Biofuel and Biorefinery Technologies 3

Vijai Kumar Gupta *Editor*

Microbial Enzymes in Bioconversions of Biomass

Biofuel and Biorefinery Technologies

Volume 3

Series editors

Vijai Kumar Gupta, Molecular Glycobiotechnology Group, Department of Biochemistry, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland
Maria G. Tuohy, Molecular Glycobiotechnology Group, Department of Biochemistry, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

More information about this series at http://www.springer.com/series/11833

Vijai Kumar Gupta
Editor

Microbial Enzymes in Bioconversions of Biomass

Editor
Vijai Kumar Gupta
Department of Biochemistry, School of Natural Sciences
National University of Ireland Galway
Galway
Ireland

ISSN 2363-7609 ISSN 2363-7617 (electronic)
Biofuel and Biorefinery Technologies
ISBN 978-3-319-43677-7 ISBN 978-3-319-43679-1 (eBook)
DOI 10.1007/978-3-319-43679-1

Library of Congress Control Number: 2016947366

© Springer International Publishing Switzerland 2016
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made.

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Preface

This book summarizes the information on different biomass converting enzymes and their potential use in the bioconversion of biomass into simple sugar to generate bioenergy and other value added co-/by products.

Biofuel produced from agricultural crops, e.g. cereals, maize, sugarcane, sugar beet, and sweet sorghum, is referred to as first generation biofuels while that produced from lignocellulosic materials is referred to as second generation biofuels. The global production of first and second generation biofuels is dependent upon the lignocellulosic materials available for bioconversion.

Microbes have been established as important mediators in the production of biofuels and other valuable biorefinery products. It is prevalent that microbes have been playing a key role by producing key enzymes for bioconversion of various biomasses for biorefinery applications. This selection of microbes is based upon their ability to selectively or simultaneously degrade lignocellulosic materials, the high redox potential of their enzymes, their engineering capabilities and/or their thermostability. The key to their degradative capabilities is the extracellular enzymes they secrete. These microorganisms are capable of producing an array of enzymes such as 1) Cellulase systems, comprised of endo-1,4-β-glucanase, cellobiohydrolase and β-glucosidases; 2) Hemicellulases include endo-1,4-β-xylanases, β-xylosidases, endo-1,4-β-mannanase and β-mannosidase; 3) Lignin degrading enzymes comprising laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase. There are also accessory enzymes which aid the hemicellulases in their degradation of hemicellulose polymers through the cleavage of side chain residues; these enzymes include α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, ferulic acid esterase and β-galactosidase.

This book summarizes the information on different biomass converting enzymes and their potential use in the bioconversion of biomass into simple sugar to generate bioenergy and other value added co-/by products. The chapters have been contributed by the experts of the area across the globe and these chapters offer clear and concise information on both standard and new technologies and will serve as an

invaluable reference for undergraduates, post-graduates, researchers and practitioners studying and working in the field of microbial enzymes for biofuel and biorefinery applications.

Galway, Ireland Vijai Kumar Gupta

Contents

1 Microbial Enzymes for Conversion of Biomass to Bioenergy 1
M.P. Raghavendra, S. Chandra Nayaka and Vijai Kumar Gupta

Part I Cellulases

2 Cellobiohydrolases: Role, Mechanism, and Recent Developments 29
Neelamegam Annamalai, Mayavan Veeramuthu Rajeswari and Nallusamy Sivakumar

3 Endo-1,4-β-glucanases: Role, Applications and Recent Developments 37
Neelamegam Annamalai, Mayavan Veeramuthu Rajeswari and Thangavel Balasubramanian

4 The Role and Applications of β-Glucosidases in Biomass Degradation and Bioconversion 47
Hanlin Ouyang and Feng Xu

Part II Hemicellulases

5 Role and Applications of Feruloyl Esterases in Biomass Bioconversion 79
Constantinos Katsimpouras, Io Antonopoulou, Paul Christakopoulos and Evangelos Topakas

6 Endo-β-1,4-xylanase: An Overview of Recent Developments 125
Alexandre Gomes Rodrigues

7 Microbial Xylanases: Sources, Types, and Their Applications 151
Hesham Ali El Enshasy, Subeesh Kunhi Kandiyil, Roslinda Malek and Nor Zalina Othman

8 Mannanase 215
Suttipun Keawsompong

9 The Role and Applications of Xyloglucan Hydrolase in Biomass Degradation/Bioconversion 231
M. Saritha, Anju Arora, Jairam Choudhary, Vijaya Rani, Surender Singh, Anamika Sharma, Shalley Sharma and Lata Nain

Part III Lignocellulose Oxidureductases

10 Role of Mushroom Mn-Oxidizing Peroxidases in Biomass Conversion 251
Mirjana Stajić, Jelena Vukojević, Ivan Milovanović, Jasmina Ćilerdžić and Aleksandar Knežević

11 Role and Application of Versatile Peroxidase (VP) for Utilizing Lignocellulose in Biorefineries 271
Nadine Busse and Peter Czermak

12 Fungal Aryl-Alcohol Oxidase in Lignocellulose Degradation and Bioconversion 301
Juan Carro, Ana Serrano, Patricia Ferreira and Angel T. Martínez

13 Monosaccharide Oxygenase 323
Avnish Kumar, Monika Asthana, Hirawati Deval and Sarika Amdekar

Chapter 1
Microbial Enzymes for Conversion of Biomass to Bioenergy

M.P. Raghavendra, S. Chandra Nayaka and Vijai Kumar Gupta

Abstract Microbial enzymes are capable of degrading a wide range of complex substrates including carbohydrates into more useful energy source. The simple sugars then can be converted into ethanol or other liquid biofuels by a large group of fermentative microbes. Even though cellulose serves as an abundant source of carbon and energy in the ecosystem, its exploitation as a source of biofuel is hindered due to lack of effective microbial systems to break it down, including other carbohydrates to simple sugars leading to more production of biofuels. If these materials could be exploited, they would represent a massive new energy resource for biofuel production. In continuous search for alternative energy sources, it is now proven that electricity can be produced directly from the degradation of organic matter in a microbial fuel cell and fermentation of lignocellulosic biomass to ethanol, which is an attractive route to fuels that supplements the fossil fuels. Studies have revealed that special group enzymes known as feruloyl esterases produced by microorganisms are capable of breaking apart key links between the polymers and helps in effective degradation of plant materials. This review covers various known microbial approaches to convert different carbon sources to simple soluble sugars en route to production of biofuels. The importance of the biofuel in future is highlighted by the Renewable Fuel Standard of the United States Energy Independence and Security Act (EISA) of 2007, which mandates that 36 billion gallons of biofuels are to be produced annually by 2022, of which 16 billion gallons are expected to come from cellulosic feed stocks. It is obvious fact that microor-

M.P. Raghavendra
Postgraduate Department of Microbiology, Maharani's Science College for Women,
JLB Road, Mysore, Karnataka 570 005, India

S.C. Nayaka (✉)
Department of Studies in Biotechnology, University of Mysore, Mysore,
Karnataka 570 005, India
e-mail: moonnayak@gmail.com

V.K. Gupta
Department of Biochemistry, School of Natural Sciences,
National University of Ireland, Galway, Ireland

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_1

ganisms and its array of enzymes need to be effectively screened, identified and employed in developing effective strategies for converting biomass to biofuel.

