.g. ß-lactamase). Although the dual selection system can not avoid the translation of C-terminal protein fragments from internal ribosomal binding sites, the lack of the signal sequence in those construct prevents allocation

of the protein product into the periplasm and therefore prevents the manifestation of selectable phenotype by the truncated product.

5. IMPROVED BIOCATALYSTS BY DIRECTED EVOLUTION AND RATIONAL PROTEIN DESIGN

5.1 Changing enzymatic function

Changing enzymatic function through genetic engineering presents a challenge to molecular biologist. Xu et al. (2001) described an example in which changing the oligomerization state of an enzyme changes its function. Type IIs restriction endonucleases such as *Alw*I usually fold into two separate domains: a DNA-binding domain and a catalytic/dimerization domain. The authors have swapped the putative dimerization domain of *Alw*I with a non-functional dimerization domain from a nicking enzyme N.*Bst*NBI. The resulting chimeric enzyme, N.*Alw*I, no longer forms a dimer. Interestingly, the monomeric N.*Alw*I still recognizes the same sequence as *Alw*I but only cleaves the DNA strand containing the sequence 5′-GGATC-3′(top strand). In contrast, the wild type *Alw*I exists as a dimer in solution and cleaves two DNA strands; the top strand is cleaved by an enzyme binding to that sequence, and its complementary bottom strand is cleaved by a second enzyme dimerized with the first enzyme. N.*Alw*I is unable to form a dimer and therefore nicks DNA as a monomer. In addition, the strand-specific nicking endonuclease is as active as the wild type *Alw*I. Approximately 50% of 1 pmol of DNA could be cleaved by 0.004 pmol of N.*Alw*I in 1h. N.*Alw*I was expressed in *E. coli* and purified by using Heparin Hyper D, Source Q and Heparin TSQ. On SDS-PAGE gels, it migrated as a 65 kDa protein.

5.2 Improvement of enzyme optimum temperature

Laboratory evolution can generate large increases in thermostability with very few amino acid substitutions. Kohno et al. (2001) succeeded in creating a lipase that could maintain its activity at a high temperature by random mutagenesis. *Rhizopus niveus* lipase gene was mutated using ep-PCR technique. One desirable mutant was isolated and three amino acids were substituted in this mutant (P18H, A36T and E218V). The specific activity of the mutant lipase was 80% that of the wild type. However, the optimum temperature of the mutant lipase (50°C) was higher by 15°C than that of the wild type. To confirm which substitution contributed to enhancing the optimum temperature, the authors constructed two chimeric lipases from the wild type and the randomly mutated gene. Chimeric lipase 1 (CL1, P18H and A36T) and chimeric lipase 2 (CL2, E218V) were produced and purified. CL1 had a similar optimum temperature to that of the wild type; whist CL2 had a higher optimum temperature like the randomly mutated lipase.

5.3 Enhancement of catalytic activity and substrate-binding capacity

Some bacterial and fungal xylanases are modular proteins consisting of a distinct cellulose-binding domain (CBD) connected to a catalytic domain (CD). These binding molecules, which are found at either terminus of the enzyme as well as internally, range in size from the small fungal CBDs (36 residues) to the larger bacterial CBDs (up to 200 residues). The exact biological function of these domains *in vivo* is unknown. However, they have been shown to enhance the catalytic activity either by increasing the enzyme concentration around the substrate or by non-covalent disruption of the substrate polysaccharide structure. Mangala et al. (2003) reported a fusion of a tandem repeat of the family VI CBD from *Clostridium stercorarium* xylanase (XylA) at the carboxyl-terminus of *Bacillus halodurans* xylanase (XylA). *B. halodurans* C-125 grows at 55°C, produces a highly alkalophilic family 10 xylanase

and lacks a CBD. The constructed chimera (XylA-CBD) was expressed in *E. coli* and was purified to homogeneity. Native *B. halodurans* XylA has a molecular weight (M_w) of approximately 43 kDa, whereas the chimera displayed a M_w of about 76kDa, indicating the presence of the CBDs. The chimeric enzyme displayed pH activity and stability profiles similar to those of the parental enzyme. The optimal temperature of the chimera was 60°C and the enzyme was stable up to 50°C. Binding studies with insoluble polysaccharides indicated that the chimera had acquired an increased affinity for oat spelt xylan and acid-swollen cellulose. The bound chimeric enzyme was desorbed from insoluble substrates with sugars and insoluble polysaccharides, indicating that the CBDs also posses affinity for soluble sugars. Overall, the chimera displayed a higher level of hydrolytic activity toward insoluble oat spelt xylan than its parental enzyme and a similar level of activity toward soluble xylan.

5.4 Bi-functional proteins

Khang et al. (2003) constructed an artificial flavohemoprotein by fusing *Vitreoscilla* hemoglobin (VHb) with D-amino acid oxidase (DAO) of *Rhodotorula gracilis* to determine whether bacterial hemoglobin can be used as an oxygen donor to immobilized flavoenzyme. Both DAO and VHb-DAO genes were cloned in pET30 vectors and overexpressed in *E. coli*. Both proteins were purified and immobilized in support by crosslinking. The chimeric enzyme significantly enhanced DAO activity and stability in the bioconversion process of cephalosporin C. In a 200 ml bioreactor, the catalytic efficiency of immobilized VHb-DAO against cephalosporin C was 12.5-fold higher than that of immobilized DAO, and the operational stability of the immobilized VHb-DAO was approximately threefold better than that of the immobilized DAO. In the scale-up bioprocess with a 5 L bioreactor, immobilized VHb-DAO (2500 U/L) resulted in 99% bioconversion of 120 mM cephalosporin C within 60 min at an oxygen flow rate of 0.2 (v/v) x min. Ninety percent of the initial activity of immobilized VHb-DAO could be maintained at up to 50 cycles of the enzymatic reaction without exogenous addition of H_2O_2 and flavin adenine dinucleotide (FAD). The purity of the final product, glutaryl-7-aminocephalosporanic acid, was confirmed to be 99.77% by HPLC analysis.

5.5 Hybrid enzyme for determining structure-function relationships

Understanding structure-function relationships is also an important task in order to improve bi-functional proteins stabilities. An excellent example is bi-functional fusion protein GluXyn-1 produced to test whether *Bacillus macerans* 1,3-1,4-β-glucanase (wtGLU) jellyroll can adopt a native-like structure *in vivo* spite of the insertion of an autonomous folding unit derived from the *Bacillus subtilis* 1,4-β-xylanase (wtXYN). The jellyroll belongs to those domain folds (superfolds) that most frequently are found in globular proteins. It is characterized by two strictly antiparallel β-sheets forming a distorted β barrel. In the fusion protein GluXyn-1, the two proteins are joined by insertion of the entire XYN domain into a surface loop of cpMAC-57, a circularly permuted variant of wtGLU. GluXyn-1 was produced in *E. coli* and shown to fold spontaneously and have both enzymatic activities at wild-type level. The crystal structure of GluXyn-1 was determined at 2.1 Å resolution and refined to R=17.7% and R (free)=22.4%. It shows nearly ideal, native-like folding of both protein domains and a small, but significant hinge bending between the domains. The active sites are independent and accessible explaining the observed enzymatic activity. Because in GluXyn-1 the complete XYN domain is inserted into the compact folding unit of GLU, the wild-type-like activity and tertiary structure of the latter proves that the folding process of GLU does not depend on intramolecular interactions that are short-ranged in the sequence.

5.6 Improvement substrate specificity and enantioselectivity

Enantiomerically pure compounds are of rapidly increasing importance to the chemical industry being used as pharmaceuticals, agrochemicals, flavors and fragrances. This trend has several reasons: a) biological activity is often restricted to one of the two enantiomers, b) the wrong enantiomer may exert undesirable effects, and c) registration of new drugs requires the production of the desired enantiomer in pure form. Thus, the biocatalytic production of the enantiopure compounds is of steadily increasing importance to the chemical and biotechnological industry. Liebeton et al. (2000) demonstrated that the combination of random and directed mutagenesis can be used to evolve an enzyme displaying increased enantioselectivity over a significant range starting from E=1.1 for the wild-type enzyme to E=25.8 for the best mutant. The enzyme used in this model is the lipase from *Pseudomona aeruginosa* (PAL), which catalyzes the hydrolysis of a chiral ester 2-methyldecanoic acid *p*-nitrophenyl. The authors have shown that a successful directed evolution strategy based solely on point mutations can be developed. Its involves the initial localization of residues important for enantioselectivity by random mutagenesis using ep-PCR followed by the introduction of the optimal residue by saturation mutagenesis using megaprimer PCR method. In the variant with the highest enantioselectivity, five substitutions have occurred, four of which involve the introduction of a glycine. This remarkable accumulation of glycine residues may increase the conformational flexibility of PAL. Flexibility has been identified as one of the possible factors that determining the substrate selectivity.

6. CONCLUSIONS AND PERSPECTIVES

Recent work in the area of protein engineering, summarized as above shows that rational design and directed evolution, both are applicable to creating desired mutant enzymes, although the positions of mutations often differ considerably. For rational design, those amino acid residues that appear logical to the researcher examining the three-dimensional structure are usually modified (i.e. they are close to the active site, the binding pocket etc.). In sharp contrast, sequencing of variants obtained by directed evolution followed by their structural analysis very often reveals that mutations are far away from the place where the reaction takes place.

Choosing the most effective approach for a particular enzyme-engineering task depends on the level that the mechanistic base of the desired property is understood, and if an effective selection scheme is available. With the rapidly increasing number of 3-D protein structures available in databases and the development of powerful protein modeling tools, rational redesign will become more efficient and broadly applicable. Meanwhile, emergence of novel high-throughput screening processes and strategies for increasing sequence diversity will extend the application of directed evolution to many more industrial enzymes and increase the feasibility for creating new functions. Although either rational redesign or directed evolution can be very effective, a combination of both strategies will probably represent the most successful route to improving the properties and function of an enzyme.

A potential projection of this powerful emerging field is the creation of totally artificial new biocatalysts not only with selected properties required for special type of reaction or process, but also with new and previously unknown catalytic properties.

Acknowledgements

The authors gratefully acknowledgement the financial support from CABBIO (2000, '0012), CONICET (Argentina), and Prof. David Kaplan (Tufts University) for his help.

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Diagnostic Enzymes

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1. INTRODUCTION

Applications of enzymes in industrial chemical conversions, various forms of catalysis, and more recently in pharmaceutical manufacture are widely known. In medicine, enzymes are used in analytical tests, disease diagnosis, treatment of enzyme deficiencies and wound therapies. In clinical pathology, enzymes are invaluable tools in diagnosing tissue damage and cellular disorders from increased activity or concentration of specific tissue enzymes in body fluids. In this chapter the definition of "diagnostic enzymology" will be assumed to be the detection of biological malfunctions by changes in enzyme levels or activity in body fluids. Enzymes subject for detection are called "diagnostic enzymes". In the last section, a brief overview of the techniques in ELISA and biosensors is made and the enzyme tags in such processes discussed.

2. ENZYMES IN DISEASE DIAGNOSIS

Enzymes are found in all body tissues and involved in most forms of metabolic activity. The regulation of enzymes allows metabolism to adapt rapidly to changing conditions. The activity of an enzyme in blood serum represents a balance between its rate of liberation into the extracellular space and its rate of uptake from the extracellular space. Factors such as age, sex and body weight affect this balance in individual subjects. Enzymes frequently appear in the serum following cellular injury or sometimes in smaller amounts from degraded cells or storage areas. Damage to or proliferation of cells from which enzyme originates leads to increased activity of enzyme in the plasma. Such increased or decreased activity levels tissue specific enzymes in the serum serve are references in the diagnosis of disease or cellular disorder.

Isoenzymes are proteins that possess similar catalytic activity but genetically determined differences in structure result in differing properties such as electrophoretic mobility, allowing identification. Isoenzyme distribution pattern also helps in diagnosis. For example, alkaline phosphatase (ALP) isoenzymes found in human serum originate from several sources with the greatest activity found in bone, liver, intestine, and the placenta. Tissue sources of elevated alkaline phosphatase in serum can be determined by identifying the isoenzyme.

Diagnostic enzymology measures increase in plasma enzymes resulting from damage or increased turnover. Enzyme tests measure activity or concentration using fixed reaction conditions. An important point that has to be considered in selecting an appropriate enzyme test is that it should determine the extent of tissue damage and the type of tissues that have been damaged. However, some diagnostic

protocols that lack specificity provide very sensitive means of detecting tissue damage and can be invaluable in following the course of a disease.

3. DIAGNOSTICALLY RELEVANT ENZYMES

Along with the research advances in clinical biochemistry and diagnostic enzymology, large advances have been made in increased capability and refinement of the identification of disease-associated enzymes and factors. While fertile markets for clinical enzymes and test kits indeed prove that enzymes make powerful diagnostic tools, international efforts to standardize analyses are continually fostered. In pathology tests, enzymes in different kinds of tissues and biological fluids are the analytes while other diagnostically relevant applications include those in which enzymes are used as antigens, standard references, or labels. Some typical examples of enzymes used in disease diagnostics are described below.

3.1 Adenosine deaminase [EC 3.5.4.4 *Adenosine aminohydrolase*, ADA]

ADA catalyzes the following reaction:

$$\text{Adenosine} + H_2O \leftrightarrow \text{Inosine} + NH_3$$

ADA is a purine salvage pathway enzyme ubiquitous in mammalian tissue, which catalyzes the irreversible deamination of adenosine into inosine and ammonia. The lack of erythrocyte ADA has been shown to be associated with inherited severe combined immunodeficiency (Hirschhorn et al 1976, Trotta et al 1976). They are characterized by the deficiencies in both B and T cell-mediated immunity and with the onset of severe infections early in life. Increased ADA levels in cerebrospinal fluid (CSF) have been observed in tuberculous meningitis (Rohani et al 1995). It is hypothesized that since RBC-ADA is similar to that produced by normal lymphocytes, its deficiency in inherited immune disease is due to impaired lymphocyte function (Giblett et al 1972). Spectrophotometry is the primary assay method (RCPA 2001). The normal ranges of ADA from pleural transudate, pleural exudates, peritoneal exudates or transudate, and cerebrospinal fluid are 0.0-6.7, 1.6-9.2, 0.0-7.6, and <10.0 U/L, respectively (ARUP Laboratories 2003). ADA is inhibited by Ag^+, Hg^{2+}, Cu^{2+}, *N*-tosyl-*L*-phenylalanine-chloromethyl ketone (TPCK), di-isopropyl fluorophosphates (DFP), and sulfhydryl reagents. Commercial sources of the enzyme are obtained from bovine spleen, calf intestinal mucosa, and calf spleen (White and White 1997).

3.2 Alkaline Phosphatase [EC 3.1.3.1 Orthophosphoric-monoester phosphohydrolase (alkaline optimum), ALP]

ALP, associated with non-specific phosphomonesterases with activity optima at alkaline pH, catalyzes the following reaction:

$$\text{Orthophosphoric monoester} + H_2O \leftrightarrow \text{Alcohol} + H_3PO_4$$

Alkaline phosphatase (ALP) has at least nine recognized isoenzymes that includes fast (pre-liver), liver, bone, placental, regan, nagao, renal, intestinal, and PA. Each has varying properties of heat stability, chemical inhibition, and electrophoretic mobility (ARUP Laboratories 2003). In an electrophoretic gel, ADA isoenzymes from different tissue origins migrates in the order Liver>Bone>Placenta>Intestine (Goldberg 1986). High or low levels of ALP are associated with many disorders (Walmsley et al 1993). Increased levels are associated with liver disease (cholestasis), bone disease (increased osteoblastic activity in Paget's disease; some bony metastases, especially in prostate and breast) and at times in

malignancy without liver or bone metastases (Regan isoenzyme). Alkaline phosphatase isoenzymes are rarely necessary to identify the source of an elevated ALP. Marked but transient elevation of ALP may be seen in children, probably attributable to viral infection. Abnormal dentition and fragile bones with decreased ALP characterize the autosomal recessive disease hypophosphatasia. Assay methods include spectrophotometry (RCPA 2001) and kinetic heat inactivation (ARUP Laboratories 2003). The latter is the most convenient method to distinguish bone from liver isozyme. By spectrophotometry, the normal range of ALP in plasma or serum is 30-120 U/L, 50-300 U/L in neonate, 70-350 U/L in growing child and 25-100 U/L in non-pregnant adult (RCPA 2001). ALP activators include $CaCl_2$, Na^+, Mg^{2+}, Zn^{2+} and alcohol (Tris) compounds. Inhibitors include Ca^{2+} chelating agents, low Zn^{2+} (0.1 mM) orthophosphate and potassium-glutamate buffer. Commercial sources include calf intestine or mucosa, bovine kidney or intestinal mucosa, bovine liver, chicken intestine, human (bone, intestine, kidney or placenta), *Escherichia coli* and some bacterium (White and White 1997).