1.1 Introduction

Due to over reliance on petrochemicals, rising oil prices, pollution associated with its usage, world is looking forward for alternative renewable resources of energy from different sources. Biofuel obtained from carbon sources are gaining momentum as a source of biofuel but the concern over production of ethanol from food crops is creating a imbalance and there is a search for a resources, which are not being used as food and should be available plenty in the ecosystem so that the problems associated with the starchy material can be addressed. In this connection cellulosic feed stock is considered to be excellent source for ethanol production, but there are problems associated with degradation of cellulose also due to its recalcitrance to biodegradation at least at the early stages of degradation. Liquid fuel derived from biomass feedstock has been considered as a low-carbon substitute for fossil hydrocarbons in vehicles, but many of the existing approaches are encountering significant challenges.

1.2 Importance of Biofuel Research

Search for new energy supplies is an important agenda for several countries in the coming years. They are developing new energy infrastructure capable of meeting growing demands for electric power and transportation fuels. Even unrest in oil producing countries will have great impact on national security and economic strength of several oil dependent countries. India in particular currently imports 80 % of its oils and it is expected to increase to 91 % by 2020. This overreliance on oil import is the greatest burden on Indian economy and its security. On the other hand, India is an agrarian country and known for its biodiversity that can be exploited to convert biomass to biofuel. Biofuels employ recycling of agricultural byproducts and dedicated energy crops, which offer opportunities for mitigation of greenhouse gas emission as growing these leads to C sequestration through photosynthesis.

Various plants and plant-derived materials are used for biofuels manufacturing including grains (1st generation) and lignocellulosic biomass (2nd generation). The second generation of biofuels is more important as they are based on the cheap and abundant lignocellulosic biomass and do not compete with food crops. Wheat straw and bagasse are considered that best example, which are inexpensive and widely available ligonocellulosic resources containing 75–80 % polysaccharides (cellulose and hemicelluloses). The monosaccharides glucose and xylose obtained from hydrolysis of these can be used as substrate for production of industrially important

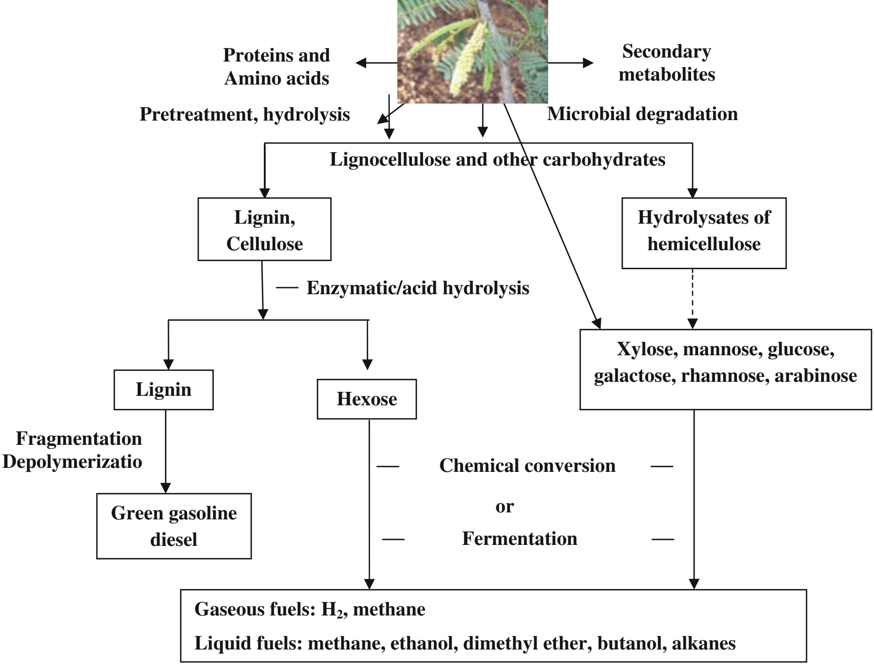


Fig. 1.1 Conversion of carbohydrate biomass into biofuel

products (Fig. 1.1). These products will have more potential economical, environmental, and strategic advantages over traditional fossil-based products.

The era of advanced biofuels—cellulosic ethanol, biomass-based diesel, biobutanol, bio-oil, green gasoline, and bio-based jet fuel—is also drawing nearer and nearer. According to the International Energy Agency, biofuels have the potential to meet more than a quarter of world demand for transportation fuels by 2050. Bioethanol is by far the most widely used biofuel for transportation worldwide (Mohanram et al. 2013). It has been estimated that widespread use of biofuels, biopower, and other bio-based products has the potential to conserve 1.26 billion barrels of oil, 58 million tons of coal, and 682 million tons of carbon dioxide from 2020 to 2030.

1.3 Lignocelluloses and Its Structural Complexity

The cell walls of plants represent an enormous nutrient source, yet highly variable in terms of amount, diversity, and botanical source. Plant carbohydrates are chemically and structurally highly complex, and are arranged in a three-dimensional network that has evolved to be intrinsically resistant to enzymatic

breakdown (Fontes and Gilbert 2010). Thus high molecular weight crystalline cellulose microfibrils are intertwinned with hemicelluloses and pectins, which are a whole range of homo- and heteropolysaccharides composed of dozens of different monosaccharide units linked in a multitude of ways to resist degradation. Ester substituents or non-carbohydrate, polymers, such as lignin, proteins, cutin, and suberin, add a further layer of complexity. As a result, a single vegetable contains hundreds of different bonds that need to be cleaved in order to unlock the assimilable carbon of the cell wall constituents.

In general cell walls are intricate assemblages of celluloses, hemicelluloses (i.e., xyloglucans, arabinoxylans, and glucomannans), pectins (i.e., homogalacturonan, rhamnogalacturonan I and II, and xylogalacturonans), lignins, and proteoglycans (e.g., arabinogalactan-proteins, extensins, and proline-rich proteins). Most mass in the plant cell wall is in the form of polysaccharides (cellulose and hemicelluloses). The next most abundant polymer is lignin, which is composed predominantly of phenylpropane building blocks.