3.3. α-Amylase [EC 3.2.1.1 1, 4-α-D-Glucan glucanohydrolase]

The activity of alpha amylase is present in all living organisms. α-Amylase catalyzes the endohydrolysis of internal α-1,4-glucan links in polysaccharides containing three or more α-1,4-linked *D*-glucose units yielding a mixture of maltose and glucose. This enzyme is very significant in the diagnosis of many physiological disorders (ARUP Laboratories 2003). Patients with acute pancreatitis have a marked rise in serum amylase within 2-12 hours of onset, highest in 12-72 hours after onset, and returns to normal in 4-8 days. Acute non-pancreatic conditions, which may also elevate amylase levels, include acute parotitis, peritonitis, small intestine obstruction, perforated peptic ulcer, rupture of a tubal pregnancy, contraction of the sphincter of Oddi following morphine administration, and mesenteric thrombosis. There are several assay methods for α-amylase, including spectrophometric and enzymatic methods. Saccharogenic method measures sugar produced. Iodometric or amyloclastic method measures starch remaining. Chromogenic method measures dye released from breakdown of polysaccharides. The kinetic method measures change of NAD to NADH at 340 nm, requiring Ca^{2+}. Enzymatic test is also used for the quantitative determination of serum pancreatic (p-) and salivary (s-) amylase activity (Tietz and Shuey 1986, Huang and Tietz 1982). Salivary amylase inhibitor will selectively inhibit s-amylase to a greater extent than p-amylase activity. Serum samples are incubated with and without the inhibitor and the amylase activity measured. Salivary amylase is calculated as the difference between the total and pancreatic amylase. While the reference interval is method dependent (ARUP Laboratories 2003, RCPA 2001), in acute pancreatitis, values exceeding 8% are common, while the amylase/creatinine clearance ratio is usually less than 2% in macroamylasemia. The highest levels of amylase in serum (ca. 2500 Somogyi U/dL) and urine (>2900 Somogyi U/dL) occurs 24 hours and 48 hours, respectively, after the onset of symptoms for acute pancreatitis. The normal levels in serum and urine are 300 and 800 Somogyi U/dL (Goldberg 1986). α-Amylase is activated and stabilized by Ca^{2+} while inhibited by urea and other amides. Commercial sources of the enzyme are obtained from some fungus and bacteria, porcine pancreas, human tissue and barley malt (White and White 1997).

3.4 Creatine kinase [EC 2.7.3.2 *ATP: creatine* N-*phosphotransferase*, CK; also creatine prophokinase (CPK)]

CK catalyzes the following reaction:

$$\text{ATP} + \text{Creatine} \leftrightarrow \text{ADP} + \text{Phosphocreatine}$$

CK, which primarily functions in ATP regeneration is widely distributed and may represent 10-20% (w/v) of muscle cytoplasmic protein (Watts 1973). This dimeric enzyme exists as three isozymes (CK-isoenzymes), namely: CK-MM fraction predominant in skeletal muscle, CK-MB fraction found in high concentration in myocardium and CK-BB fraction present primarily in brain tissue. These forms are measurable in serum. CK-isoenzymes determinations are useful for differentiating CK released by skeletal muscle from CK released by cardiac muscle and their activity significant in a variety of clinical circumstances (ARUP Laboratories 2003). Skeletal muscle contains less than 5% CK-MB. CK-MB is a sensitive indicator of acute myocardial infarction. Damaged cardiac tissue releases CK-MB 2-6 hours following infarction. CK-MB values peak at 12-24 hours after infarction and return to normal within 24-48 hours. CK isoenzymes can also be used to evaluate disorders other than those of cardiac or skeletal muscle. CK-BB activity can be evaluated in a variety of clinical circumstances, including normal childbirth, brain-damaged and/or low- birth-weight neonates, multi-organ system failure, cardiopulmonary resuscitation, hypothermia, gastrointestinal infarction, a variety of tumors, and following head trauma. CK-MM activity is elevated in a variety of skeletal muscle disorders, including muscular dystrophy, myositis, and polymyositis. Elevated CK-MM activity also results in acute rhabdomyolysis of a variety of causes. Assay methods for CK include spectrophotometry, immunoassay, and electrophoresis (RCPA 2001). Diagnostic test of myocardial infarction has been superseded by cardiac troponin T or I (Adams et al 1994). Normal reference values for C-MM, CK-MB and CK-BB by electrophoresis are 96-100%, 4% and 0%, respectively (ARUP Laboratories 2003). Normal range for total creatine kinase in serum is <200 U/L (MedIndia 2002). In the case of Duchenne muscular dystrophy, serum creatine kinase almost steadily falls from 75 times the normal value at an age below five years to less than 25 times the normal value at age less than 20 years (Goldberg 1986). CK is activated by Mg^{2+}, Ca^{2+} and Mn^{2+}. Among the inhibitors are sulfhydryl-binding reagents, chelating agents, some adenosine phosphate compounds, acetate, Cl^-, SO_4^{2-} and orthophosphate. Commercial sources of the enzyme are obtained from brain and heart from human and rabbit, human and rabbit muscle and other human tissues for MB isozymes (White and White 1997).

3.5 Lactate dehydrogenase [EC 1.1.1.27 *(S)-Lactate: NAD^+ oxidoreductase*, LDH]

LDH catalyzes the following reaction:

$$(S)\text{-Lactate} + NAD^+ \leftrightarrow \text{Pyruvate} + NADH$$

Mammalian lactate dehydrogenase (LDH) has five tetrameric isozymes that differ in catalytic, physical and immunological properties. Polypeptide subunits are referred to as "H" and "M", which combine to form two pure types of isozymes, H_4 and M_4, and three hybrids, H_3M, H_2M_2, and HM_3 (Cahn et al 1962). At pH 7 type H_4 is the most negatively charged and appears nearest the anode in zone electrophoresis. Subunit "H" predominates in heart muscle LDH that functions in the aerobic oxidation of pyruvate. The "M" subunit predominates in skeletal muscle and liver and is concerned more with anaerobic metabolism and pyruvate reduction (Fritz 1965). LDH is of limited value in the diagnosis of myocardial infarction when testing is required >48 hours after the onset of chest pain. It is occasionally useful in the assessment of patients with liver disease or malignancy (lymphoma, seminoma, hepatic metastases); anaemia when haemolysis or ineffective erythropoiesis suspected. Although it may be elevated in patients with skeletal muscle damage, it is

not a useful diagnostic test in this situation (RCPA 2001). Spectrophotometric and enzymatic assays are used for total LDH while electrophoresis is used for the isozymes. The normal range in plasma or serum is method and age dependent. On the other hand, in a densitometric analysis of LDH isozymes, the amounts goes in the order of $LDH_2>LDH_1>LDH_3>LDH_4>LDH_5$ in normal serum, $LDH_1>LDH_4>LDH_3>LDH_5>LDH_2$ in acute myocardial infarction, and $LDH_1>LDH_3>LDH_4>LDH_2>LDH_1$ in acute hepatitis (Huijgen et al 1997). LDH is activated by dimethyl sulfoxide, ethanol and methanol. It is inhibited by Ag^+, Cu^{2+}, Hg^{2+}, hydroxylamine, I^-, *p*-chloromercuribenzoate, oxalate and oxamic acid. Commercial enzymes are obtained from *Bacillus* species, bovine heart and muscle, chicken heart, human erythrocytes, human heart, human muscle, porcine heart and muscle, rabbit muscle, *Staphylococcus epidermis*, yeast and trout muscle (White and White 1997).

Table 1 contains some of the other diagnostic enzymes that are commonly used in disease detection. Commercial sources of some such diagnostic enzymes that are used as controls and in kits are available from suppliers listed in Table 2.

Table 1. Enzymes of significance in disease diagnosis

Enzyme	Significance	Source/Tissue	Normal range
Acetylcholinesterase [EC 3.1.1.7 *Acetylcholine acetylhydrolase*, AChE]	Neural tube defect including spina bifida, anencephaly or microcephaly	Synaptic junction, endoplasmic reticulum, erythrocytes	7-7.8 U/L in amnioti fluid
Alanine Transaminase [EC 2.1.6.2 L-*alanine:* 2-*oxoglutarate aminotransferase*, ALT; also serum glutamic pyruvic transaminase (SGPT)]	Hepatitis, liver necrosis, obstrutive jaundice, chronic hepatitis, neoplastic liver disease, cirrhosis, myocardial infarction, infectious mononucleosis and Reye's syndrome, extrahepatic obstruction	Liver, heart, kidney, muscle and erythrocytes	3-60 U/L in serum; <35 U/L in adult; and <50 U/L in neonate
Aldolase [EC 4.1.2.13 *D-Fructose 1,6-biphosphate D-glyceraldehyde-3-phosphate lyase*, also Fructose-biphosphate aldolase, ALD]	Myocardial infarction, acute hepatitis, megaloblastic anemia, myeloid leukemias, Duchenne muscular dystrophy	Type A (the major form, in muscle), Type B (liver and kidney) and Type C (plus some A in brain)	1-8 U/L in serum
Amine Oxidase [EC 1.4.3.6 *Amine:* oxygen oxidoreductase (deaminating) (copper-containing)]	Cancer	Plasma	-

contd...

Enzyme	Significance	Source/Tissue	Normal range
Aspartate Transaminase [EC 2.6.1.1 L-*Aspartate: 2-oxoglutarate aminotransferase,* AST; also Glutamic oxaloacetic transaminase (GOT)]	Obstructive jaundice, acute hepatitis, cirrhosis, myocardial infarct, intrahepatic neoplasm, haemolytic jaundice, trauma, Reye's syndrome, skeletal muscle trauma, alcoholism, some anaesthetics, exercise, paracetamol overdose	Heart, liver	AST/ALT ratio is typically >1 in alcoholic liver disease and <1 in non-alcoholic liver disease; <40 U/L in plasma or serum
Cholinesterase [EC 3.1.1.8 *Acylcholine acylhydrolase,* ChE; also Butyryl cholinesterase (ButChE) or Pseudocholinesterase (PChE)]	Acute hepatitis, chronic hepatitis, cirrhosis, carcinoma, organophosphate poisoning, chronic renal disease, late stages of pregnancy, and estrogen therapy	Erythrocytes and nervous tissues; pseudocholinesterase is found in the plasma	25-52 U/g Hgb for cholin-esterase (RBC/Hgb Ratio), 2900-7100 U/L for total pseudocholin-esterase and 2900-7100 U/L pseudocholin-esterase after dibucaine inhibition
Elastase [EC 2.4.21.36 *Pancreatic elastase;* also Pancreatopeptidase E, Pancreatic elastase I]	Emphysema, atherosclerosis, acute hemorrhagic pancreatitis	Pancreas, duodenum, blood components	-
Galactose-1-Phosphate Uridyltransferase [EC 2.7.7.10 UTP: *α-D-Hexose-1-phosphate uridylyltransferase,* G-1-PUT]	Transferase-deficient galactosemia; results in liver damage, cataracts, mental retardation, and sometimes death if transferase-deficient galactosemia goes undiagnosed beyond the first few weeks of life	Blood, liver, brain, and lens	14.7-25.4 U/g hemoglobin using enzymatic assay; 0.26-0.52 U/g haemoglobin by spectrophotometry
Glucose-6-Phosphate Dehydrogenase [EC 1.1.1.49 D-*Glucose 6-phosphate: $NADP^+$ 1-oxidoreductase, G-6-PD]*	Non-spherocytic hemolytic anemia	Erythrocytes	4.6-13.5 U/g hemoglobin
γ-Glutamyl Transferase [EC 2.3.2.2 *(5-L-Glutamyl)-peptide: amino-acid 5-glutamyltansferase,* GGT]	Cholestatic liver disease, chronic intake of excess alcohol and with certain drugs (*esp* phenytoin), pancreatitis, prostatitis	Liver, kidneys and pancreas	<50 U/L in plasma or serum in male and <30 U/L in female
Glutathione S-Transferase [EC 2.5.1.18 *RX: Glutathione R-tansferases,* GST]	Renal damage (rejection of a transplanted kidney) or hepatic parenchymal damage	All	<10 g/L in serum

contd...

Enzyme	Significance	Source/Tissue	Normal range
Lipase [EC 3.1.1.3 *Triacylglycerol acylhydrolase*]	Gallstone colic, perforated hollow viscus, strangulated or infarcted bowel, pancreatic cyst or pseudocyst, and peritonitis	Pancreatic acinar cells, salivary glands, pulmonary and gastrointestinal mucosa	0.2-1.5 U/L in serum
Lysozyme [EC 3.2.1.17 *-Peptidoglycan N-acetylmuramoyl-hydrolase*]	Acute myelomonocytic leukemia, chronic myelomonocytic leukemia, chronic myelocytic leukemia, tuberculosis, sarcoidosis, megaloblastic anemias, acute bacterial infections, ulcerative colitis, Crohn's disease, renal insufficiency, renal transplant rejection, urinary tract infections, pyelonephritis, glomerulonephritis, and nephrosis	Serum, urine, tears, seminal fluid, and milk	4-13 µg/mL in serum or body fluid and <4 µg/mL in urine
Myeloperoxidase [EC 1.11.1.7 *Donor: hydrogen-peroxide oxidoreductase,* MPO; also peroxidase]	Acute leukemias, airway inflammation caused either by disease, such as asthma, or environmental irritants; markers for several systemic vasculitis diseases	Released from cytoplasmic granules of neutrophils to the extracellular space and phagosome	MPO stain is generally performed on smears of peripheral blood or bone marrow
5'-Nucleotidase [EC 3.1.3.5 *5'-Ribonucleotide phosphohydrolase*]	Liver disease; helpful in the differential diagnosis between disorders affecting bone and liver	All	0-15 U/L in serum
Thiopurine Methyltransferase [EC 2.1.1.67 *S-adenosyl-L-methionine:thiopurine S-methyltransferase,* TPMT]	Fatal myelo-suppression from usual doses of thiopurines	Erythrocytes	0.5-0.9 U/g hemoglobin
Terminal Deoxynucleotidyl Transferase [EC 2.7.7.31 *Nucleoside-triphosphate: deoxynucleotidylexo-transferase,* TdT; also DNA nucleotidylexotransferase]	Marker for the classification of leukemias and for the recognition of lymphoblastic lymphoma	Lymphocytes, bone marrow	Qualitative and quantitative test via immunofluorescent stain (microscopic) should be negative or 0.0%, respectively
Thyroid Peroxidase [EC 1.11.1.8 *Iodide: hydrogen-peroxide*	Autoimmune thyroiditis, idiopathic hypothyroidism, Graves' disease	Thyroid microsome	0.0-2.0 U/mL in serum by chemiluminescent immunoassay

Table 2. Suppliers of enzyme immunoassay kits and enzymes used as standard diagnostic references

Enzyme immunoassay kits		Enzymes
5 Prime – 3 Prime Inc.	Idexx Laboratories	AGS Heidlb
AAI-Abtech	Immuno-Dynamics Inc.	Amano Novo
AMAC Inc.	ImmunoSystems Inc.	Nordisk
American	ImmunoVision	Amersham
Amersham Corp.	Integrated	AMRESCO
Biomedical	Kronus	Asahi, ProZyme
Bio-Rad Laboratories	Lampire Biological	Biozyme
Biosolutions Inc.	Life Technologies	Boehringer
BioSource International Inc.	MGM Instruments Inc.	Calbiochem
Biotecx Laboratories Inc.	Nordic Immunological Labs	Calzyme
BioWhittaker Inc.	OrganonTeknika/Biotechnology	CHIMERx
Boehringer Mannheim	Oxford Biomedical Research Inc.	Epicenter
Biochemicals	Paracelsian	Fermentas
Calypte		
Cayman Chemical Co.	Perceptive Diagnostics	Fluka
Ciba-Corning	Pharmacia	Genzyme
Diagnostics		
Cistron Biotechnology	Pierce Chemical Co	ICN
Endogen Inc.	R & D Systems	Life Technol
Exocell Inc.	Repligen Corp.	MINOTECH
Genzyme Diagnostics	Scimedex Corp.	NBL Gene
Granbio Inc.	Serex Inc.	NE Biolabs
Hepar Inc.	Syva	Novozymes
Hercules	TAGO Inc.	Oncor
Human Biologicals Inc.	Takara Biochemical Inc.	Pharmacia
Hycor	The Binding Site Inc.	Promega
ICN Pharmaceuticals	Tosoh Medics Inc.	Scripps
ICN Plaza	USA Scientific Plastics	Stratagene
	Vector Laboratories	TaKaRa
		Toyobo
		Wako
		Worthington

4. ASSAY METHODS EMPLOYED IN DIAGNOSTIC ENZYMOLOGY

There are several methods available to analyze the catalytic activity of enzymes. The sensitivity of the analyses is also enhanced by an amplification effect provided by the ability of a single enzyme molecule to catalyze the reaction of numerous substrate molecules. Most enzyme-catalyzed reactions can be monitored by simple, widely available spectroscopic and electrochemical (*e.g.* amperometric, potentiometric) methods. Spectrometric assay methods include fluorescence spectrometry, chemiluminescence, and bioluminescence. Immunoassays, in which enzymes are used for determining the extent of antibody-antigen reaction is also widely employed today.

4.1 Spectrometric assay methods

Many oxidoreductase enzymes involve NAD/NADH or NADP/NADPH as cofactor systems. Such reactions are readily followed by absorption measurements at 340 nm, where the absorption of NADH and NADPH is strong, but that of NAD and NADP negligible. Another approach using UV–visible spectrometry involves colorigenic substrates. These molecules change color when hydrolyzed in the enzymatic reaction under study. A good example is provided by *p*-nitrophenol esters, which are colorless, but are hydrolyzed by appropriate enzymes to yellow *p*-nitrophenol, which can be determined at about 405 nm.

Fluorescence spectrometry is widely used in enzymology, usually because of its extra sensitivity compared with absorption methods. Fluorophores are characterized by two specific wavelengths, absorption and emission. The latter is the longer of the two, excited molecules having lost some energy between the processes of photon absorption and emission, and this allows the fluorescence to be determined (at 90° to the incident light beam in most instruments) against a dark background. This provides limits of detection unattainable by absorption spectrometry. A second important characteristic of fluorescence spectrometry is its versatility; it is just as easy to study concentrated solutions, suspensions, solid surfaces, flowing systems, etc., as it is to measure dilute solutions.

Recent years have seen an explosion of interest in chemiluminescence (CL) and bioluminescence (BL) methods in enzymatic analyses. As in fluorescence, these methods measure photon emission from excited molecules, but here the latter are generated chemically, as reaction products. The reactions concerned are oxidations, often in mildly alkaline solutions in the presence of a catalyst.