Among these different carbon sources lignocellulosic biomass is the most abundant, low-cost and unexploited biomass that is considered as a source for industrial production of fuel ethanol. Considering its availability over the last decades, researchers have been devoted to converting lignocellulosic materials to bioethanol. Lignocellulosics are composed of heterogeneous complex of carbohydrate polymers (cellulose, hemicelluloses, and lignin). Majority of plant biomass is locked up in 5- and 6-carbon sugars, comprised of mainly cellulose (a glucose homopolymer, 40–60 % by wt.); less so, hemicelluloses (a sugar heteropolymer, 20–40 % by wt.); and least of all lignin (a complex aromatic polymer, 10–30 % by wt). The major component cellulose, which is organized into microfibrils, each containing up to 36 glucan chains having thousands of glucose residues linked by β-(1,4) glycosidic bonds, is largely responsible for the plant cell wall's mechanical strength. Hemicelluloses are built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. These include β-glucan, xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan, etc. Hardwood contains mainly xylans, while in softwood glucomannans are most common. Both the cellulose and hemicelluloses can be broken down enzymatically into the component sugars which may be then fermented to ethanol.

Cellulose can be classified into two types based on the arrangement of chains. In type I cellulose chains are arranged in parallel direction of the long axis of the microfibril, whereas in type II chains are in antiparrellel position. Among these, type I is considered to be dominant that can be converted to type II by alkali treatment. Studies show that cellulose I actually contain two distinct crystal lattices: cellulose Iα, with triclinic symmetry, and cellulose Iβ, with monoclinic symmetry. These two forms differ in the organization of their intermolecular hydrogen bonds and lead to further complexity of cellulose structure. The percentage crystallinity of cellulose varies from very low to almost 100 % and it depends on cellulose source. However, it is clear that cellulose microfibrils are not uniformly crystalline; imperfections in packing or mechanical damage result in a proportion of substrate

in which the lattice is disordered or paracrystalline (Ranjan 2014). Hence, microbes have to produce cellulases with range of structures, activities, and stabilities.

1.4 Enzymatic Hydrolysis of Lignocelluloses

Even though lignocellulose is considered as the most abundant and renewable natural resource available to man throughout the world, massive technological difficulties must be overcome to exploit this for commercial purpose. In order that cellulose hydrolysis becomes economically feasible, it is important to identify methods that increase enzyme effectiveness and overcome barriers of enzymatic hydrolysis (Kristensen et al. 2007). Major factors that influence enzymatic conversion of lignocelluloses to fermentable sugars include accessible surface area of lignocelluloses (Mansfield et al. 1998), enzyme loading, and presence of inhibitors (Eriksson et al. 2002).

Pretreatment is a necessary step generally carried out to produce ethanol from lignocellulosic materials by dilute sulfuric acid or enzymatically. The polysaccharide chains, being tightly packed, require additional factors that would make the substrate more accessible, as has been suggested since the 1950s (Horn et al. 2012). The enzymatic hydrolysis is generally carried out by cellulolytic enzymes. Without pretreatment, conversion is tedious and very slow. Enzymatic hydrolysis is considered to be effective because it happens in mild hydrolysis conditions and high yields of hydrolysis, but it results in accumulation of inhibitory byproducts, costly, and hydrolysis require long time and also results in product inhibition during hydrolysis. The biological treatments can be achieved by enzymes of fungi and actinomycetes, which results in delignification and reduction in degree of polymerization of hemicellulose and cellulose.

By the use of enzymatic hydrolysis, pure cellulose can be degraded to soluble sugars, which can be fermented to form ethanol, butanol, acetone, single cell protein, methane, and many other products. Enzymatic saccharification remains one of the most costly steps in conversion of cellulosic biomass to ethanol and cellulase preparations dedicated for bioethanol industry are hardly available. It has been estimated that the greatest returns in cost savings will be realized by improving conversions of biomass to sugars, increasing hydrolysis yields, reducing enzyme loadings, and eliminating or reducing pretreatment (Lynd et al. 2008). A broader suite of enzymes is required for hydrolysis of cellulose and hemicelluloses to fermentable sugars (McMillan et al. 2011). Thus enzymatic hydrolysis can be effectively carried out if a mixture of different cellulolytic and accessory enzymes is used.

Enzyme hydrolysis is generally considered the most sustainable technology for saccharification. Enzymatic hydrolysis of cellulose consists of three steps viz.,

1. Adsorption of cellulase enzymes onto the surface of the cellulose,
2. Biodegradation of cellulose to fermentable sugars, and
3. Desorption of cellulase (Sun and Cheng 2002).

Different strategies are also available for enzymatic hydrolysis and fermentation of lignocelluloses such as separate enzymatic hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), nonisothermal saccharification and fermentation (NSSF), simultaneous saccarification, and co-fermentation (SSCF) and consolidated bioprocessing (CBP) (Taherzadeh and Karimi 2007). Among different strategies CBP is considered to posses outstanding potential, ethanol together with all of the required enzymes is produced by single microbial communities is also referred to as direct microbial conversion (DMC). It may be involved single or multiple cultures of microorganism to directly convert cellulose to ethanol in a single bioreactor (Wyman 2007).

The classical model for degradation of cellulose to glucose involves the cooperative action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and betaglucosidases (EC 3.2.1.21). Endocellulases hydrolyze internal glycosidic linkages in a random fashion, which results in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. Exocellulases hydrolyze cellulose chains by removing mainly cellobiose either from the reducing or the nonreducing ends, which lead to a rapid release of reducing sugars but little change in polymer length. Endocellulases and exocellulases act synergistically on cellulose to produce cello-oligosaccharides and cellobiose, which are then cleaved by beta-glucosidase to glucose (Kumar et al. 2008).

Hydrolysis of hemicelluloses involve enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endohemicellulases, and others, the concerted action of which hydrolyze glycosidic bonds, ester bonds, and remove the chain's substituents or side chains (Sweeney and Xu 2012). These include endo-1, 4-β-xylanase, β,-xylosidase, β-mannanase, β mannosidase α-glucuronidase, α- L-arabino furanosidase, acetylxylan esterase and other enzymes.

Glycohydrolases viz., cellulase and hemicellulase enzymes are involved in hydrolysis of cellulose and hemicelluloses respectively. These are known to posses 15 protein families with other subfamilies. Hydrolysis of cellulose begins with adsorption of it on to the surface of the enzyme then its biodegradation to glucose with the synergistic action of three major classes of enzymes: endoglucanases, exoglucanases and β-glucosidases which are generally represented as cellulase or cellulolytic enzymes.

All these enzymes are available in the cellulosome complexes, which are intricate multienzyme machines produced by many cellulolytic microorganisms and are designed for efficient degradation of plant cell wall polysaccharides. In the past several years, selected studies have addressed the conformational flexibility of the cellulosome and its possible significance to biomass degradation. Early on, it was

evident that isolated cellulosomes could attain an overall tight or loose conformation (Mayer et al. 1987; Lamed et al. 1983a). The cellulolytic enzymes are either secreted into the substrate or attached to the cell wall of the microorganism. The former system is called noncomplexed and the latter is complexed cellulase system. Noncomplexed cellulase systems are mostly found in filamentous fungi and actinomycete bacteria, because they can penetrate the lignocellulosic material with their hyphal extensions. The enzymes of noncomplexed cellulase systems are then just released into the substrate and the free enzymes start hydrolyzing the cellulose. The glucose and cellodextrins of a length of maximal four glucose molecules are taken up by the microorganism and either used directly or cleaved further via intracellular hydrolases. Organisms that produce noncomplexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested (Lynd et al. 2002).

Cellulosome contains cellulose-binding domains, also called carbohydrate binding modules (CBM), which bind to the cellulosic material (Fontes and Gilbert 2010; Sweeney and Xu 2012; Shoseyov et al. 2006). CBMs can also be found in noncomplexed cellulase systems, where they are especially important for binding and processing of exoglucanases. The main function of the cellulosome is to hold the microorganism tight to the cellulose, and therefore reduce diffusion distances and losses and to arrange the different cellulolytic enzymes in a favorable way. More recently (Garcia-Alvarez et al. 2013), ultrastructural studies of a homogeneous mini-cellulosome containing three cohesin modules attached to three matching cellulases suggested that the flexibility of the linkers connecting consecutive cohesin modules could control structural transitions and thus regulate substrate recognition and degradation (Vazana et al. 2013).

One of the most potent cellulose degrading microorganisms is the well-studied bacterium *Clostridium thermocellum*. This anaerobic thermophilic cellulolytic bacterium secretes a multienzymatic complex called the cellulosome, first discovered in 1983 (Lamed et al. 1983a, b). Later there are several reports on cellulosomal oraganisms which possess an array of cellulosomal architectures (Belaich et al. 1997; Gal et al. 1997; Ding et al. 1999, 2000; Ohara et al. 2000; Rincon et al. 2003, 2004, 2010; Xu et al. 2003, 2004; Bayer et al. 2004, 2013; Dassa et al. 2012).

1.5 Microbial Enzymes for Degradation of Cellulose

The isolation and characterization of novel glycoside hydrolases from eubacteria are now becoming widely exploited. There are several reasons for these shifts, for one, bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes. Secondly, bacterial glycoside hydrolases are often more complex and are often expressed in multienzyme complexes providing increased function and synergy. Most importantly, bacteria inhabit a wide variety of

environmental and industrial niches, which produce cellulolytic strains that are extremely resistant to environmental stresses. These include strains that are thermophilic or psychrophilic, alkaliphilic, or acidophilic, and, strains that are halophilic. Not only can these strains survive the harsh conditions found in the bioconversion process, but also they can produce enzymes that are stable under extreme conditions which may be present in the bioconversion process and this may increase rates of enzymatic hydrolysis, fermentation, and, product recovery (Maki et al. 2009). Along with these due to availability of novel techniques for gene transfer, microbes can be genetically modified easily to suit the need of the industrial processes and also the different substrates employed in the fermentation.

Considering these, decomposition of lignocelluloses by enzymes produced by complex microbial communities represents a promising alternative for biomass conversion. In particular, thermophilic consortia are a potential source of enzymes adapted to adverse reaction conditions (Wongwilaiwalina et al. 2010; Gladden et al. 2011). Members of the genus *Caldicellulosiruptor* can grow on and degrade biomass containing high lignin content as well as highly crystalline cellulose without conventional pretreatment (Blumer-Schuette et al. 2008; Yang et al. 2009) and are also known to ferment all primary C5 and C6 sugars from plant biomass and are the most thermophilic cellulolytic bacteria known, with growth temperature optima between 78 and 80 °C raising the possibility of further economic improvement of biofuel production from plant biomass by reducing or eliminating the pretreatment step (Hamilton-Brehm et al. 2010).

In comparison with the anaerobic thermophilic bacteria few aerobic have been described to produce cellulases and xylanses. The aerobic thermophiles *R. marinus* produces a highly thermostable cellulose (Cel 2A), and three glycoside hydrolases belonging to family GH10 of the carbohydrate active enzyme (CAZy) database (http://www.cazy.org) (Cantarel et al. 2009). *Aquifex aeolicus*, isolated in the Aeolic Islands in Sicily (Italy), represents one of the most thermophilic bacteria since its growth temperature can reach 95 °C (Deckert et al. 1998).

Bacteria belonging to *Clostridium*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetovibrio*, *Microbispora,* and *Streptomyces* can produce cellulases (Bisaria 1991). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Similarly, other bacterial strains have the ability to produce cellulase complexes aerobically as well as anaerobically. Cellulase producing bacterial strains of *Rhodospirillum rubrum*, *Cellulomonas fimi*, *Clostridium stercorarium*, *Bacillus polymyxa*, *Pyrococcus furiosus*, *Acidothermus cellulolyticus*, and *Saccharophagus degradans* have been extensively reviewed (Kumar et al. 2008; Weber et al. 2001; Kato et al. 2005; Taylor et al. 2006). An extracellular alkaline carboxy methyl cellulase (CMCase) from *Bacillus subtilis* strain AS3 has been purified and characterized by Deka et al. (2013) for utilization of cellulosic biomass. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce

cellulases with high specific activity, they do not produce high enzyme titres (Lynd et al. 2002).

Apart from the cellulolytic fungus *T. reesei*, many other fungi produce cellulases and degrade treated cellulosic material or soluble cellulose derivatives such as carboxymethylcellulose. However, they are not very effective on crystalline cellulosic substrates. Mesophilic strains producing cellulases like *Fusarium oxysporum*, *Piptoporus betulinus*, *Penicillium echinulatum*, *P. purpurogenum*, *Aspergillus niger* and *A. fumigatus* have also been reported (Martins et al. 2008; Valaskova and Baldrian 2006). The cellulases from *Aspergillus* usually have high β-glucosidase activity but lower endoglucanase levels, whereas, *Trichoderma* has high endo and exoglucanase components with lower β-glucosidase levels, and hence has limited efficiency in cellulose hydrolysis. Thermophillic microorganisms such as *Sporotrichum thermophile*, *Scytalidium thermophilum*, *Clostridium straminisolvens* and *Thermomonospora curvata* also produce the cellulase complex and can degrade native cellulose (Kato et al. 2005; Kaur et al. 2004).