4.2. Electrochemical assay method: Biosensors

Electrochemical methods of following enzyme-catalyzed reactions are rapidly growing in popularity, reflecting the growth of these methods across analytical science in general. Biosensors, portable or disposable devices which combine the specificity of an enzyme reaction with the simplicity and compactness of an electrical transducer, have gained much popularity. A biosensor is defined as compact analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer. The usual aim of biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes. Selective electrodes have been adapted to form such sensors: for example, oxygen electrodes can monitor any enzyme-catalyzed reaction in which oxygen is consumed or produced. Other potentiometric and amperometric methods have achieved impressive successes, exemplified by the variety of glucose sensors now available. A major advance in the *in vivo* application of glucose biosensors using glucose oxidase was the needle-type enzyme electrode for subcutaneous implantation. Biosensors today include a variety of devices exploiting enzymes, nucleic acids, cell receptors, antibodies and intact cells, in combination with electrochemical, optical, piezoelectric and thermometric transducers. Pearson et al (2000) provides schematic representations of a biosensor and immunoassay combined with biosensor. Developments of such methods have made measurement of glucose in solution (blood samples) like a pH meter type dip test.

4.3. Enzymes and immunoassays methods

Antibody-antigen binding, used to detect contamination or infection, is highly specific but the direct detection of this reaction is cumbersome and difficult. Therefore, labels are used for measuring the

complexation product. Enzymes, fluorescent molecules, and radioactive labels are used for the labeling of the complexation. There are two categories of immunoassays utilizing enzymes, namely nonisotopic homogenous and heterogenous immunoassays. Nonisotopic homogenous assays include enzyme-multiplied immunoassay technique (EMIT), apoenzyme reconstitution immunoassay system (ARIS), and substrate-labeled fluorescence immunoassay (SLFIA). A widely known nonisotopic heterogeneous method is enzyme-linked immunosorbent assay (ELISA). The enzymes used are not part of the specific antigen-antibody reaction, but assist quick assessment of the immune reaction.

ELISA is based on the fact that antigen or antibody can be attached to a solid phase support yet retains immunological activity, and either antigen or antibody can be linked to an enzyme while the complex retains immunological and enzymatic activity. In this method, a known amount of enzyme-labeled antigen is added to a sample with unknown antigen concentration. When this mixture reacts with antibody, enzyme-labeled and non-labeled antigens compete for the binding sites of the antibody. The more antigens there are in the sample, the lower is the fraction of enzyme-labeled antigen in the antigen-antibody complex. After removal of unbound antigen the amount of bound enzyme-labeled antigen is determined via the enzyme-catalyzed reaction. Colorimetric, fluorescent, and luminescent are three types of detection system most commonly used in ELISA. ELISA has two available techniques, the sandwich technique and the competitive technique.

The sandwich technique begins with an antibody bound to polystyrene well plus the antigen to be measured. An enzyme conjugate is then added to the well with bound antigen-antibody or immune complex. A substrate is added to the enzyme conjugate which is bounded to the immune complex. If there are changes due to the presence of the enzyme conjugate which is bound to the immune complex, a positive test or color change will occur.

The competitive technique begins with an antibody bound to a polystyrene well. A test sample containing an antigen mixture to which an antigen-enzyme conjugate is added. At this point, competitive inhibition occurs between the antigen-enzyme conjugate and an unlabeled antigen. Depending on which antigen type is in excess, two different outcomes can follow when binding to a specific antibody occurs. The reagents are separated by washing. Next a substrate is added to the immune complex. If the antigen-enzyme conjugate is the antigen in excess a color change will occur indicating that the substrate was chemically changed as a result of the enzyme conjugate being bound to the immune complex. If it is the unlabeled antigen that is in excess, there will be little to no change in color because the test sample would be containing antibody-types specific antigen.

The most common enzymes used as labels for ELISA are horseradish peroxidase, calf intestine alkaline phosphatase, and *E. coli* β-D-galactosidase. These enzymes are typically used because they each meet most, if not all, of the criteria necessary to produce a sensitive, inexpensive, and easily performed assay. These criteria include stability at typical assay temperatures (4, 25, and 37 °C), greater than six months shelf life when stored at 4 °C, commercially available, capable of being conjugated to an antigen or antibody, inexpensive, easily measurable activity, high substrate turnover number, and unaffected by biological components of the assay.

4.4. Enzyme immunoassay of myeloperoxidase (MPO)

This is an example of ELISA in the diagnosis of leukemia or air way inflammation in humans caused by disease or environmental irritants. In the indirect sandwich immunoassay of MPO, human sample (antigen

from blood) is added to the mouse anti-MPO (antibody) coated well and incubated at room temperature for one hour and washed. Rabbit anti-MPO is added and the mixture is incubated for one hour with shaking. Unbound rabbit anti-MPO is then washed. Horseradish peroxidase (HRP) labeled goat anti-rabbit antibody is added followed by 30-minute incubation at room temperature with shaking. Unbound HRP-labeled goat anti-rabbit antibody is washed and 3, 3', 5, 5'- tetramethylbenzidine (TMB) substrate for HRO is added followed by 30-minute incubation. The reaction is halted by denaturing the enzyme with 1N HCl. Colored product is spectrometrically measured at 450 nm.

5. CONCLUSIONS

With high throughput methods for testing and evaluation now available there is huge thrust for developing methods for quick, easy and early diagnosis. New developments with the complete knowledge of the human genome will change human health methods dramatically. A quantum improvement in enzyme and enzyme based diagnosis is on the anvil.

6. PERSPECTIVE

It is clear that no human being has the perfect genome. The search for the "golden genome" is on. Similarly the enzymes and enzyme levels in human sera will also be fully quantified and methods standardized in the near future. With high throughput machines, deviations from normal levels would be detected quickly. As these enzymes control all body functions there early quantification could lead to faster control of the ailment, while a more permanent cure strategy is made effective. There is no doubt that human genomic, proteomic and subsequent enzyme activity control using designer drugs will lead to dramatic changes in human health care in the near future.

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Therapeutic Enzymes

35

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1. INTRODUCTION

The concept of catalysis, the process by which the rate at which chemical reactions proceeds is speeded up by the presence of another chemical compound that classically does not undergo chemical change, was developed in the early part of the nineteenth century. In biological system, enzymes act as catalysts; they are protein catalysts synthesized by living systems and are important in synthetic as well as degradative processes.

Enzymes or biocatalysts are produced in the human body from amino acids that the body obtains by digesting food proteins. They accelerate and control all biochemical processes in the body. In a single second, several millions of enzyme mediated chemical reactions occur in a human body. Each enzyme is programmed to carry out one special task. The immense number of enzymes acts as a perfectly matched orchestra to ensure that enormously complex life mechanisms and processes occur in a right direction. Sufficient amount and optimal function of enzymes present in the human body is essential for life and health.

In the 21st century having the knowledge on human genome, enzymes particularly of microbial origin, are envisaged to play a crucial role in the diagnosis, curing, biochemical investigation and monitoring of many dreaded diseases.

1.1. Historical developments

The manufacture or processing of enzymes for use as drugs is an important facet of today's pharmaceutical industry (Cassileth 1998). Attempts to exploit the advantages of enzymes as drugs are now being done at every pharmaceutical research center. By the end of 19th century, people started using crude proteolytic enzymes for gastrointestinal disorders. In fact the therapeutic use of enzymes were largely ignored until Emmerich and his associates observed in 1902 that an extracellular secretion of *Bacillus pyocyaneus* was capable of killing anthrax bacilli. Emmerich deduced that the secretion in question was a nuclease, which acted by enzymatically degrading nucleic acids. This milestone study gradually opened the way for the use of parenteral enzymes in the treatment of a diverse spectrum of diseases. John Beard, an English scientist, reportedly first used pancreatic enzymes to treat cancer in 1902 (Gonzalez & Isaacs 1999). In 1906, John Beard proposed that pancreatic proteolytic enzymes, in addition to their well-known digestive function, represented the body's main defense against cancer. During the first two decades of the last century, a number of physicians used injectable pancreatic enzymes to treat advanced

human cancer, often times with great success. German researchers later used enzyme therapy to treat patients with multiple sclerosis, cancer, and viral infections. Dr. Edward Howell introduced enzyme therapy to the United States in the 1920s. He believed that by eating raw meat, people created an enzyme surplus, which resulted in better health and increased resistance to diseases.

There are several studies from the 1960s showing that orally ingested pancreatic enzymes have an anti-cancer effect, and might work through immune modulation. Enzyme supplements are available in pills, capsules, and powders that often consist of combinations of several different enzymes.

2. APPLICATIONS

2.1 General Applications

In contrast to the industrial use of enzymes, therapeutically useful enzymes are required in relatively tiny amounts but at very high degree of purity and specificity. The costs of such enzymes may be quite high but still very much comparable to those of competing therapeutic agents or treatments. Therapeutic enzymes have a broad variety of applications as oncolytics, anticoagulants or thrombolytics, and replacements for metabolic deficiencies. Additionally, there is a growing group of miscellaneous enzymes of diverse function. Enzymes are being used to treat many diseases such as cancer, cardiac problems, cystic fibrosis, dermal ulcers, inflammation, digestive disorders, etc. Proteolytic enzymes have been widely used as anti-inflammatory agents. Reduction of inflammation and edema is ascribed to the dissolution of soft fibrin and to the clearance of proteinaceous debris found in inflammatory exudates. Information on the utilization of microbial enzymes for therapeutic purpose is scarce and the available reports are largely on some anti cancer enzymes and on some enzymes, which are active against cystic fibrosis. A selection of those enzymes, which have realised this potential to become important therapeutic agents, is shown in Table 1.

2.2 Specific Applications of few selected enzymes

2.2.1. Collagenase

Collagenase is a unique enzyme; it hydrolyses native collagen but not the other proteins. It has been used in the debridement of dermal ulcers and burns.

2.2.2. Protease

Protease has a very different and powerful function when taken on an empty stomach. It is a tremendous all natural blood enhancer, able to break down protein invaders in the blood supply so that the natural immune system can destroy them. Parasites, fungal forms, and bacteria are protein. Viruses are nucleic acids covered by a protein film. Since protease can break down undigested protein, cellular debris, and toxins in the blood, it frees up the immune system for the more important work of destroying the unnatural invaders such as bacteria. Cancer cells are more sensitive to enzymes than normal cells and enzymes dissolve the fibrous coating on cancer cells, allowing the immune system to work. Also enzymes can diminish the ability for cancer cells to attach to healthy organs or tissue.

Lysostaphin is effective on coagulase-positive *Staphylococcus aureus* and is presently under considerable study. Lysostaphin is a protease that lyses susceptible cells in a highly efficient manner probably by peptidase-like cleavage of the glycoprotein of the bacterial wall. At present, lysostaphin has been administered in humans only topically for reduction of staphylococcal carrier rate in the nose and throat

Table 1. Therapeutic enzymes in current use

Enzyme	EC Number	Application	Reaction
L- Asparaginase	3.5.1.1	Anti-tumour/ Leuk-aemia	L-Aspargine $H_20 \rightarrow$ L-Aspartate + NH_3
L-Glutaminase	3.5.1.2	Anti-tumour/ Leuk-aemia	L-glutamine $H_20 \rightarrow$ L-Glutamate + NH_3
Superoxide dismutase	1.15.1.1	Antioxidant, anti-inflammatory	$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2$
Amino peptidases e.g.leucine aminopeptidase	3.4.11.	Anti-inflammatory	Hydrolysis of peptides
Penicillin acylase	3.5.1.11	Antibiotic	Penicillin+H_20 = Fatty acid anion + 6-aminopenicillanate
Lipase	3.1.1.3	Digests lipids	Lipid hydrolysis
Laccase	1.10.3.2	Detoxifier	Oxidoreductase. Acting on diphenols and related substrates as donars.
Glucosidase (α and β)	3.2.1.20 3.2.1.21	Anti-tumour	Carbohydrate metabolism
β-lactamase	3.5.2.6	Penicillin allergy	Penicillin $\rightarrow$ Penicicollate
Collagenase	3.4.24.3	Skin ulcers	Collagen hydrolysis
Protease e.g. Serine protease Cysteine protease	3.4.21 3.4.22	Anti-tumour, Digestive aid	Breakdown of complex proteins in to smaller peptides and amino acids
Hyaluronidase	3.2.1.35	Heart attack	Hyaluronate hydrolysis
Lysozyme	3.2.1.17	Antibacterial	Bacterial cell wall hydrolysis
Ribonuclease	3.1.26.4	Antiviral	RNA hydrolysis
Streptokinase	3.4.22.10	Blood clots	Plasminogen $\rightarrow$ plasmin
Trypsin	3.4.21.4	Inflammation	Protein hydrolysis
Uricase	1.7.3.3	Gout	Urate $+O_2 \rightarrow$ allantoin
Urokinase	3.4.21.31	Blood clots	Plasminogen $\rightarrow$ plasmin

where it has been found to be effective and nontoxic. The *in vivo* effectiveness of this enzyme against methicillin-resistant strains of *S. aureus* has been demonstrated. Lysostaphin might prove useful in the treatment of methicillin-resistant staphylococcal infections.

Trypsin and chymotrypsin are proteolytic enzymes, which have been very often used successfully in the treatment of postoperative hand trauma, athletic injuries, and sciatica. Papain is another protease, which results in marked reduction of obstetrical inflammation and swelling. Papain is also effective to treat the edema following dental surgery.

Serrapeptase is a proteolytic enzyme and is also known as serratiopeptidase. For over 30 years serrapeptase

has been gaining wide acceptance in Europe and Asia as a potent analgesic and anti-inflammatory drug (Yamasaki et al. 1967, Mazzone et al. 1990). It's been used to promote wound healing and surgical recovery. Recent Japanese patents even suggest that oral serrapeptase may help to treat or prevent such viral diseases as AIDS and hepatitis B and C. But perhaps its most spectacular application is in reversing cardiovascular disease. Serrapeptase is effective in unblocking carotid arteries. The mechanism behind the action of this enzyme is the ability of the enzyme to cleave a protein target into two or more pieces. The same mechanism makes it possible for peptidases to inactivate HIV, the AIDS-associated virus, by pruning the viral proteins necessary for infectivity (Tang et al. 1991). Serrapeptase is commercially obtained from *Serratia marcescens* cultures.

2.2.3. Deoxyribonuclease

Deoxyribonuclease is an enzyme that degrades nucleic acid. Recently it has been investigated as a mucolytic agent for use in patients with chronic bronchitis.

2.2.4. Lysozyme

Lysozyme is a group of enzymes, which hydrolyzes chitins and mucopeptides of bacterial cell walls. It has been used as an antibacterial agent usually in combination with standard antibiotics.

2.2.5. Hyaluronidase

Hyaluronidase exerts its action by destroying the intracellular ground substance hyaluronic acid, thus allowing diffusion of vital molecules through this normally impermeable connective tissue barrier.

2.2.6 Superoxide dismutase

Most organisms are exposed to oxygen for their lives and oxygen can be converted to form extremely reactive radicals that bind to DNA, proteins and lipids, causing permanent loss of structure to such molecules. Superoxide radicals are the most dangerous. To protect from such danger, cells have superoxide dismutase (SOD) and catalase enzymes. A number of tumour cells have been found to be deficient in SOD and re-expression of SOD gene cancels immortality. Absence of SOD activity seems to support cancer. Phagocytes destroy cells by pumping superoxide radicals into cells and tissues, and other systems such as Ab-Ag complexes. This can trigger phagocytes to dump superoxide and it seems to be a general alert signal to attract white blood cells to the scene causing swelling, inflammation etc. SOD mops up the superoxide. SOD is also an effective defense weapon. *Mycobacterium* and *Nocadia* have SOD, which enables them to resist the injection of superoxide by phagocytes.

2.2.7. Oncolytic enzymes

The oncolytic enzymes fall into two major classes, those that degrade small molecules for which neoplastic tissues have a requirement, and those that degrade macromolecules such as membrane polysaccharides, structural and functional protein, or nucleic acids. At present, tumor-cell specificity observed only in the former category. An example is the typical oncolytic enzymes L- glutaminase and L-asparaginase. Certain tumor cells are deficient in their ability to synthesize the nonessential amino acids L-glutamine and L-asparagine, and are forced to extract it from body fluids; by contrast, most normal cells can produce their own aminoacids. L-glutaminase and asparaginase given parenterally act in this way in many susceptible tumors. However, only acute lymphocytic leukemia ordinarily responds to chemotherapy with these enzymes. Nevertheless, the response of this one tumor type is promising.

L-methioninase, which effectively dismantles L-methionine to yield methanethiol, ammonia, and a ketobutyric acid, is effective against several murine tumors, but no clinical trials have been undertaken. The same is true for L-phenylalanine ammonialyase, which deaminates both L-phenylalanine and L-tyrosine yielding trans-cinnamic and trans-coumaric acids, respectively. In the case of both of these enzymes, mammalian cells are incapable of reconstructing the substrate from the products, so the reaction is effectively irreversible in vivo. Other amino acid degrading enzymes with oncolytic activity in experimental tumors include: L-arginase; L-tyrosinase; L-serine dehydratase; L-threonine deaminase; and, indolyl-3-alkane hydroxylase, which decompose L-tryptophan.

Diphtheria toxin, a different type of oncolytic enzyme still in the experimental stage, catalyzes transfer of the adenosine diphosphate ribose (ADP-ribose) moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor 2. This enzyme process halts protein synthesis. Most important from a chemotherapeutic standpoint is the observation that protein synthesis in tumor cells is one hundred to ten thousand times more sensitive to this toxin than the analogous process in normal cells.