Even human gut microbiota encodes a huge diversity of enzymes for the digestion of all components of plant cell wall polysaccharides including cellulose. Turnbaugh et al. (2010) have shown that the distal gut microbiota of humans also encodes dockerin-containing cellulolytic enzymes that indicate the presence of cellulosomes (Fontes and Gilbert 2010; Bayer et al. 2004). The presence of dockerin-containing proteins is also observed in the oral, nasal and vaginal samples (Cantarel et al. 2012). In nondigestive sites, however, these dockerin domains may have another role than attaching cellulases to form a cellulosome.

The interaction between loads of bacteria in gut with other bacteria has lead to development of novel CAZymes. The recent outcome of the research by Hehemann et al. (2010) proved the novel CAZymes added due to flexible microbial interactions. They characterized the first porphyranases from a member of the marine Bacteroidetes, *Zobellia galactanivorans*, active on the sulphated polysaccharide porphyran from marine red algae of the genus *Porphyra*. Furthermore, they showed that genes coding for these porphyranases, agarases and associated proteins have been transferred to the gut bacterium *Bacteroides plebeius* isolated from Japanese individuals. Their comparative gut metagenome analyses showed that porphyranases and agarases are frequent in the Japanese population and that they are absent in metagenome data from North American individuals.

White Rot Fungi (WRF) are microorganisms of great interest that secrete complex suites of nonspecific extracellular ligninolytic enzymes, i.e., lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2) to biodegrade lignin or digest substrates required for their proliferation and growth. β-glucosidase is among the suite of enzymes produced by WRF such as *Pleurotus ostreatus*, *Auricularia auricula*, *Polyporus squamosus*, *Trametes versicolor*, *Lentinula edodes*, and *Grifola frondosa* to biodegrade plant biomass. The β-glucosidase family (EC 3.2.1.21) is a wide-spread group of enzymes that catalyze

the hydrolysis of a broad variety of glycosides (Berrin et al. 2003). While some organisms secrete either endoglucanase or β-glucosidase, in other organisms; β-glucosidase is either lacking or produced in insufficient quantities (Kumar et al. 2008). When β-glucosidase secretion is low, cellobiose accumulates instead of glucose. Cellobiose accumulation acts as a feedback-inhibitor of cellulose depolymerization by endo- and exoglucanases (Howell and Stuck 1975; Morais et al. 2010), which is a critical factor in the industrial scale conversion of cellulose to glucose (Cai et al. 1997). This situation can be alleviated during industrial scale conversion of cellulosic biomass by exogenous incorporation of β-glucosidase enzyme.

Among known cellulolytic microorganisms, cellulases of *Trichoderma reesei* or *T. viride* are well studied and characterized. Cellulase of *T. reesei* comprises of two major cellobiohydrolases, (CBHI and CBHII), two major endoglucanases, (EGI and EGII) and at least two low molecular weight endoglucanases, EGIII, and EGV. The mixture of these enzymes is capable of solubilizing native cellulose. Many other aerobic filamentous fungi, including *Agaricus bisporus*, *Humicola* spp., *Irpex lacteus,* and *Sclerotium roysii*, also use similar cellulase systems.

1.6 Novel Enzymes for Cellulose Degradation

In addition to enzymes that act directly on polysaccharides, lignocelluloses degrading microorganisms are also found to secrete several proteins, which modify cellulose and enhance its hydrolysis by cellulase. These include the no-enzymatic proteins such as 'expansins' and their fungal and bacterial homologs, the 'swollenins' expressed by *T. reesei* with sequence homology to plant expansins and the similar 'loosenins' produced by *Bjerkandera adusta*. These interact with cellulosic substrates resulting in expansion, slippage, or lengthening of the components, thereby facilitating the access of glycosyl hydrolases (Sweeney and Xu 2012; Banerjee et al. 2010). Recently, cellulose-induced proteins (CIP1 and CIP2) have been found in a transcriptional analysis of *T. reesei*, with some synergistic activity with both GH61 and swollenin and are thought to play a role in the cleavage of hemicellulose–lignin crosslinks (Sweeney and Xu 2012; Scott et al. 2009). There are various hydrolytic enzymes and proteins produced by fungi and bacteria, which help to facilitate the lignocellulose bioconversion process.

Along with the catalytic core, many of the enzymes also possess noncatalytic domains including CBM and dockerins which, respectively, anchor the enzyme to targeted substrate or onto scaffolding to assemble a cellulosome and disrupt the crystalline cellulose microfibrils (Kumar et al. 2008; Sweeney and Xu 2012). Recently, a new type of bacterial proteins currently classified as CBM33 (family 33 CBM) in the CAZy database and fungal proteins classified as GH61 (family 61

glycoside hydrolases) that catalyze oxidative cleavage of polysaccharides have been discovered (Horn et al. 2012; Sweeney and Xu 2012). These copper dependent monooxygenases act on the surfaces of insoluble substrates where they introduce chain breaks in the polysaccharide chains thereby increasing substrate accessibility and potentiating hydrolytic enzymes (Horn et al. 2012). Recent data obtained from CBM33 and GH61 families revealed that these enzymes disrupt the more recalcitrant portions of the cellulose and make the substrate more accessible to digestion by cellulases. The emerging research on disruptive proteins from the CBM33 and GH61 is exciting because these proteins appear to have novel mechanism to attack cellulose and synergize with cellulase system (Kostylev and Wilson 2012). Studies related to synergistic activity with high throughput advanced screening will yield efficient method to degrade cellulose with these enzymes.

Inspired by the findings for CBP21 and earlier indications that certain CBM33s may act synergistically with cellulases (Moser et al. 2008) studies were conducted to prove if certain CBM33s act on cellulose like CBP21 acts on chitin. In 2011 it was then shown that CelS2, a CBM33 protein from *Streptomyces coelicolor*, indeed cleaves cellulose, producing aldonic acids (Forsberg et al. 2011). Like CBP21, the activity of CelS2 depended on the presence of divalent metal ions as shown by the inhibitory effect of EDTA and the ability to restore activity by adding divalent metal ions. Again like CBP21, purified CelS2 was active without the addition of metals, probably due to high affinity binding. Both the initial study on CBP21 and the study on CelS2 concluded that the enzymes could use several divalent metal ions, but the most recent work clearly shows that these enzymes in fact are copper dependent monooxygenases.

At the same time, a series of elegant studies showed that GH61s are functionally very similar to CBM33s (Quinlan et al. 2011; Beeson et al. 2012; Phillips et al. 2011; Westereng et al. 2011; Li et al. 2012). Quinlan et al. (2011) described the crystal structure of a GH61 from *Thermoascus aurantiacus* (TaGH61A) and showed that this protein catalyzes oxidative cleavage of cellulose in the presence of an external electron donor such as gallic acid. These authors were the first to convincingly show that enzyme activity is copper dependent. These findings were confirmed by work on a GH61 from *Phanerochaete chrysosporium* (PcGH61D) by Westereng et al. (2011) and work on several GH61 proteins from *Neurospora crassa* (Beeson et al. 2012; Phillips et al. 2011; Li et al. 2012). Thus, GH61s are copper dependent lytic polysaccharide monooxygenases. Recent work on a chitin-active CBM33, using experimental conditions that ruled out possible effects of metal ions trapped in the substrate, showed that this CBM33 was copper dependent too (Vaaje-Kolstad et al. 2005).

The list of enzymes and noncatalytic proteins associated with degradation of lignocellulose is increasing every year, the recent list of novel microbial enzymes and its functions are given in Table 1.1 (Mohanram et al. 2013).

Table 1.1 Novel enzymes and other proteins involved in degradation of biomass

Enzymes	Function	Microorganisms
Endo-1,4 β-D-glucanglucanohydrolase (EC. 3. 2. 1.4)	Hydrolysis of the internal glycosidic linkages in a random fashion, generating oligosaccharides of varying lengths	*Acidothermus cellulolyticus, Pectobacterium chrysanthami, Thermobifida fusca, Thermotoga maritime, Bacillus, Pseudomonas, Ruminococcus, Fibrobacter, Clostrdium, Halomonas, Streptomyces, Cellulomonas, Mycobacterium, Aspergillus, Trichoderma, Anaeromyces, Pestalotiopsis, Phanerochaete, Fusarium, Orpinomyces, Piromyce*
Exoglucanase or 1,4-β-D-glucan cellobiohydrolase (EC3.2.1.91)	Hydrolysis of beta-D-glucosidic linkages by releasing mainly cellobiose either from the reducing or nonreducing ends of the chains	*Bacillus, Pseudomonas, Clostridium, Paenibacillus, Thermobifida, Cellulomonas, Mycobacterium, Ralstonia, Trichoderma, Penicillium, Aspergillus, Chaetomium, Fusarium, Pestalotiopsis, Orpinomyces, Piromyces, Rhizopus*
β-glucosidases or β-D-glucoside glucohydrolase (EC 3.2.1.21)	Hydrolysis of terminal, nonreducing β-D-glucosyl residues with release of β-D-glucose	*Clostridium, Cellulomonas, Aerobacter, Leuconostoc, Aspergillus, Monilia, Phanerochaete, Sclerotium, Saccharomyces, Kluyveromyces*
Cellodextrin phosphorylase or (1 → 4)-β-D-glucan: phosphate α-D-glucosyltransferase (EC 2.4.1.49)	Catalysis of the reversible phosphorolytic cleavage of cellodextrins ranging from cellotriose to cellohexoses	*Clostridium, Cellvibrio*
Cellobiose phosphorylase or cellobiose: phosphate α-D-glucosyltransferase (EC 2.4.1.20)	Catalysis of the reversible phosphorolytic cleavage of cellobiose	*Cellulomonas, Clostridium, Ruminococcus, Thermotoga, Cellvibrio, Fomes annosus*
Endo-1,4-β-D-xylanase or 1,4-beta-xylan xylanohydrolase (EC 3.2.1.8)	Endohydrolysis of (1 → 4)-beta-D-xylosidic linkages in xylans to release xylose	*Bacillus circulans, Thermoanaerobacterium, Ruminococcus, Geobacillus, Thermopolyspora, Cellulomonas, Streptomyces, Aspergillus, Trichoderma, Thermomyces, Fusarium, Anaeromyces, Neocallimastix, Dictyoglomus Thermophilum, Streptomyces halstedii*

(continued)

Table 1.1 (continued)

Enzymes	Function	Microorganisms
Xylan β-1,4-xylosidase (EC 3.2.1.37)	Hydrolysis of (1 → 4)-beta-D-xylans, to remove successive D-xylose residues from the non-reducing termini	*Bacillus, Thermoanaerobacterium, Geobacillus, Aspergillus, Fusarium, Talaromyces, Trichoderma*
Mannan endo-1,4-β-mannosidase (EC 3.2.1.78)	Random hydrolysis of (1 → 4)-beta-D-mannosidic linkages in mannans, galactomannans and glucomannans	*Cellulomonas, Bacillus, Clostridium, Rhodothermus, Aspergillus, Trichoderma*
α-L-arabinofuranosidase (EC 3.2.1.55)	Hydrolysis of terminal nonreducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides	*Bifidobacterium, Thermobacillus, Bacillus, Clostridium, Streptomyces, Aspergillus, Penicillium, Fusarium, Trichoderma*
Acetyl (xylan) esterase (EC 3.1.1.72)	Hydrolysis of ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of natural acetylated xylan	*Bacillus, Clostridium, Streptomyces, Fibrobacter, Pseudomonas, Thermoanaerobacterium, Penicillium, Aspergillus, Trichoderma, Phanerochaete, Chrysosporium*
α-L-Fucoside fucohydrolase or α-L-fucosidase (EC 3.2.1.51)	Hydrolysis of *O*-glycosyl bond in xyloglucan to release l-fucose residues	*Thermotoga, Bifidobacterium, Streptococcus, Bacillus, Cellulomonas, Clostridium, Fusarium, Beauveria, Penicillium, Trichoderma, Neurospora, Aspergillus*
α-D-Glucosiduronate glucuronohydrolase or α-glucuronidase (EC 3.2.1.139)	Hydrolysis of *O*-glycosyl bond to release 4-*O*-methylglucuronic acid from xylan	*Thermotoga, Cellvibrio, Bacteroides, Bacillus, Aspergillus, Trichoderma, Thermoascus*
Novel proteins		
Swollenins	Homologous to plant expansins which rapidly induce extension of plant cell walls by weakening the noncovalent interactions; contain an N-terminal carbohydrate binding module family 1domain (CBD) with cellulose-binding function and a C-terminal expansin-like domain	*Bacillus, Cellulomonas, Clostridium, Myceliophthora, Thermomonospora, Streptomyces, Fibrobacter, Trichoderma, Aspergillus, Neosartorya, Humicola, Fusarium, Penicillium, Neurospora, Gliocladium, Candida, Pichia, Rhodotorula, Sporobolomyces, Bjerkandera adusta*