Among the oncolytic enzymes that degrade macromolecules neuraminidase, ribonuclease, and a diverse group of proteases are the most prominent examples. Neuraminidase removes sialic acid residues from the surface of neoplastic cells, thereby altering their immunogenicity, and rendering them sensitive to immune response. To date this effect has been studied mainly in experimental trials. In addition, several ribonucleases have shown modest activity against experimental murine neoplasms, but their use is beset by the problem of forcing these molecules into the cytoplasm where the substrate ribonucleic acid (RNA) is present. Pepsin, given intralesionally, was one of the first enzymes used for the chemotherapy of cancer, but its clinical use was surrounded by controversy and has ceased. On the other hand, a mixture of vitamins and proteolytic enzymes, marked under the name Wobe Mugos, is widely prescribed for the control of cancer in Europe and appears to be of some use in the palliation of the disease. The carboxypeptidases are catalysts that cleave the carboxyl-terminal residue of many peptides; certain of these enzymes also are capable of hydrolyzing the L-glutamyl moiety of folic acid. In so doing, they achieve a state of folic acid deficiency deleterious to the tumor cell. Use of this approach has, so far, been restricted to test animals, but human trials are beginning with a preparation designated carboxypeptidase G1. Because carboxypeptidase G1 can decompose the drug methotrexate -a folic acid analogue and antagonist, the enzyme is also envisaged as an antidote to overdose of methotrexate.

2.2.8. Enzymes to treat cystic fibrosis

Cystic fibrosis is the most common fatal hereditary disease among Caucasians. This dreaded disease affects ~30,000 people in US. Affected persons are susceptible to bacterial infections in lungs and the infecting bacteria cause accumulation of thick mucus. Bacterial DNA and polysaccharides induce the secretion of mucus. Enzymes are currently available for the treatment of cystic fibrosis. Genentech produces recombinant human DNase I under the trade name Pulmozyme®. Cloned and over- expressed DNase I is delivered to patients as aerosol, which digests the DNA in mucus, and hence reduces viscosity of mucus.

Mucus also contains the polysaccharide alginate, which is produced by seaweeds, and soil and marine bacteria. *Pseudomonas aeruginosa* is one among them and is the main infectious agent in cystic fibrosis affected lungs. Alginate lyase in combination with DNase is used to degrade alginate as well as DNA. Alginate lyase gene from the soil bacteria *Flavobacterium* was isolated and the alginate degradation domain was amplified. This was then cloned in to an expression vector.

2.2.9. β-lactamases and antibiotic resistance

The β-lactam family of antibiotics includes many of the most commonly used antibacterials in clinical medicines and majority of them belongs to either penicillin or the cephalosporin group. The β-lactam ring structure is being exploited by many drug development groups in the search for new drugs with improved efficiency against resistant bacterial strains. The β-lactams also include the moxalactam, the carbapenems (e.g. imipenem), the monobactams (aztreonam), and the β-lactamase inhibitors (e.g. clavulanic acid). One of the major mechanisms of resistance to β-lactams is the expression of β-lacatmases which inactivate the antibiotics by hydrolysis of the β-lactam ring. β-lactamase have been found very widely in both gram positive and gram negative bacteria such as *Staphylococcus* sp., *Bacillus* sp., *Pseudomonas* sp., *Haemophilis* sp. and *Neisseria* sp. (Livermore 1995). Many members of the enterobacteriaceae including *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Serratia marcescens*, *Klebsiella pneumoniae,* etc. are generally resistant to amoxycillin and early generation cephalosporins and have variable resistance profiles to second-generation cephalosporins. *Mycobacterium tuberculosis* has been considered intrinsically resistant to β-lactam antibiotics, owing to its beta lactamase activity (Nampoothiri 2003). Most of these bacterial species produce a chromosomally encoded β-lactamase belonging to ambler class C (amb C) gene (Bush 1988). β-lactamases hydrolyze the cyclic amide bonds in the β-lactam ring of penicillins, cephalosporins and related compounds. β-lactam drugs combined with β-lactamase inhibitors have been used in recent years to treat infections caused by other β-lactamase producing bacteria.

3. SOURCES

Therapeutic enzymes are widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi. Although microorganisms are considered potential source of enzymes, utilization of microbial enzymes for therapeutic purpose is limited because of their incompatibility with the human body, although there is an increased focus on utilization of microbial enzymes because of their economic feasibility. One other major problem is the large molecular size of the biological catalysts, which prevents its distribution within the somatic cells. Another important problem related to enzyme therapy is the elicitation of immune response in the host cells after injecting the foreign enzyme protein. Through the technique of drug targeting and disguising the enzyme as an apparently non-proteinaceous molecule by covalent modification can alleviate these problems. These inherent problems necessitate the requirement of therapeutic enzymes with a very high degree of purity and specificity (Sabu 2003). In general, the favoured kinetic properties of these enzymes are low K_m and high V_{max} in order to be maximally efficient even at very low enzyme and substrate concentrations. Table 2 describes the reports on some microbial therapeutic enzyme.

4. MICROBIAL PRODUCTION

As is the case with other enzymes, production of therapeutic enzymes too have most been studied in submerged fermentation (SmF), although there are few reports on solid-state fermentation (SSF) and immobilized systems. Modern biotechnology based techniques such as transfecting the desired gene to animal cells and production of the desired metabolite by cell culture is also an important production strategy. Screening and evaluation of nutritional and environmental requirements of microorganisms is an important step for bioprocess development. As a part of the search for value added enzymes, researchers turned their attention to alternate production systems such as solid-state fermentation (SSF).

Table 2 Microbial sources of therapeutic enzymes

Enzyme	Micro organisms
L-glutaminase	*Beauveria bassiana, Vibrio costicola, Zygosaccharomyces rouxii*
L-asparaginase	*Pseudomonas acidovorans, Acinetobacter* sp.
β-Lactamase	*Citrobacter freundii, Serratia marcescens, Klebsiella pneumoniae*
Serratia peptidase	*Serratia marcescens*
Lipase	*Candida lipolytica, Candida rugosa, Aspergillus oryzae*
Alginate lyase	*Pseudomonas aeruginosa*
L-arabinofuranosidase	*Aspergillus niger*
Protease	*Bacillus polymyxa, Beauveria bassiana*
Superoxide dismutase	*Mycobacterium sp, Nocadia* sp.
Glucosidase	*Aspergillus niger*
Amylase	*Aspergillus oryzae, Bacillus* sp.
Serrapeptase	*Serratia marcescens*
Penicillin acylase	*Penicillium* sp
Laccase	*Trametes versicolor*

Extracellular L-glutaminase production was carried out in SSF by *Beauvaria* sp isolated from marine sediments. Polystyrene beads were used as inert solid support. Maximal enzyme production (49.9 U/ml) occurred in a seawater based medium supplemented with L-glutamine (0.25 %w/v) as substrate and D-glucose (0.5 % w/v) as additional carbon source (Sabu et al. 2000). SSF was also carried out for the production of extracellular L-glutaminase using saline tolerant yeast *Zygosaccharomyces rouxii* where wheat bran and seasame oil cake were employed as substrates (Kashyap et al. 2002).

The antilymphomic and antineoplastic activities of L-asparginase have been well documented. Asparginase has been generally produced by submerged fermentation (SmF), although a few attempts have also been made in SSF. Statistically based experimental designs were applied to optimize SSF for the production of L-asparginase by *Pseudomonas aeroginosa*. Fifteen culture conditions were examined for their significance on enzyme production and specific activity using Plackett-Burman factorial design. pH (7.9), casein hydrolysate (3.11%), and corn steep liquor(3.68%) were the most significant factors improving enzyme production (142.8IU) process (Yasser et al. 2002)

Good success have been achieved in developing a recombinant *E. coli* strain for the production of Penicillin G amidase. However, some problems were faced as the post translational proteolytic conversion of its polypeptide precursor in to mature enzyme was a complex process. Recently, this limitation has been overcome by expressing the *pac* gene from *Providencia rettgeri* in the yeast *S. cerevisiae,* yielding a high amount of rPACpr. (Ljubijanki et al. 1999). Similarly, over- production of glycosylated thermostable *Providencia rettgeri* penicillin G amidase in *P. pastoris* was attained by Sevo et al. (2002).

β-lactamases are distributed ubiquitously in both gram positive and gram-negative bacteria (Massova et al. 1998). These are also produced by non-pathogenic bacteria such as *Streptomyces* sp (Ogwara et al. 1999). Aminopeptidases are found to participate in biological processes such as protein

maturation, activation, hormone production and peptide digestion. A fermentation broth supernatant of the *Aspergillus oryzae* strain ATCC 20386 contained aminopeptidase activities that released a wide variety of amino acids from natural peptides (Blinkovski et al. 2000). For this purpose, the culture was grown in a mineral salt medium containing maltodextrin and soy flour at pH 6.5 and 34 ^{0}C. Chein et al. (2002) produced extracellular leucine aminopeptidase (LAP) from *A. sojae*.

4.1. rDNA technology for the production of therapeutic enzymes

Advents of genetic engineering have facilitated the production of highly purified proteins such as recombinant human DNase (rhDNase), which is used for the treatment of cystic fibrosis. rDNA technology allows production of large quantities of pharmaceutical proteins, which have been previously difficult and costly to produce. Protein activity is often modified by rDNA technology and can be overcome by shuffling functional domains and site directed mutagenesis. This is done to modify activities, regulation and avoid unwanted side effects. About 400 human proteins have been produced by rDNA technology for therapeutic use. Present global market for therapeutic recombinant proteins is around $200 billion. Since there is a need for large quantity of therapeutic enzymes for clinical trials and for sales once approved, the gene expression process must be optimized (Christensen 2000). Recombinant DNA technology has fueled an explosion of new applications and techniques and the availability of numerous complete microbial genome sequences has profoundly altered our understandings of a number of fundamental biological processes. For example, the enzyme involved in aminoacyl- tRNA (AA_tRNA) synthesis, the key process responsible for the accuracy of protein synthesis, has been found to be highly species-specific (Raczniak et al. 2001).

5. PRODRUG

An innovative use of enzymes as therapeutic agents entails their administration to tumor-bearing subjects along with a prodrug conjugated to a functional group that is susceptible to attack by an enzyme. To achieve the requisite selectivity advantage is taken of two features: the acidic intracellular environment of many neoplasms as compared to normal tissues, and an enzyme with an acidic pH-activity optimum. Using a combination of L-arabinofuranosidase from *A. niger* and Peltatin-L-arabinofuranoside, scientists have successfully developed a technique to depress thymidine incorporation by mammary adenocarcinomas.

6. ENZYME REPLACEMENT THERAPY

The treatment of enzyme deficiency state represents an obvious use of enzymes. More intriguing is the treatment of the in-born errors of metabolism in which deficiency of a single enzyme leads to accumulation of abnormal amounts of substrate. With the recognition that many of these errors are owing to inadequacies of lysosomal enzymatic catabolism, it was reasoned that exogenously administered enzyme might react with and dispose of such accumulations. The infusion of crude glucosidase from *Aspergillus niger* into patients with type II glycogenolysis, a condition attributed to a deficiency of this enzyme, was reported in the mid 1960s.

7. THE CONCEPT OF BIODRUG

The biodrug concept involves the use of orally administered recombinant microorganisms as a new drug delivery route to prevent or treat diseases. The tools used for genetic engineering that have been

developed to date have led to the emergence of novel applications using genetically modified microorganisms to produce drugs in large-scale bioprocesses (Primrose, 1986). An innovative extension of these approaches is drug production directly in the digestive environment by ingested living recombinant microorganisms. For this purpose, recombinant bacteria, mainly lactic acid bacteria, have been studied (Chang & Prakash 1988). Yeast is a convenient host and a good alternative for the production of biodrug. The most common yeasts, *S. cerevisiae* and *S. boulardii*, have a 'GRAS' status and have recently been used as in both animals and humans, and as in some human digestive pathologies, such as antibiotic-associated diarrhea and Clostridium difficile-disease. In the past few decades, *S. cerevisiae* has become an attractive host for the production of recombinant proteins and bioconversion owing to its high productivity and ease of genetic engineering. The biodrug concept was later validated (Alric 2000) using a recombinant model of *S. cerevisiae*. This enzyme provides a relevant model of bioconversion for potential therapeutic applications, such as biodetoxication' in the digestive environment. The yeasts have been studied in an artificial digestive system, which simulates human digestion.

The potential medical applications of this new generation of biodrugs are numerous: for example, the correction of enzyme deficiencies, the control of the activation of pro-drug to drug or the production of therapeutic proteins, such as vaccines, directly in the digestive tract. In particular, by increasing the body's protection against environmental xenobiotics, these biodrugs can offer an innovative way to prevent or treat diseases that escape traditional drug action, such as cancer or other widespread multifactorial diseases.

8. CONCLUSIONS

Pharmaceutical industry is being recognized as an important consumer for commercial enzymes. Development of medical applications for enzymes has been at least as extensive as those of industrial applications, reflecting the magnitude of the potential rewards. Microbial enzymes offer potential to treat many important diseases, which are resurging after acquiring resistance to antibiotics. There is an urgent need for the accelerated and in depth studies to exploit the various microbial resources for this novel purpose.

9. PERSPECTIVES

The emerging field of genomics in combination with rDNA technology, biochemistry and structural biology affords a new perspective in developing novel drug targets in the future. Emergence of lethal diseases is a global problem and in spite of sincere efforts paid in the past, search for efficient drugs to solve this problem is being continued worldwide. Enzymes play a major role in each and every aspect of human metabolism and hence they can be applied to combat most of the metabolic disorders. Increased awareness of the use of biocatalytic capabilities of enzymes and microorganisms has made possible the creation of a new generation of rationally developed biologically based processes and products. The majority of enzymes currently used in therapeutic applications are of microbial origin. Since incompatibility of these enzymes to the human system is a major hurdle in therapeutic application, there is a need for in depth study to over come these hurdles by way of rDNA techniques. Enzyme engineering coupled with biomolecular modeling will be best tool for the creation of custom designed drugs for enzyme therapy.

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Inteins

Isabelle Saves

1. INTRODUCTION

1.1. What is an intein?

Inteins, also named protein introns, are invading proteins, which are post-translationally excised from their host protein by a self-catalytic phenomenon of protein splicing. Intein coding sequences, 400 to 1800 bp long, are inserted in-frame in the ORF of host genes, which are generally essential for the cellular life. By contrast with ribonucleic introns, these invading DNA sequences are not spliced at the RNA level but are translated within the host protein sequence, also named extein. That leads to the production of a large peptide precursor from which the intein has to be excised to release the mature and functional host protein. At the protein level, the intein is able of self-excision concomitant with the generation of a peptide bound between the N- and C-terminal halves of the extein (Fig 1) (Shao & Kent 1997).

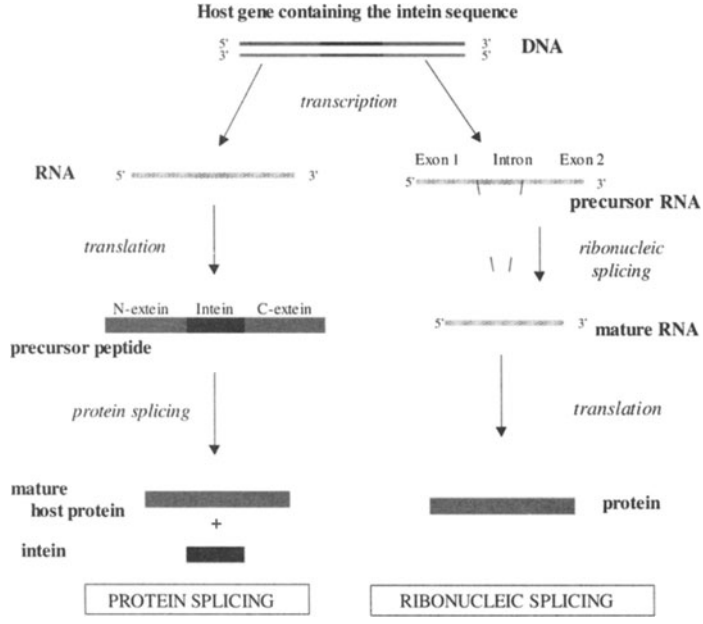

Figure 1. Two different ways of maturation by splicing (adapted from Shao & Kent 1997).

1.2. SceVMA, the first described intein

The first intein was described in 1990 in the vacuolar H^+-ATPase of *Saccharomyces cerevisiae* (Kane et al. 1990, Hirata et al. 1990). Its encoding *VMA1* gene was shown to be translated into a 119 kDa protein while the yeast ATPase was previously described as a 69 kDa protein. Within this protein, the region of homology with other ATPases from several species maps with the N- and C-terminal thirds of the sequence and is interrupted by a "spacer domain" sharing 31 % identity with one region of the yeast HO endonuclease. Afterwards, it was shown that the 50 kDa internal protein, named *Sce*VMA intein, was excised at the protein level leading to the production of the mature ATPase, this protein splicing event occurring either in the yeast and in heterologous context (Chong et al. 1996).

1.3. Inteins and exteins recensed to date

Currently, around 160 intein sequences have been found in 70 living species among the all three domains of life (Inbase, the New England Biolabs intein database ; Inteins-Protein introns web site). Inteins are present in 38 different host proteins at 52 different insertion sites, the same host protein can thus contain more than one intein (Fig 2). Moreover, orthologous host proteins in different species can contain similar inteins at the same insertion site ; these orthologous inteins are classified in 27 allelic families (Perler et al. 1997; Inbase; Inteins-Protein introns web site). As specified in figure 2, 3 of these families are found in archaeal DNA polymerase α.