(continued)

Table 1.1 (continued)

Enzymes	Function	Microorganisms
Cellulose induced proteins (CIP1 and CIP2)	Contain a carbohydrate binding module (CBM); Hydrolysis of the ester linkage between 4-*O*-methyl-D-glucuronic acid of glucuronoxylan and lignin alcohols	*Streptomyces coelicolor, Trichoderma, Schizophyllum, Pyrenophora*
Lossenins	Facilitate acess of glycosyl hydrolases	*Bjerkandera adusta*
Dockerin and Cohesin	Assembly of cellulase and hemicellulase proteins to structural scaffoldings on microbial surface	Anaerobic microorganisms

1.7 Swollenins: A Novel Protein for Cellulose Degradation

Expansins in plants have been proposed to disrupt hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides without having hydrolytic activity (McQueen-Mason and Cosgrove 1994; Whitney et al. 2000). In this way they are thought to allow the sliding of cellulose fibers and enlargement of the cell wall. Saloheimo et al. (2002) reported the discovery of a novel fungal protein having significant sequence identity to plant expansins. Unlike plant expansins, this protein has a modular structure with an N-terminal CBD. The protein was named swollenin due to its ability to swell cotton fibers and loosening effect of swollenin makes lignocellulosic biomass more accessible and readily hydrolyzable by cellulase, thereby promoting the degradation of lignocellulose during fermentation (Arantes and Saddler 2010; Chen et al. 2010) without producing detectable amounts of reducing sugars. Swollenin was later shown to be capable of weakening and disrupting hydrogen-bond networks in lignocellulose (Wang et al. 2011). Swollenin has an N-terminal fungal type cellulose-binding domain connected by a linker region to the expansin-like domain. The protein also contains regions similar to mammalian fibronectin type III repeats, found for the first time in a fungal protein. The swollenin gene is regulated in a largely similar manner as the *T. reesei* cellulase genes.

Efforts were made by different groups to express swollenin gene heterologously in *Saccharomyces cerevisiae* and *Aspergillus niger* (Saloheimo et al. 2002) and *Kluyveromyces lactis* (Jäger et al. 2011). In continuation to improve the direct conversion of cellulose to ethanol, yeast *Saccharomyces cerevisiae* co-displaying cellulase and expansin-like protein on the cell surface were constructed and examined for direct ethanol fermentation performance. The cellulase and expansin-like protein co-expressing strain showed 246 mU/g-wet cell of

phosphoric acid swollen cellulose degradation activity, which corresponded to 2.9-fold higher activity than that of a cellulase-expressing strain. This result clearly demonstrated that yeast cell surface expressed cellulase and expansin-like protein act synergistically to breakdown cellulose. In fermentation experiments examining direct ethanol production from PASC, the cellulase and expansin-like protein co-expressing strain produced 3.4 g/L ethanol after 96 h of fermentation, a concentration that was 1.4-fold higher than that achieved by the cellulase-expressing strain (2.5 g/L) (Nakatani et al. 2013).

Its exploitation for removal of detectable amounts of reducing sugars has been recently reported by Kang et al. (2013). They have isolated a novel gene encoding a swollenin-like protein, POSWOI, from the filamentous fungus *Penicillium oxalicum* by Thermal Asymmetric Interlaced PCR (TAIL-PCR). It consisted of a family 1 carbohydrate binding module (CBM1) followed by a linker connected to a family 45 endoglucanase-like domain. Using the cellobiohydrolase I promoter, recombinant POSWOI was efficiently produced in *T. reesei* with a yield of 105 mg/L, and showed significant disruptive activity on crystalline cellulose. Simultaneous reaction with both POSWOI and cellulases enhanced the hydrolysis of crystalline cellulose Avicel by approximately 50 %. Using a POSWOI-pretreatment procedure, cellulases can produce nearly twice as many reducing sugars as without pretreatment. The mechanism by which POSWOI facilitates the saccharification of cellulose was also studied using a cellulase binding assay.

1.8 CIP1 and CIP2

Cellulose-induced protein 1 (Cip1) protein consists of a glycoside hydrolase family 1 carbohydrate binding module connected via a linker region to a domain. A calcium ion binding site was identified in a sequence conserved region of Cip1 and is also seen in other proteins with the same general fold as Cip1, such as many carbohydrate binding modules. The Cip1 structure was analyzed and a structural homology search was performed to identify structurally related proteins. The two published structures with highest overall structural similarity to Cip1 found were two poly-lyases: CsGL, a glucuronan lyase from H. jecorina and vAL-1, an alginate lyase from the Chlorella virus. This indicates that Cip1 may be a lyase. However, initial trials did not detect significant lyase activity for Cip1 (Jacobson et al. 2013). The function of CIP1 is not known yet. BLAST searches of CIP1 in public databases show that it is similar to glycosyl hydrolases found in bacteria but that are not yet characterized in detail, indicating that CIP1 could have a novel undescribed enzymatic activity or mechanism. CIP2 was described to encode a CE15 glucuronoyl esterase, probably cleaving the ester linkage between 4-*O*-methyl-D-glucuronic acid of glucuronoxylan and lignin alcohols (Li et al. 2007).

1.9 Consolidated Bioprocessing (CBP) for Enhanced Biofuel Production

CBP can be achieved by two ways by modifying cellulase producer to ferment glucose or to modify glucose producer to produce celulase. CBP may result in poor yield of alcohol and requires more incubation period and hence thermophilic strains are considered to be best alternative and above said combination is appears to be practically viable. It represents an alternative method with outstanding potential for low-cost processing of lignocellulosic biomass (Lynd et al. 2008). Fujita et al. (2004) constructed a whole-cell biocatalyst with the ability to induce synergistic and sequential cellulose hydrolysis reaction through co display of three types of the cellulololytic enzymes on the cell surface of *S. cerevisiae*. This strain achieved successful conversion of cellulose to ethanol with good yield. In another attempt Den et al. (2007) developed recombinant strain of *S. cerevisiae* which can be used for CBP. Two cellulase-encoding genes, an endoglucanse of *T. reesei* and β-glucosidase of *Saccharomyces fibuligera* in combination were expressed in *S. cerevisiae*.

No ideal CBP microbe able to degrade efficiently lignocelluloses and at the same time, to utilize the released sugars to produce ethanol is currently available. A newly discovered thermophilic microorganism, *Geobacillus* sp. R7 (T 60 °C), is a facultative anaerobic bacterium isolated from soil samples of the Homestake gold mine, South Dakota. It produces a thermostable cellulose when grown on extrusion-pretreated agricultural residues such as corn stover and prairie cord grass, and ferments lignocellulosic substrates to ethanol in a single step (Zambare et al. 2011).