The host proteins have various but generally essential functions, including metabolic enzymes, DNA polymerases, DNA helicases, proteases, ribonucleoside reductases, ATPases, etc. Within these proteins, the inteins are generally located in highly conserved domains, meaning that they probably interrupt the host protein in important functional domains rendering the splicing event imperative (Saves et al. 2000b; Inteins-Protein introns web site).

1.4. Intein motifs and domains

Excepting the inteins belonging to the same allelic family, the inteins sequences are highly divergent. Nevertheless, at least 8 conserved peptide motifs can be defined in the majority of inteins (Figure 3A) (Dalgaard et al. 1997; Pietrokovski 1994; Inbase; Inteins-Protein introns web site). These motifs are not strictly conserved from one intein to another, but they are rather composed of related residues. Four motifs, named blocks A, B, F and G, are absolutely necessary. They are located in the N- and C-terminal regions of the intein sequence and are responsible for the splicing activity. Four other motifs, named blocks C, D, E and H, are located in the central region of the intein sequence and are associated with a putative endonuclease activity. Few small size inteins, named mini-inteins, do not possess this central domain meaning that the endonuclease activity of intein is optional.

Even if the activities associated with inteins have been demonstrated for only a few cases, it appears that the majority of inteins are bifunctional enzymes since they possess an activity of protein splicing, which can be considered as a protein ligation activity, and a specific activity of double-stranded DNA cleavage.

The structural analysis of inteins, in particular the crystallographic resolution of the tridimensional structures of the *Sce*VMA intein also named PI-*Sce*I (Duan et al. 1997), and the *Pfu*Pol-1 intein, also known as PI-*Pfu*I, from *Pyrococcus furiosus* (Ichiyanagi et al. 2000), showed that the two activities are borne by two separate structural domains with different folds (Figure 4). The splicing domain, which has an elongated form and is largely composed of β-sheets, harbours the N- and C-terminal residues

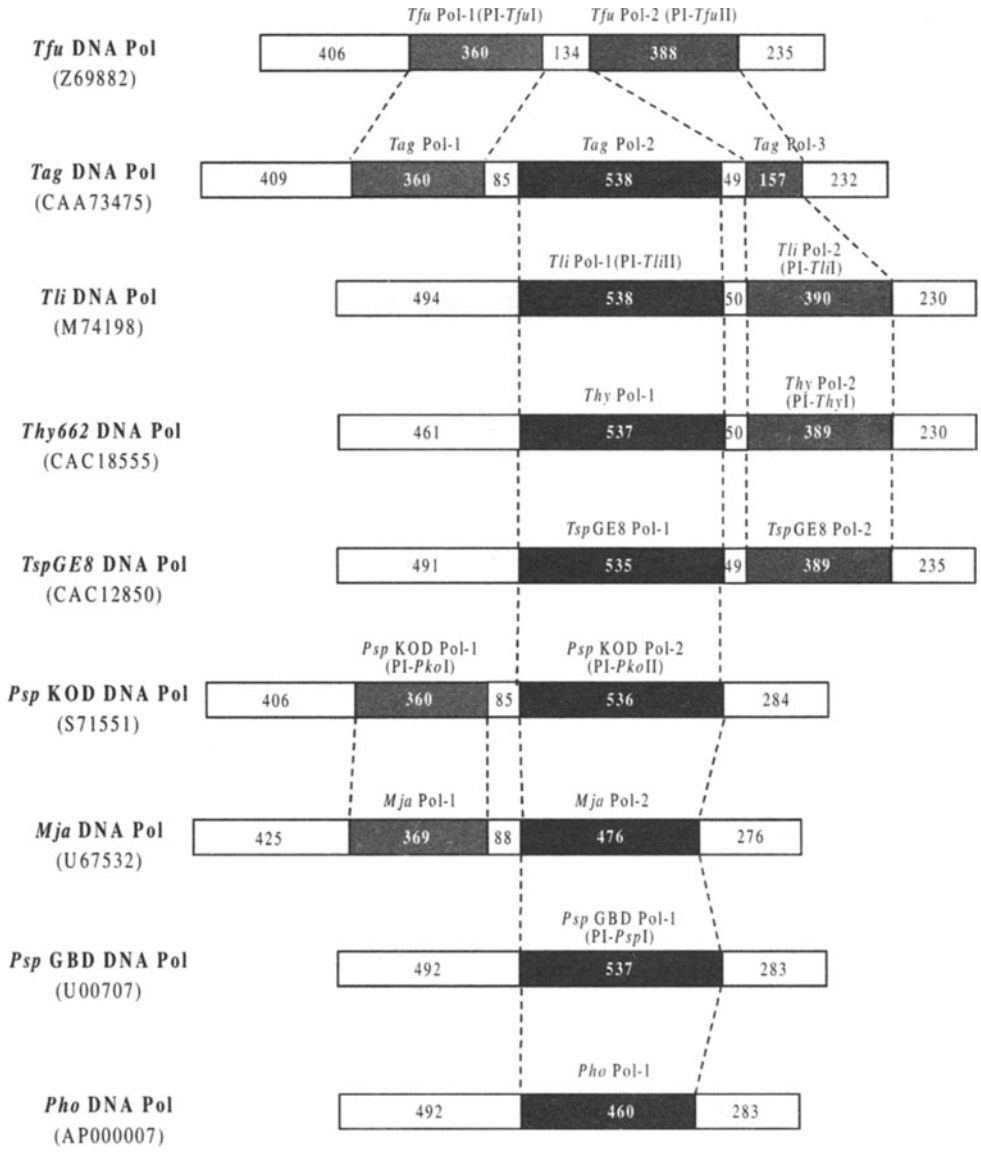

Figure 2. Inteins in DNA polymerases of archaebacteria. The 3 families of allelic inteins are presented in different colours. Names of inteins, lengths in amino acids of each exteins and inteins and GenBank accession numbers of DNA polymerase are indicated. Names of the endonuclease activities, which have been experimentally demonstrated, are indicated in parentheses after the intein names. *Tfu* DNA Pol, *Tag* DNA Pol, *Tli* DNA Pol, *Thy662* DNA Pol, *TspGE8* DNA Pol, *Psp* KOD DNA Pol, *Mja* DNA Pol, *Psp* GBD DNA Pol and *Pho* DNA Pol are the abreviations for the DNA polymerases from *Thermococcus fumicolans*, *Thermococcus agregans*, *Thermococcus litoralis*, *Thermococcus hydrothermalis*, *Thermococcus spp.* GE8, *Pyrococcus* spp. KOD, *Methanococcus jannaschii*, *Pyrococcus* spp. GBD and *Pyrococcus horikosshi* OT3, respectively. Adapted from Saves et al. 2000a.

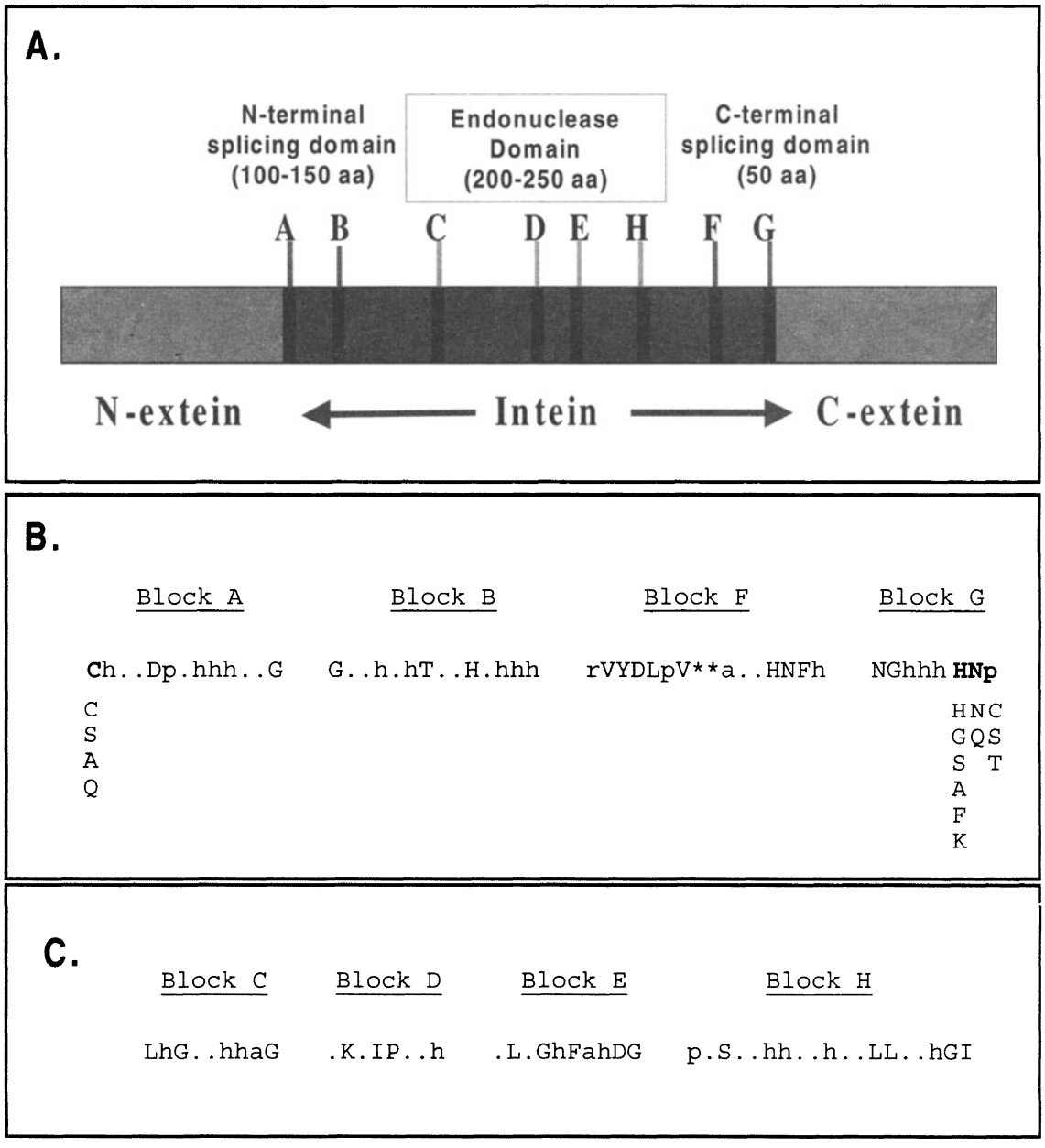

Figure 3. Intein conserved motifs and domains. A. Position of the 8 conserved motifs and the 2 domains of inteins. B. Consensus sequence of the 4 splicing motifs. Residues directly implied in the splicing reaction appear in bold. The possible variations of these residues are indicated below. C. Consensus sequence of the endonuclease motifs. Blocks C and E are dodecapeptides motifs. In B and C, the consensus line keys are a : acidic residues, h : hydrophobic residues (G,V,L,I,A,M), p : polar residues (S,T,C), r : aromatic residues (F,Y,W).

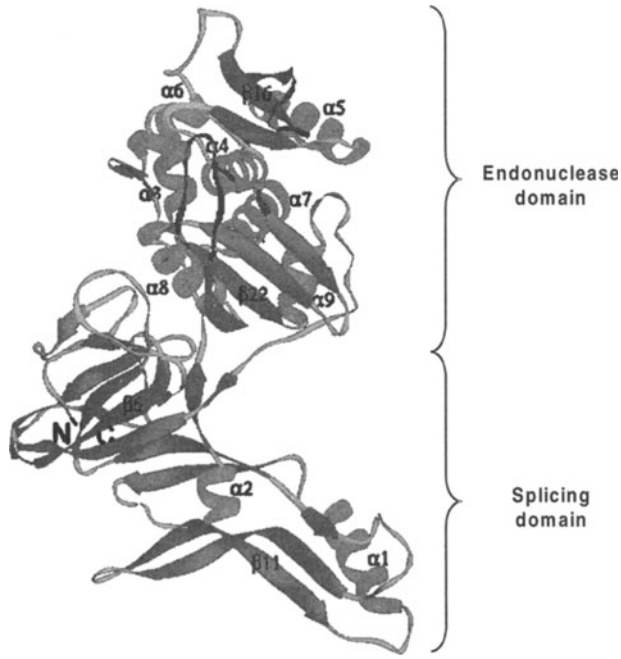

Figure 4. Bipartite structure of *Sce*VMA intein, taken from Duan et al. 1997.

that are implied in the protein splicing activity. In both inteins, this domain has the same horse-shoe shaped tertiary structure than the mycobacterial *Mxe*GyrA mini-intein (Klabunde et al. 1998). In corroboration these essential structural features are also found in other self-processing proteins as hedgehog proteins (Hall et al. 1997). The second domain, more compact and formed by the central part of the intein sequence, contains the putative endonuclease active site and its structure is very similar to that of the homodimeric endonuclease I-*Cre*I encoded by a self-splicing Group I intron (Heath et al. 1997).

The bipartite domain structure of bifunctional inteins is likely paralleled by the independence of the two catalytic activities that was demonstrated by directed mutagenesis studies of few inteins (Hodges et al. 1992; Chong & Xu 1997). Effectively, mutation of important residues abolishing one or the other activity has little or no effect on the second activity. That is coherent with the fact that mini-inteins conserve an efficient protein splicing activity (Telenti et al. 1997). Moreover, substantial evidence suggests that the two domains and activities have evolved independently.

2. INTEIN ACTIVITY OF PROTEIN SPLICING AND PROTEIN LIGATION

The description of *Sce*VMA intein splicing immediately provoked a kind of emulation among protein engineering scientists because it was the first report of a protein able to self-catalyse the ligation between two peptides ends. Indeed, extein ligation differentiates protein splicing from the other forms of self-proteolysis such as the autocleavage reaction of glycosylasparaginase, the hedgehog protein or the pyruvoyl-dependent histidine decarboxylase. Hence, the fine elucidation of the molecular events handling

the splicing pathway enabled scientists to modulate the successive reactions and thus to design inteins able to manage highly specific cleavages and protein ligation reactions. Nowadays, the protein engineering tools using such inteins are currently and successfully used.

2.1. Protein splicing pathway

The splicing activities of few inteins (Chong et al. 1996; Chong & Xu 1997; Kawasaki et al. 1997; Chong et al. 1998; Nogami et al. 1997; Hodges et al. 1992; Perler et al. 1992; Xu & Perler 1996) were fully studied. The substitution of residues among the intein and the extein allowed defining the residues directly implied in the splicing reaction. In parallel, the analyses of the precursors, the splicing intermediates and the splicing products of either wild type inteins, point mutated inteins or chimeric splicing systems (Chong et al. 1996 ; Xu et al. 1993) allowed understanding the mechanism of splicing at the molecular level. This mechanism was recently reviewed by Noren et al. (2000) and Paulus (2000).

2.1.1. Splicing key residues and common protein splicing mechanism

All the critical information necessary for the splicing reaction is present in the intein and the first residue of the C-extein. Indeed, four highly conserved residues in blocks A and G, located at the borders between the intein and the N- and C-exteins are directly implied in the reaction. These residues are the most often a Ser or a Cys at the first N-terminal position of the intein, a doublet formed by an His and an Asn at the C-terminal extremity of the intein and a Ser, a Cys or a Thr at the first N-terminal position of the C-extein (Fig 3B) (Pietrokovski 1994 ; Perler et al. 1997). However, the recent finding and sequencing of a large number of inteins highlighted some possible divergences among these residues as shown in Figure 3B (Inbase).

In spite of the variability of sequence at the intein borders, a common intramolecular splicing pathway, based on the mechanisms described in 1996 by Xu and Perler for the *Psp*Pol-1 intein and by Chong et al. for the *Sce*VMA intein, can be generalised to all inteins sequenced to date.

Four successive and independent nucleophilic displacements by 3 of the 4 conserved splice junction residues are responsible for the splicing of the intein and the peptide ligation of the N- and C-exteins (Fig 5). The first step is the activation of the N-terminal junction between the N-extein and the intein. It consists in a N-O or N-S acyl shift of the first Ser or Cys of the intein and leads to a linear esterified intermediate. During the second step, the nucleophilic attack of the N-terminal junction by the first residue of the C-extein results in a trans-esterification : the N-terminal junction is cleaved and the N-extein is transferred to the lateral chain of the first residue of the C-extein, leading to a branched intermediate. The third step consists in the cyclisation of the Asn residue at the C-terminal extremity, which results in the cleavage of the peptide bound between the intein and the extein. It is assisted by the intein penultimate His by hydrogen bonding to the Asn carboxyl oxygen, which makes the peptide bond more labile. Finally, the fourth step of O-N or S-N-acyl rearrangement spontaneously converts the thioester linkage between the N- and C-exteins into a native peptide bound.

Since only the last step is a rapid and spontaneous chemical reaction, inteins can be termed enzymes which are able to catalyse the first three steps of the splicing reaction, the substrate of such enzymes being the splicing junction residues to be linked.

2.1.2. Possible variations of the protein splicing mechanism

The residue divergences at the intein borders (Figure 3B) imply an adaptation of the prevailing protein

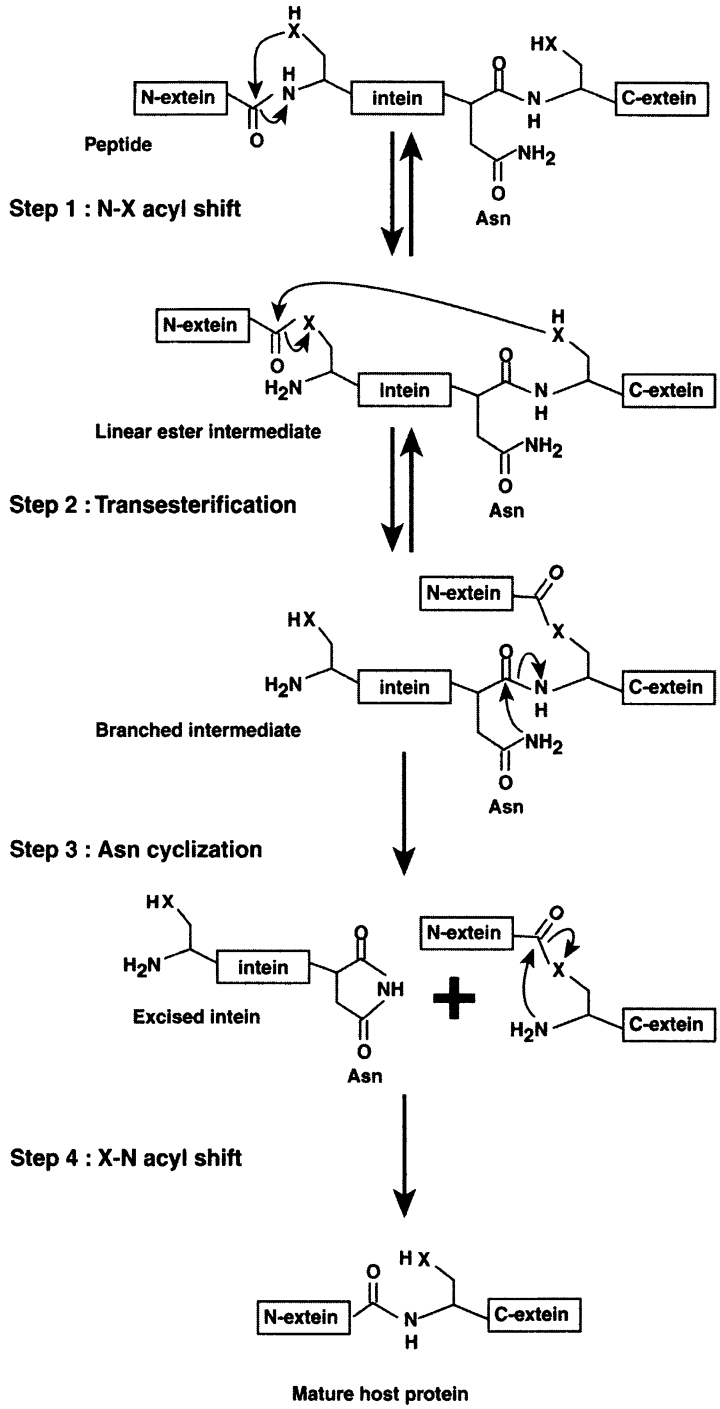

Figure 5. Prevaling protein splicing pathway. X= S or O, adapted from Xu & Perler 1996 and Chong et al. 1996.

splicing pathway described above. Southworth et al. (2000) proposed a slightly variant mechanism for inteins beginning with Ala rather than the consensus Ser or Cys. In such cases, since the N-terminal Ala cannot perform the initial activation reaction leading to the requisite linear ester intermediate of the typical mechanism, the C-extein nucleophile directly attacks the peptide bound at the N-terminal splice junction rather than an ester bound (Fig 6A), the following splicing steps remaining unchanged.

In the case of inteins which possess a Gln instead of the conserved Asn at the C-terminal extremity (Fig 3B), the release of the cleaved intein from the ligated N- and C- exteins during the penultimate step of the reaction probably involve a cyclisation of the glutamine, either analogous to that of the Asn leading to the formation of a glutarimide ring (Fig 6B middle) (Pietrokovski et al. 1998) or forming an anhydride such as glutaranhydride (Fig 6B right) (Amitai et al. 2003).

Recently, an atypical intein from *Carboxydothermus hydrogenoformans* (*chy*) with a C-terminal Asp residue was described by Amitai et al. (2003). This intein was shown to splice and two variations for the C-terminal cleavage step and extein ligation were proposed (Fig 6C). One possibility is that the C-terminal Asp could cyclise into a succinic anhydride (Fig 6C left). The second possibility is that the C-terminal cleavage would result from the nucleophilic attack of the peptide bond linking the intein and the C-extein by the free N-terminal residue of the intein (Figure 6C right).

Among the intein conserved residues, the penultimate C-terminal His residue facilitates the splicing reaction by assisting the Asn or Gln cyclisation. The structural analysis of *Mxe*GyrA and *Sce*VMA inteins (Duan et al. 1997; Klabunde et al. 1998) showed that the His, which is in hydrogen bonding distance to an Asn carboxylate oxygen, facilitates the cyclisation by making the Asn carboxyl group more electrophilic. Around 10% of known inteins possess an other residue at the penultimate position (Inbase), including Gly, Ala and Phe which are unlikely to assist in Asn or Gln cyclisation. It was proposed that modifications in the active site of these inteins could lead to the use of another residue to assist the cyclisation step (Chen et al. 2000).

2.1.3. Trans-*splicing reaction*

One atypical intein, described in *Synechocystis sp.* PCC6803 genome, allowed to demonstrate the phenomenon of *trans*-splicing *in vivo* (Wu et al. 1998). Effectively, in this organism, the *dna*E gene encoding the a DNA polymerase III is split within its intein sequence by a 745 kbp insertion and a genome inversion. Thus, two precursor fragments, each being composed of one half of the DnaE polymerase in fusion with one half of the intein, are expressed independently and the mature polymerase is produced by *trans*-splicing between these two proteins (Fig 7). This first description of the *trans*-splicing reaction *in vivo* allowed to show that the protein splicing reaction can process intermolecularly.

Protein *trans*-splicing was reconstituted *in vitro* by splitting precursor genes within the intein coding sequence. The two parts of the precursor genes were thus expressed and purified independently and mixed to reassemble the intein splicing element, leading to the ligation of the exteins and the release of the intein fragments (Mills et al. 1998; Southworth et al. 1998). Even more, Lew et al. (1998) and Shingledecker et al. (1998) conceived minimal synthetic splicing elements using as short as possible intein fragments.

2.2. Control and applications of protein splicing

2.2.1. Control of the protein splicing reaction

The elucidation of the protein splicing mechanism allowed to modulate the intein activity in order to use

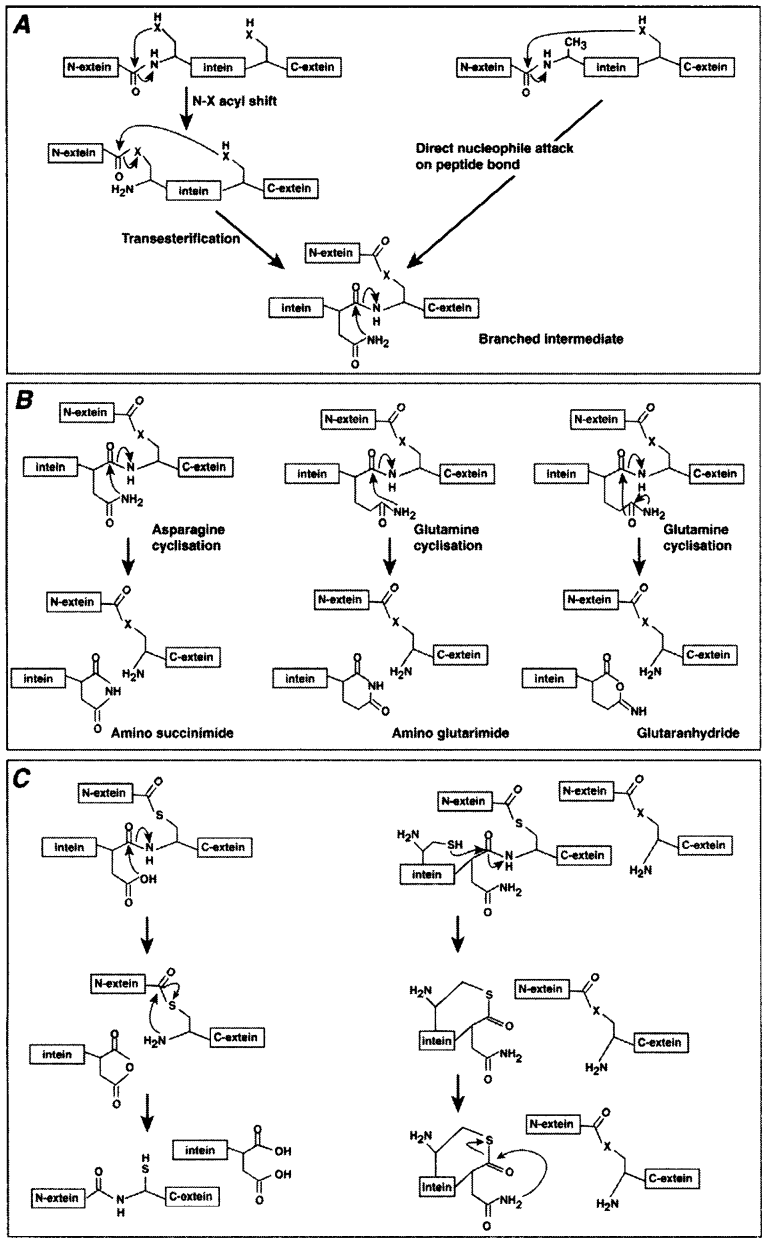

Figure 6. Alternatives in the protein splicing mechanism. A. Standard (right) and alternative (left) pathways to form the branched intermediate in the protein splicing mechanism. This alternative pathway may be used by inteins beginning by an Ala residue instead of the conserved Ser or Cys at the N-terminal extremity. Taken from Southworth et al. 2000. B. Standard (rigth) and alternative (middle and left) pathways for the release of the cleaved intein in the protein splicing mechanism. These alternative pathways may be used by inteins ending by a Gln residue instead of the conserved Asn. Adapted from Pietrokovski 1998 and Amitai et al. 2003. C. Alternative reactions for C-terminal autocleavage of the *Chy* intein ending by an Asp residue instead of the conserved Asn. Adapted from Amitai et al. 2003.

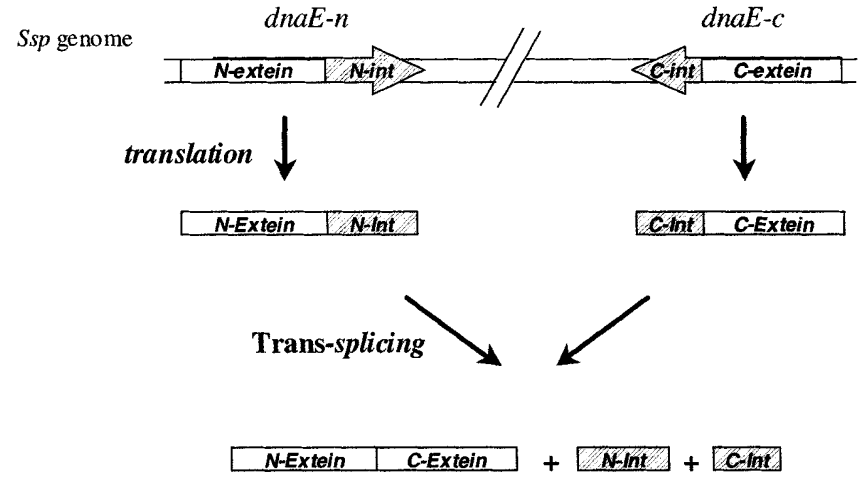

Figure 7. Protein *trans*-splicing. In the *Ssp* genome, the gene encoding for the DnaE polymerase is split in two halves *dna*E-n and *dna*E-c, which are in the opposite direction and separated by 745 kbp. *dna*E-n gene codes for the N-terminal part of the polymerase fused to the 123 N-terminal residues of the intein and *dna*E-c gene codes for the 36 C-terminal residues of the intein fused to C-terminal part of the polymerase. When expressed, these two proteins are submitted to an intermolecular *trans*-splicing process leading to the production of the mature DNA polymerase (adapted from Wu et al. 1998).

it for various applications, particularly in the field of protein engineering. Since all the information necessary for protein splicing is borne by the intein sequence, an intein conserves its splicing activity in a foreign protein, provided that it is inserted upstream the accurate residue (a Cys, a Ser or a Thr). That was demonstrated with hybrid peptide precursors containing two foreign exteins halves interrupted by an intein (Xu et al. 1993; Chong et al. 1996 ; Telenti et al. 1997 ; Shingledecker et al. 1998). In all cases, the protein splicing activity is conserved even though its efficiency can be affected by the extein sequence and abortive side reactions can lead to single splice junction products.

Hybrid constructs, in which the intein was inserted in suboptimal extein contexts, and mutations within the intein sequence were used to modulate the splicing activity of inteins (Chong et al. 1998; Chong et al. 1997; Southworth et al. 1999; Telenti et al. 1997; Xu & Perler 1996; Paulus, 2000). Cleavage at only the N-terminal intein junction can effectively be achieved by mutation of one or more residues at the C-terminal intein border, preventing Asn cyclisation while allowing the formation of thio-ester at the N-terminal intein junction, which can thus be cleaved in the presence of thiol agents or nitrogen nucleophiles. Similarly, the cleavage at only the C-terminal intein junction in a thiol- or temperature-controlled way can be achieved by mutation of one or more residues at the N-terminal intein border or modulated by substituting the penultimate His. On the other side, the use of minimal *trans*-splicing elements is another way to control a splicing reaction.

2.2.2. Application to recombinant protein purification

A method of purification of recombinant proteins based on the use of the protein splicing activity of an intein is commercialised by New England Biolabs (New England Biolabs Home page). This method, named IMPACT (Intein Mediated Purification with an Affinity Chitin-domain Tag), implies the fusion of

a modified intein sequence, designed to undergo self-splicing under controlled conditions, between a chitin binding domain as an affinity tag and the target protein as the N- or C-extein. In the first IMPACT purification system (Fig 8), the substitution of the *Sce*VMA intein C-terminal Asn by an Ala residue hinders the intein splicing leading to the accumulation of the entire fusion protein which can be purified by affinity with chitin. Since the substitution of the Asn has no effect on the two first steps of the splicing pathway, the cleavage of the intein N-terminal junction is induced in presence of thiol agents, such as DTT or Cys (Chong & Xu 1997). This cleavage directly induced on the affinity chromatography column results in the release of the pure target protein while the intein fusion is retained, avoiding problems of protease specificity and additional steps of purification.

Other similar systems allowing the expression of peptide precursors containing a chitin binding domain, an intein and the recombinant protein to be purified as a N- or C-terminal fusion were designed. They present various characteristics (Table 1) ; they notably differ in the way to induce its release. Such systems allowing to purify recombinant protein with high yields are now currently used in purification processes and many examples of successful use could be cited (Inbase).

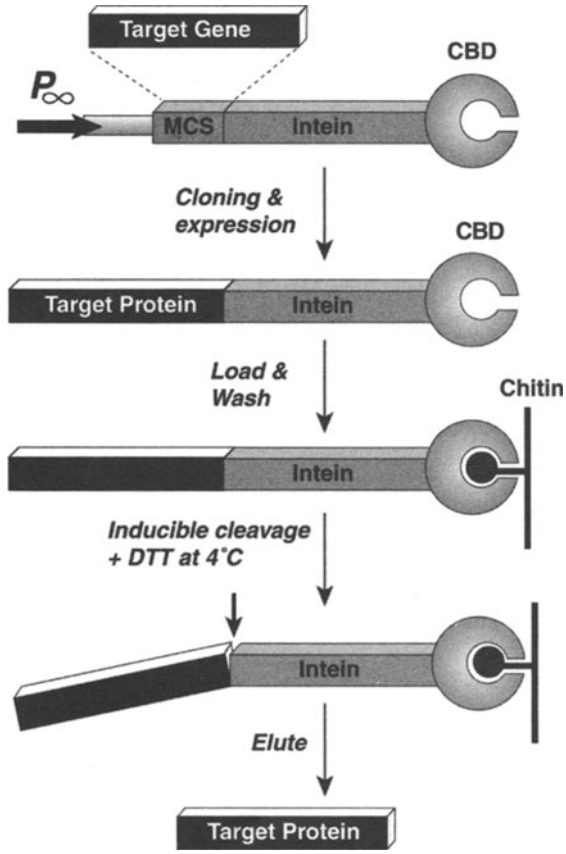

Figure 8. The first generation IMPACT system, commercialised by New England Biolabs is described. CBD = Chitin binding domain. Taken from Chong et al. 1997.

Table 1 : Characteristics of the intein-mediated purification systems. Intein length is indicated in number of amino acids. Position –1 corresponds to the last residue of the N-extein.* A chitin binding domain was inserted in-frame in a loop of the endonuclease domain of the intein

Intein used (intein length)	Intein mutation	Position of the target protein	Active Splice junction	Induction of cleavage	References
*Sce*VMA (454)	N454A	N-extein	N-terminal	Thiol reagents	Chong et al, 1997
*Mxe*GyrA (198)	N198A	N-extein	N-terminal	Thiol reagents	Evans et al, 1998
*Mth*RIR1(134)	P-1G N134A	N-extein	N-terminal	Thiol reagents	Evans et al, 1999; Southworth et al, 1999
*Ssp*DnaB (154)	N154A	N-extein	N-terminal	Thiol reagents	Mathys et al, 1999
*Sce*VMA (510*)	H509Q	C-extein	C-terminal	Thiol reagents	Chong et al, 1998
*Mxe*GyrA (198)	Y-1S 12V	C-extein	C-terminal	Temperature shift	Southworth et al, 1999
*Mth*RIR1 (134)	P-1G C1A	C-extein	C-terminal	pH and temperature shifts	Evans et al, 1999
*Ssp*DnaB (154)	C1A	C-extein	C-terminal	pH and temperature shifts	Mathys et al, 1999

2.2.3. Application to protein ligation

The elucidation of the protein splicing mechanism gave also rise to important advances in the protein semisynthesis through the amelioration of polypeptides ligation techniques. Effectively, the procedure of "native chemical ligation" described by Dawson et al. (1994) was significantly improved by the use of the intein-mediated protein expression. This new protein engineering technology was named "expressed protein ligation" or "Intein mediated protein ligation".

The native chemical ligation (Dawson et al. 1994), which consists in a protein ligation through a transesterification step between a polypeptide carrying a C-terminal reactive thioester and the N-terminal Cys residue of a second polypeptide, follows the steps of the protein splicing pathway, excepted the final Asn cyclisation (Fig 9A). This procedure is limited by the ability to make the two precursor peptides to be ligated. In particular, it is limited by the difficulty to generate a polypeptide with a C-terminal thioester moiety and by the necessity to generate a peptide with a N-terminal Cys residue by protease digestion. More generally, the limitation is to synthesise large polypeptides. A way to circumvent these technological barriers consists in using IMPACT or similar intein vectors to produce either only the polypeptide with C-terminal thioester moiety (protein 1; Fig 9B) (Evans et al. 1998 ; Muir et al. 1998 ; Severinov & Muir 1998) or both partners of the condensation reaction (Fig 9C) (Evans et al. 1999).

As recently reviewed by Hoffmann and Muir (2002), protein ligation allows differential modification of defined regions of a protein prior to ligation of these regions. This was usefully applied to the segmental labelling for NMR studies. In fact, an ideal approach to solve the structure of large proteins by NMR is to label only a region of the protein. That reduces the number of signals in spectra to a manageable number, allowing the resolution of the labelled region in the context of the entire folded protein. This technique also allows to directly observe the effect on the labelled domain of ligand binding or domain interactions and thus to elucidate the structure-activity relationships. In the same way, the expressed

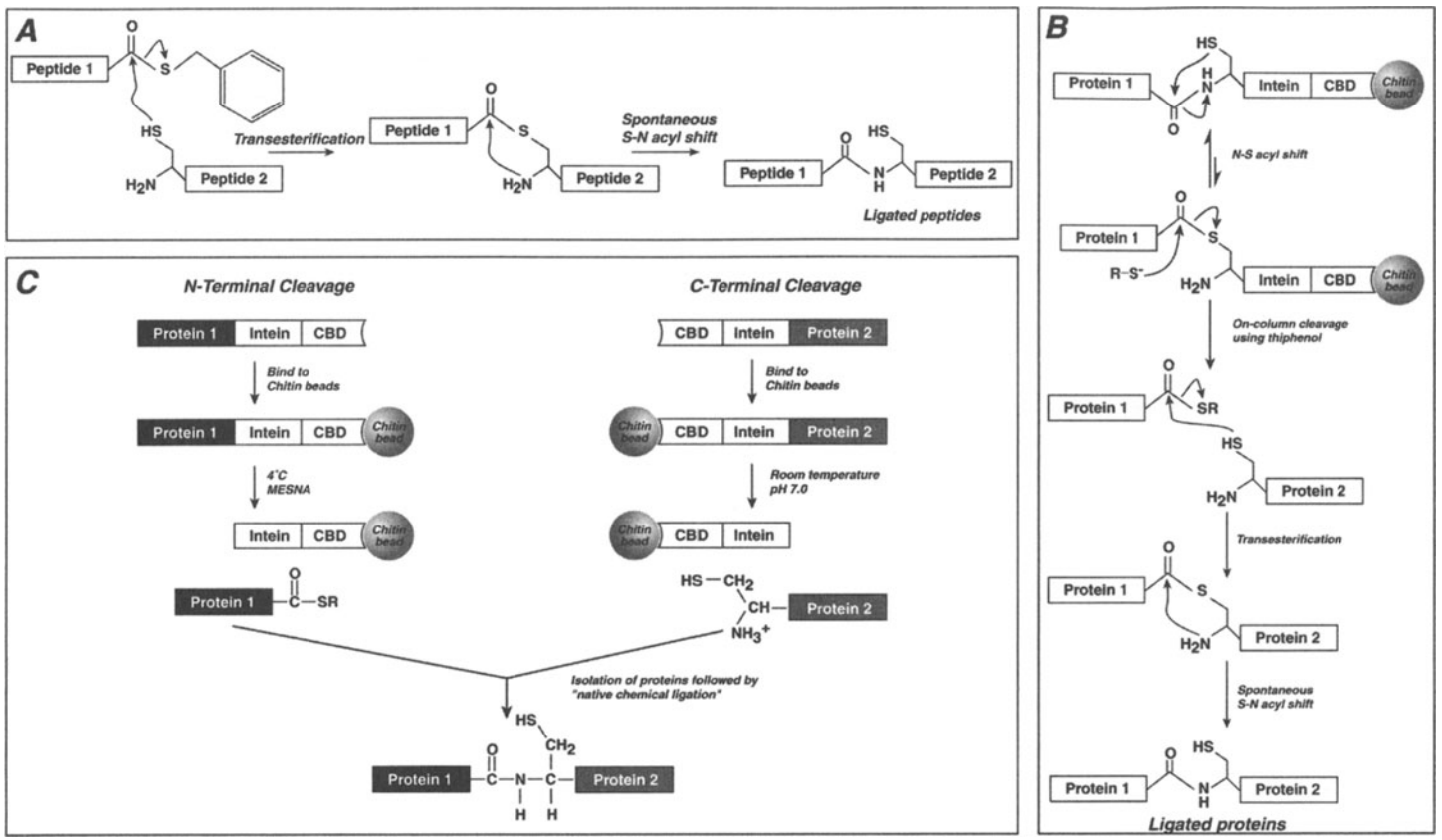

Figure 9. Native chemical ligation (A) (taken from Dawson et al. 1994) and Intein-mediated proteinligation (B and C) (adapted from Evans et al. 1998 and 1999).

protein ligation can be a tool to glycosylate or phosphorylate only a subset of sites located in a region of the target protein (Southworth et al. 1998), to introduce specific tags, fluorescent probes, photoactivable cross-linkers, unnatural amino acids or to cyclise proteins backbones of any size or sequence (Hofmann & Muir 2002). An other application is the semisynthesis of cytotoxic proteins (Evans et al. 1998).

An alternative approach to make intein-mediated protein ligation is *trans*-splicing. Effectively, *trans*-splicing makes possible the ligation of two proteins or two parts of a protein fused to the N- and C-terminal regions of an intein. This property can allow the control of protein expression. For example, an extremely cytotoxic protein can be expressed splitted in two parts fused to intein fragments and reconstituted by protein splicing. In the same order, ligation of two parts of an enzyme can also be used to induce its activation. In general, protein ligation by *trans*-splicing may find similar applications than the expressed protein ligation described before (Yamazaki et al. 1998). Moreover, it can be used to conceive modular proteins by recombining different functional domains or to search for protein-protein interactions (Ozawa et al. 2000; Paulmurugan & Gambhir 2003).

3. ENDONUCLEASE ACTIVITY AND INTEIN HOMING

The putative endonuclease activity of inteins was fairly suggested by sequence analyses (Perler et al. 1997). Effectively, among the 8 conserved intein motifs, four central blocks are expected to belong to an endonuclease domain (Fig 3). Notably, the two blocks C and E (Fig 3C) are dodecapeptides which are characteristic of the LAGLIDADG, or DOD, endonucleases forming the largest family of homing endonucleases (Chevalier & Stoddard 2001; Dalgaard et al. 1997; Mueller et al. 1993). Uncommonly in two inteins, this domain is replaced by an endonuclease domain of the HNH family (Dalgaard et al. 1997) and in about thirty mini-inteins no endonuclease domain is present (Inbase).

Even if the endonuclease activity of inteins is not systematically demonstrated, a representative number of inteins containing the DOD motifs was shown to possess such an activity. Indeed, since the identification of the double-stranded DNA cleavage activity of the *Sce*VMA and *Tli*Pol-2 inteins (Gimble & Thorner 1992; Perler et al. 1992), named PI-*Sce*I and PI-*Tli*I according to the current nomenclature of homing endonucleases, the activity of some archaeal and eubacterial inteins was researched and characterised (Table 2).

3.1. Inteins are Homing endonucleases

As previously shown for the internal ORF encoded by group I introns, the DNA cleavage activity of inteins seems responsible for the self-propagation of intein coding sequences among genomes by mediating horizontal transfer between species. This phenomenon, termed "Homing" was reviewed by Mueller et al. (1993) and Belfort and Roberts (1997).

Homing endonucleases, once expressed in the host organism, bind and cleave the non invaded allele of their host gene at or near the site of invasion by their coding sequence. This double-strand break in the genome promotes a homologous recombination repair event between the cleaved non invaded allele and the allele containing the endonuclease coding sequence, which leads to the duplication of this sequence (Fig 10). While these enzymes are highly specific, some punctual nucleotide substitutions are tolerated, which ensures the cleavage and the propagation of the homing endonuclease coding sequences among genomes despite the evolutionary deviance of their sequence.

Table 2. List of inteins tested for their endonuclease activity. The names of the host organisms and of the associated endonuclease activities are indicated. NF means that no activity was found in the conditions of the study.(*) The same name has been given to two mycobacterial intein endonuclease activities since they were found simultaneously by two different labs. Genebank accession numbers for these proteins can be found at Inbase, the intein database.

Domain of life	Organism	Intein name	Endonuclease name	Reference
eucaryotic	Saccharomyces *cerevisiae*	*Sce*VMA	PI-*Sce*I	Gimble & Thorner 1992
	Kluyveromyces lactis	*Kla*VMA	NF	Okuda et al. 2003
	Saccharomyces unisporus	*Sun*VMA	NF	Okuda et al. 2003
archaeal	*Pyrococcus spp.* G-BD	*Psp*Pol-1	PI-*Psp*I	Xu et al. 1993
	Thermococcus litoralis	*Tli*Pol-1	PI-*Tli*II	Perler et al. 1992
	Thermococcus litoralis	*Tli*Pol-2	PI-*Tli*I	Perler et al. 1992
	Thermococcus fumicolans	*Tfu*Pol-1	PI-*Tfu*I	Saves et al. 2000c
	Thermococcus fumicolans	*Tfu*Pol-2	PI-*Tfu*II	Saves et al. 2000c
	Thermococcus hydrothermalis	*Thy*Pol-2	PI-*Thy*I	Saves et al. 2000a
	Pyrococcus abyssii	*Pab*Lon	NF	Saves et al. 2002b
	Pyrococcus abyssii	*Pab*RIR1-1	NF	Saves et al. 2002b
	Pyrococcus abyssii	*Pab*RIR1-2	PI-*Pab*I	Saves et al. 2002b
	Pyrococcus abyssii	*Pab*RIR1-3	PI-*Pab*II	Saves et al. 2002b
	Pyrococcus furiosus	*Pfu*RIR1-1	PI-*Pfu*I	Komori et al. 1999a
	Pyrococcus furiosus	*Pfu*RIR1-2	PI-*Pfu*II	Komori et al. 1999a
	Pyrococcus kodakaraensis	*Psp*KODPol-1	PI-*Pko*I	Nishioka et al. 1998
	Pyrococcus kodakaraensis	*Psp*KODPol-2	PI-*Pko*II	Nishioka et al. 1998
eubacterial	*Mycobacterium gastri*	*Mga*Pps1	PI-*Mga*I	Saves et al. 2001b
	Mycobacterium tuberculosis	*Mtu*Pps1	PI-*Mtu*I (*)	Saves et al. 2002a
	Mycobacterium tuberculosis	*Mtu*RecA	PI-*Mtu*I (*)	Guhan & Muniyappa 2002
	Mycobacterium leprae	*Mle*RecA	NF	Saves et al. unpublished

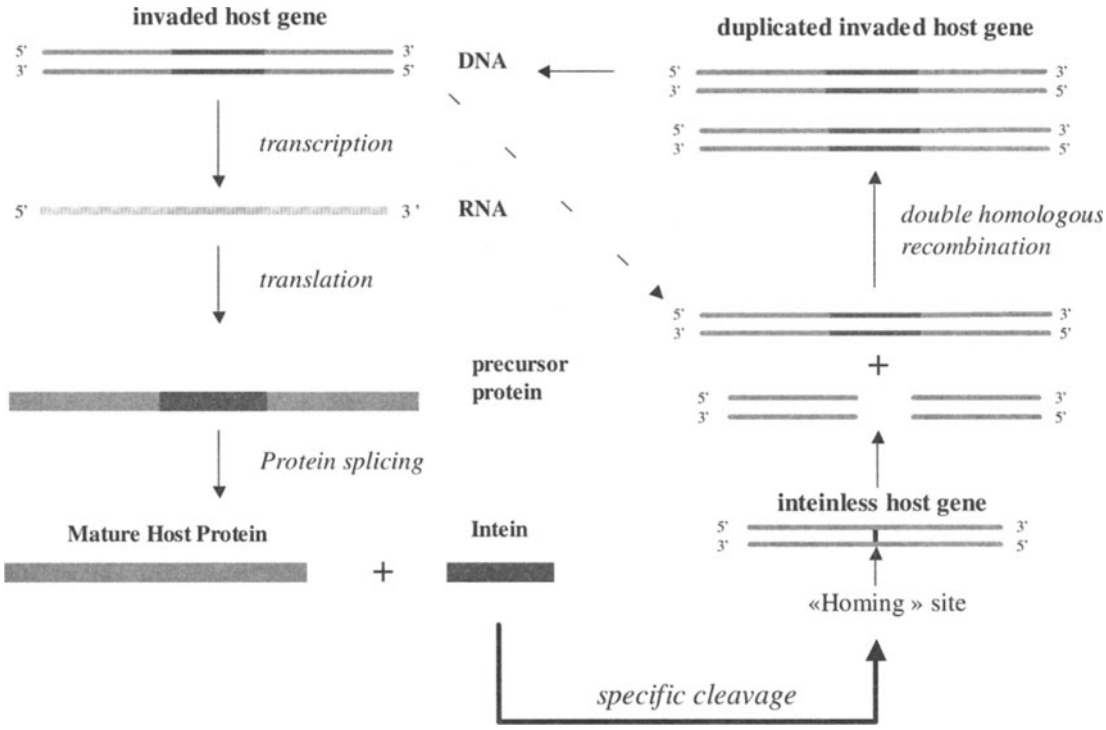

Figure 10. Intein sequence propagation by Homing.

Gimble and Thorner (1992) clearly demonstrated that PI-*Sce*I acts as a homing endonuclease in the yeast. Firstly, PI-*Sce*I was shown to cleave *in vitro* the allele of the *VMA1* gene that lacks the intein sequence, the cleavage site being located at the exact position of intein insertion. Therefore, it was shown that the intein sequence was able of self-propagation in heterozygous diploid yeasts containing one allele invaded by the intein coding sequence and one allele lacking this sequence. More recently, evidence for the horizontal transfer of this intein among the saccharomycete yeasts was provided by Okuda et al. (2003).

3.1.1. General properties of DNA cleavage

If a large amount of information concerning the molecular mechanism of double-stranded DNA cleavage by inteins comes from PI-*Sce*I intein, additional data were provided by the studies of 15 archaeal or mycobacterial inteins (Table 2). The comparison of individual enzymatic characteristics is rather difficult; nevertheless, a few general properties of DNA cleavage by inteins can be emphasised.

Some common features are observable. (1) Inteins are monomeric enzymes in solution, either in the presence or in the absence of DNA substrate. (2) Inteins are very specific endonucleases since they recognise and cleave exceptionally long asymmetrical sequences: a 16 to 31 bp sequence spanning the intein insertion site in the inteinless allele of their host gene is the substrate of the intein. (3) Both DNA strands are cleaved at a precise location, the cleavage leaving a 4-base long 3'-hydroxyl overhang. (4)

The endonuclease activity of inteins requires a divalent cation, usually Mg^{2+}. This cation is an essential cofactor for the phosphodiester bonds hydrolysis but is usually not required for DNA binding. (5) All these enzymes are active at pH about 8.0-8.5 and, in some cases, the catalysis is enhanced in the presence of monovalent ions. (6) As demonstrated for at least 4 inteins, conserved residues of the endonuclease motifs, in particular the two penultimate acidic residues of the dodecapeptide motifs and one Lys residue of block D, are directly involved in the cleavage reaction. (7) The intein cleavage specificity is somewhat relaxed by the tolerance of subtle nucleotide variations in the minimal cleavable sequence.

Besides, some important divergences between the catalytic features can also be observed. (1) While PI-*Sce*I and mycobacterial inteins cleave DNA at 37°C, all other inteins are thermophilic enzymes which are active at temperatures ranging from 50 to 100°C. (2) Inteins appear to bind DNA in different ways. (3) The two DNA strands of the substrate are not systematically cleaved simultaneously since in some cases a nicked intermediate of the reaction can be observed. (4) The intein activity can be dependent on the DNA substrate conformation and on the divalent cation used as a cofactor.

All together, these observations point to the probable variability of the ways to bind and cleave DNA and thus to the difficulty to elaborate a model of DNA cleavage by inteins.

3.1.2. Mechanism of DNA cleavage

Coherently with the study of numerous DOD endonucleases by Lucas et al. (2001), it seems that inteins use different protein-DNA interactions to achieve DNA recognition. Firstly, the DNA binding step by inteins optionally needs an ionic cofactor. As demonstrated for PI-*Pfu*II and PI-*Pab*II allele inteins (Komori et al. 1999a; Saves et al. 2002b), Mg^{2+} cations are required for the substrate binding whereas this cofactor is, in general, only required for the cleavage step. The role of the cations in the DNA recognition by these inteins remains unknown, it probably concerns the active site topology or ionisation state.

Secondly, while some inteins have been found to form only one specific complex with their target DNA (Komori et al. 1999a; Saves et al. 2000c and 2002b), PI-*Sce*I, and probably PI-*Pab*I, interacts with DNA in a biphasic pathway (Gimble & Wang 1996; Wende et al. 1996; Saves et al. 2002b). In few cases, it was shown that the binding process is linked to DNA bending aimed to settle the scissible bonds into the active site. In the different DNA-intein complexes, the DNA is bent to various degrees, probably depending on the fine architecture of the endonuclease active site, without the co-occurring nicking of the DNA substrate (Gimble & Wang 1996; Wende et al. 1996; Komori et al. 1999a).

Moreover, some inteins were shown to interact more strongly with a precise region of the target DNA, for example a strong inhibition of PI-*Sce*I and PI-*Tfu*II by one of the cleavage products argues for a probable strong binding site in the 3' region of the target DNA (Saves et al. 2000c; Wende et al. 1996). In the case of PI-*Sce*I, Gimble and Wang (1996) defined a minimal binding region in the 3' part of the 31 bp recognition site. The intein binds with a high affinity to this region using phosphate backbone and some major groove interactions. According to the proposed model, this primarily binding induces DNA conformational changes, which promote the binding to the cleavage site through interactions in the minor groove and thus an additional DNA distortion required for catalysis. Consistently with this model, the structural analysis and the modelisation of DNA docking in PI-*Sce*I structure show that the globular

endonuclease domain is not sufficiently large to bind the 31 bp long target DNA, even if bent. In fact, the DNA binding involves the splicing domain in a region containing a high concentration of positive clusters aimed to interact with the 3' extension of the target DNA sequence, the DNA bending being probably due to this particular interaction with the splicing domain while the endonuclease core interacts with a shorter DNA sequence extending through the cleavage site (Fig 11) (Duan et al. 1997 ; Grindl et al. 1998; He et al. 1998; Hu et al. 2000). Identically, it appears from docking model between DNA and PI-*Pfu*I monomer that a region adjacent to the splicing domain could also interact with the DNA target sequence (Ichiyanagi et al. 2000). It is thus conceivable that these particular endonucleases may employ DNA interactions with their splicing domain to enhance their specificity by contrast with introns encoded endonucleases, which bear no additional domains (Heath et al. 1997; Jurica et al. 1998).

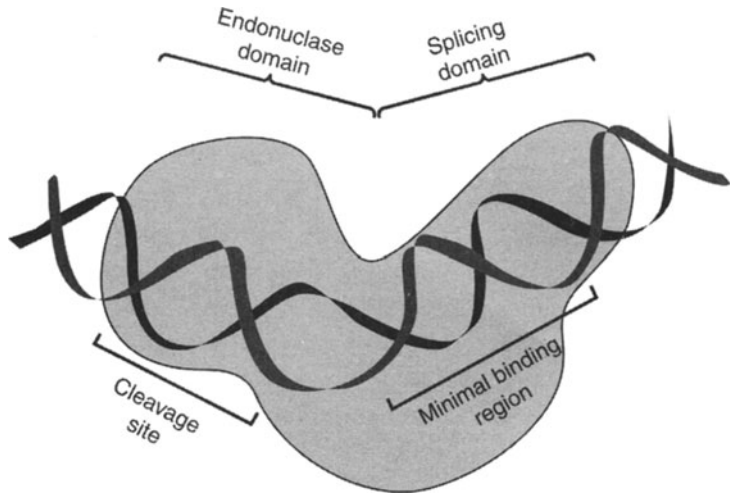

Figure 11. Model of interaction of PI-*Sce*I with its target DNA. Adapted from Duan et al. 1997.

Inteins diverge also in the way to induce the DNA cleavage. If PI-*Sce*I, after DNA binding, is able to induce the double-stranded DNA cleavage without accumulation of a nicked intermediate (Gimble & Wang 1996; Wende et al. 1996), it can not be extrapolated to all inteins. This observation raised the question of whether the cleavage reaction of the double-stranded DNA is performed by only one catalytic centre that simultaneously cleaves the two DNA strands following a single binding event or by two catalytic centres that act in a concerted reaction. The existence of two separate active sites is fairly suggested. In one hand, the 2 DOD motifs, which are spaced by approximately 100 amino acids in the primary structure of most inteins are involved in the endonuclease activity (Gimble & Stephens 1995; Hodges et al. 1992; Komori et al. 1999a). On the other hand, the structural homology between the endonuclease domain of the monomeric PI-*Sce*I (Duan et al. 1997) and the homodimer of I-*Cre*I (Heath et al. 1997; Jurica et al. 1998) is high. This intron endonuclease contains one DOD motif per subunit, each of which being involved in the phosphodiester bond cleavage in one DNA strand. These observations suggest that the 2 DOD motifs of PI-*Sce*I are part of two tightly coupled catalytic centres apparently located at the C-terminal end of the parallel helices constituted by the DOD motifs (Duan et al. 1997).

Christ and collaborators (1999) clearly supported this hypothesis by defining the catalytic residues of PI-*Sce*I, which are implied in the two active centres that specifically cleave each DNA strand of the substrate. Additionally, Schöttler et al. (2000) described the residues responsible for the metal cofactor binding in each site. Coherently, we ascertained the two-step cleavage of the DNA by PI-*Tfu*I and PI-*Pab*I, the two steps consisting in the cleavage of each strand and the top strand cleavage being the limiting step (Thion et al. 2002; Saves et al. 2002b). Moreover, in the case of PI-*Tfu*I, the two distinct active sites are none solely specialised in the cleavage of one DNA strand but also require a specific cofactor, manganese ions being absolutely necessary for the cleavage of linear DNA top strand (Thion et al. 2002). By contrast, PI-*Pab*II, as well as PI-*Tfu*II, PI-*Sce*I and PI-*Pfu*I (Gimble & Thorner 1992; Komori et al. 1999b; Saves et al. 2000c and 2002b), simultaneously process both DNA strands. It is thus conceivable that the double-stranded DNA is cleaved by these enzymes through a similar two-step mechanism, the efficiencies of the two steps being then equivalent as it was shown for PI-*Sce*I (Christ et al. 1999).

3.1.3. Specificity of DNA cleavage activity

In view of the extremely long recognition sites, the specificity of intein DNA cleavage is extremely high and the probability for an intein to cleave a genome is low. However, this specificity is reduced to some extent by the tolerance of some punctual nucleotide substitutions within the recognition sequence. Such permissiveness allows the cleavage of related sequences and thus the propagation of the homing endonuclease coding sequence among genomes.

The study of PI-*Sce*I specificity by a systematic single base change within the 31 bp recognition site showed that mutations at several positions have little or no effect on the cleavage activity whereas only nine nucleotides are of critical importance. Rationally, these nucleotides occurred in the minimal binding region, their substitution eliminating the intein binding, or overlapped the cleavage site (Gimble & Wang 1996). The cumulative effects of the mutations within the target site can lead to a significant decrease in the cleavage efficiency by PI-*Sce*I. However, despite the fact that in some target sequences nearly a quarter of the nucleotides are changed, the substrates can still be cleaved rendering the homing process possible within these divergent sequences (Gimble 2001).

In the same way, it was shown that PI-*Tli*I, PI-*Tfu*II and PI-*Thy*I, all inserted at the pol-c site of DNA polymerase a gene (Fig 2) which is approximately 70 % conserved between these species, are able to cleave the three DNA sequences spanning the pol-c site despite the sequence variability and a 24 bp consensus cleavage site was defined for the three isoschizomers (Fig 12). Within this consensus sequence, only 8 nucleotides can be substituted by any nucleotide or by a pyrimidine, meaning that the specificity of these enzymes is quite high compared to other homing endonucleases and in particular to PI-*Sce*I. The inability of the isoschizomers to cleave the inteinless DNA polymerase gene from *Pyrococcus sp.* KOD, due to point substitutions on the 5' side of the pol-c site, and the sequence comparison of DNA polymerase genes containing or not an intein at the pol-c site (Fig 12) strongly suggests that the absence of intein at this site is due to the inability of this endonuclease family to cleave these target sequences (Saves et al. 2000a).

Shaded results were obtained while considering the specificity and the invading chance of mycobacterial inteins. No intein coding sequence has been found to interrupt the *pps1* genes of *M. leprae* and *M. tuberculosis* at the pps1-c site while the *M. gastri* gene contains an intein endonuclease, named PI-*Mga*I,

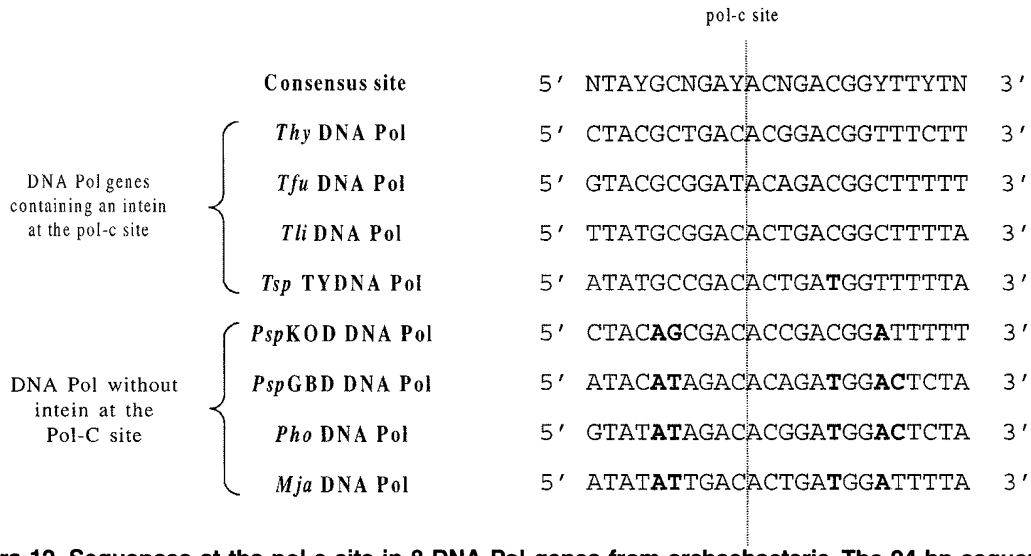

Figure 12. Sequences at the pol-c site in 8 DNA Pol genes from archaebacteria. The 24 bp sequences spanning the pol-c site in DNA pol, with or without an intein at this site, are aligned with the consensus cleavage site. The nucleotides which diverge from this consensus appear in bold. N represents any nucleotide and Y represents pyrimidine. Taken from Saves et al. 2000a.

at this site. This observation can not be only explained by a defective homing event due to the high cleavage specificity of this homing endonuclease since sequence divergences around the pps1-c site hinder the cleavage of the *M. tuberculosis pps1* gene but not that of *M. leprae* gene (Saves et al. 2001b). Similarly, we compared the *rec*A gene sequence of several mycobacterial species and strains containing or not an intein at the recA-b site and it was not possible to explain the intein distribution by target site sequence divergences (Saves et al. 2000b and 2001a). In parallel, Gimble (2001) had the same conclusions while sequencing the inteinless *VMA1* gene from the DH1-1A strain of *S. cerevisiae*.

Therefore, if the absence of intein in some target genes can be the fact of a defective homing event, in the absence of inducement by genome cleavage; it could also be the consequence of a defective genetic exchange between species and strains. Moreover, divergences of the peptide sequence of the extein can be responsible for the absence of an intein; for example in the case of the *Mtu*RecA intein, the peptide sequence of RecA in the majority of mycobacterial species harbour a Val residue at the position of the first C-extein Cys residue found in *M. tuberculosis* RecA, this residue hindering the protein splicing of an intein at this site.

3.2. Control and application of intein endonuclease activity

Since the discovery of inteins, there has been a particular interest for these uncommonly specific endonucleases. Effectively, at the time of genomic research, the need of rare-cutting enzymes focused in the engineering of enzymes able to recognise and cleave long DNA sequences. Thus, the ability of homing endonucleases to introduce double-strand breaks in extremely precise regions of genomes makes them useful tools for analysing and manipulating genomes for mapping, gene cloning, targeting and for studying double-strand-break repair (Belfort & Roberts 1997).

From structural data, it can be envisaged that single amino acid residue substitution at the DNA-intein interface, in particular in few β-strands, could lead to an alteration of base preference within the target site. Hence, it would be possible to adapt the intein sequence to a modified target site and the important variety of such enzymes would result in a profusion of potential cleavable target sites. However, few results were obtained to date in this particular field (Chevalier & Stoddard 2001).

4. INTEINS FUNCTION AND EVOLUTION

Nowadays, little is known about intein function and it is admitted that no physiological role can be attributed to inteins which are probably nothing more than opportunistic selfish invasive elements among genome. In 1991 and 1994, Davis et al. successively found inteins in RecA recombinase of *M. tuberculosis* and *M. leprae*, respectively. The occurrence of inteins in the two obligate pathogens suggested that these inteins may play a role in mycobacterial functions related to pathogenesis or virulence ; alternatively, these inteins may be responsible for the low efficiency of homologous recombination in these slow growing mycobacteria. However, the systematic research of intein coding sequences in *rec*A from a large number of mycobacterial species and strains allowed the identification of inteins in a vast range of species and thus to definitely rule out any phylogenetic or physiological relevance of RecA inteins (Saves et al. 2000b). Moreover, it was demonstrated that the recombinase activity is not regulated by the protein splicing process (Davis et al. 1994; Frischkorn et al. 1998). A similar approach excluded any role of the intein invading the mycobacterial Pps1 protein (Saves et al. 2001b and 2002a).

As stated before, a set of unoccupied sites within host genes are potential target sites for intein invasion, for example the *M. leprae pps1* gene while cleaved by PI-*Mga*I intein at the pps1-c insertion site is free of intein. Two hypotheses are reminiscent of this observation.

In the first one, inteins would have been acquired by an ancestral strain and were lost during the evolution. In the second one, inteins are individually acquired and a lot of sites have yet to be invaded. The fact that these invading elements do not apparently benefit their host favours the first hypothesis, which is also coherent with the loss of the endonuclease activity. By contrast, the difference in DNA G/C content and codon usage between the intein coding sequences and their host genes indicates that some intein genes have recently been transmitted between organisms (Fsihi et al. 1996; Liu & Hu 1997). In the same way, the homology between mycobacterial inteins is not correlated with the phylogeny of invaded species (Saves et al. 2000b) and more generally the homology between orthologous host proteins can not be compared with the homology between the invading inteins. Indeed, highly conserved proteins can contain inteins which are non homologous and located at different sites, which suggests that the inteins have been acquired at different stages of evolution.

In a structural point of view, the bipartite domain of PI-*Sce*I and PI-*Pfu*I inteins, paralleled by the independence of the two activities of bifunctional inteins and by the existence of inteins lacking the endonuclease domain and of autonomous homing endonucleases, suggests that intein coding sequences are composite genes. Such genes would result from the invasion of an endonuclease ORF into pre-existing genes, which encode splicing elements (Duan et al. 1997 ; Mueller et al. 1993), the symbiotic association benefiting both entities : the proteolytic activity ensures that the intein is safely removed from the host protein and the endonuclease activity assures the dissemination of the splicing element. Since the splicing domain of inteins appears to be involved in the endonuclease specificity, the coevolution

of the two domains, after the fusion of the genes coding for these domains, may have led to the generation of specific DNA contacts in the splicing domain (Grindl et al. 1998).

While both activities of protein splicing and DNA cleavage are necessary for intein propagation by homing, only the protein splicing activity is essential for the intein maintenance in host genes. That implies that the selection pressure for evolution is different between the two domains, which is coherent with a more rapid degeneration of the endonuclease domain than the splicing domain (Okuda et al. 2003). Effectively, alterations of the endonuclease domain have been described whereas splicing domains remain intact, mutations altering the capacity of splicing being lethal. Indeed, at least five cases of non-cutting enzymes have been reported (Table 2). In these cases, deletions in the endonuclease domain of these inteins and sequence divergences within the endonuclease motifs could easily explain the absence of activity (Gimble 2001; Saves et al. 2002b ; Okuda et al. 2003). In the same way, some other inteins display mutations within the DOD motifs that probably inactivate DNA cleavage but leave the splicing function native (Inbase).

All these cases illustrate the loss of the endonuclease activity during the evolution of inteins. It has been recently shown that horizontal transmission is critical for long-term persistence of selfish genes with little or no benefit to the host organism such as homing endonucleases (Goddard & Burt 1999). Since persistence over long evolutionary time scales probably requires cyclical gain and loss of the homing endonuclease genes, finding within a small group of closely related species some with functional inteins, others with non functional ones and species without inteins, is precisely what one would expect, reflecting the dynamic evolutionary biology of these genes (Goddard & Burt 1999).

5. CONCLUSIONS AND PERSPECTIVES

Since the discovery of inteins and protein splicing in 1990, a significant way has been done toward the comprehension of the protein splicing mechanism and the homing endonuclease activity ; even though the intein biological function is still unknown. These invading elements play no apparent role in the host functions or in the host protein regulation and they probably are selfish DNA elements invading important host genes to ensure their survival in infected genomes.

Nevertheless, the interest in studying these particular proteins appeared evident while new inteins were discovered ubiquitously in a vast range of host proteins. The attractiveness is double since the inteins can be scrutinised in a biotechnological or a pharmacological point of view. Actually, the elucidation of the protein splicing pathway has already allowed the generation of multiple powerful protein engineering applications and the very specific endonuclease activity offers attractive perspectives for creating useful tools for genomic studies. In parallel, the high frequency of inteins in mycobacterial species and the location specificity of *Mycobacterium tuberculosis* inteins could allow to combat mycobacterial infections and to diagnose tuberculosis.

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