Cha et al. (2013) demonstrated recently the utility of gene deletion in the pyruvate metabolic pathway for rational strain engineering of *Caldicellulosiruptor bescii* while simultaneously creating a platform for further strain modification for advanced production of fuels and chemicals from renewable plant feed stocks. The method for creating a deletion of the ldh gene in the *C. bescii* chromosome was efficient enough to allow targeted marker replacement using nonreplicating plasmids. The resulting mutant grew to a higher cell density and produced more hydrogen than the wild-type strain. Using the tools developed by them, *C. bescii* JWCB017 will serve as a platform for additional rational strain engineering for production of fuels and chemicals from lignocellulosic feedstocks.

Efforts were also continued to transfer genes responsible for degradation of cellulose to yeast. Cellulase genes from various kinds of microorganisms have been expressed in the ethanol-producing yeast *Saccharomyces cerevisiae* with the aim of directly producing ethanol from cellulose (Van and Van 1998; Fujita et al. 2002; Ito et al. 2009; Wen et al. 2010; Yamada et al. 2013). In addition, yeast strains displaying cellulase on the cell surface have also been developed for improving the efficiency of direct ethanol production from cellulose (Yamada et al. 2013).

1.10 Recycling of Enzymes

A key factor that prevents the commercialization of enzymatic cellulose hydrolysis is the high cost of cellulase enzymes. Enzyme cost is expected to account for more than 20 % of ethanol production (Wooley et al. 1999). As much of it remains active after hydrolysis, recycling of cellulases makes the overall conversion process more economically feasible. Various methods have been used for recycling enzymes, which include sedimentation followed by ultra filtration or micro centrifugation, cation exchange chromatography, readsorption, and immobilization.

1.11 Potential Strategies for Stabilizing Cellulases

Enzyme stabilization and lifetime improvement has been achieved through point substitution and chemical modification, of single catalytic domains, either through rational, structure-based modeling, or through directed evolution. Another approach to increase enzyme stability and enhance activity is insertion of protein domains into stable scaffolds.

An effort was made to stabilize the cellulases by embedding cellulases of extremophiles into hyperstable α-helical consensus. It was observed that catalytic domains CelA (CA, GH8; *Clostridium thermocellum*) and Cel12A (C12A, GH12; *Thermotoga maritima*) to be stable in the context of ankyrin scaffold, and to be active against both soluble and insoluble substrates. The ankyrin repeats in each fusion are folded, although it appears that for C12A catalytic domain (CD) (where the N- and C-termini are distant in the crystal structure), the two flanking ankyrin domains are independent, whereas for CA (where termini are close), the flanking ankyrin domains stabilize each other. Although the activity of CA is unchanged in the context of the ankyrin scaffold, the activity of C12A is increased between two and sixfold (for RAC and CMC substrates) at high temperatures. For C12A, activity increases by with the number of flanking ankyrin repeats. These results show ankyrin arrays to be a promising scaffold for constructing designer cellulosomes, preserving or enhancing enzymatic activity and retaining thermostability.

In this type of scaffolding strategy, the distance between the N and C-termini of the inserted protein (cellulase catalytic domains) is an important parameter. Cellulases catalytic domains belong to various Glycoside Hydrolase (GH) families, as reported in the CAZY database, with different folds, and thus have variable N-to-C distances (Cantarel et al. 2009).

Insertion into a scaffold protein requires appropriate selection of insertion sites to avoid disruption of structure of the inserted protein or scaffold (Betton et al. 1997; Cutler et al. 2009). Proteins with linear, repeated architectures, such as ankyrin repeats, have structurally modular architectures that should be able to accommodate insertions locally, without affecting more distant regions of the scaffold. Ankyrin repeat proteins are roughly linear arrays of 33 residues repeating units (Sedgwick

and Smerdon 1999; Zweifel and Barrick 2001). Each unit consists of two short helices connected by a short, conserved turn, and connects to neighboring repeats through an extended beta-hairpin containing loop (Zweifel et al. 2003). Adjacent repeats strongly stabilize each other through highly stabilizing interfaces. The repeating unit spans about 11 Å, approximately the size of each cellobiose unit (2-units of glucose 10.4 Å). Studies have found that ankyrin repeats are greatly stabilized by consensus sequence substitutions (Wetzel et al. 2008; Aksel et al. 2011). For a five repeat consensus ankyrin protein, the thermal denaturation midpoint (Tm) was around 90 °C (Aksel et al. 2011; Aksel and Barrick 2009). Therefore, consensus ankyrin seems to be an ideal scaffold to study the effects of inserting cellulase CDs with various N–C distances.

1.12 Enzyme Cocktails

Catalytic activity of an enzyme is defined and mediated by its 3D structure and its mobility. It is impossible to obtain complete comprehension of the enzymatic action without detailed molecular studies which include structural, biophysical, biochemical and genetic investigations, enzymatic assays, bioinformatics, and molecular dynamics computations. It is not possible to innovate and to consistently produce competitive enzymatic cocktails/mixtures for biomass transformation without profound understanding of function and activity of glycoside hydrolases. Therefore, we need to integrate our knowledge of biodiversity, genomics and genetics studies, structural and molecular biology, biochemistry, enzymology, and bioinformatics, focusing on bioindustrial applications.

Enzyme cocktails that hydrolyze plant cell wall polysaccharides are a critical component of bioprocessing configurations designed to transform lignocellulosic biomass into biofuels (Lynd et al. 2002; Gao et al. 2011). Specific characteristics of an enzyme (activity, thermostability, etc.) is often based on homology to known enzymes (Schnoes et al. 2009). Although free cellulases and cellulosomes employ different physical mechanisms to break down recalcitrant polysaccharides, when these system combined display dramatic synergistic enzyme activity on cellulose. Two natural enzyme systems—one produced by fungi and the other by bacteria—break down cellulose faster if used in combination. The resulting process shows promise for less expensive biofuels (Resch et al. 2013).

Current commercial cocktails consist of preparations of fungus derived glycoside hydrolases, primarily cellulases and hemicellulases (Bouws et al. 2008; King et al. 2009). However, fungal enzymes are often deactivated by elevated temperatures or by residual chemicals from pretreatment (Bouws et al. 2008). For example, ionic liquids (ILs), such as 1-ethyl- 3-methylimidazolium acetate ([C2mim][OAc]), can dissolve lignocellulosic biomass and dramatically improve cellulose hydrolysis kinetics, yet multiple studies have shown that fungal endoglucanases are deactivated at low levels of ILs that may persist in the biomass after pretreatment (Dadi et al. 2006; Turner et al. 2003). In contrast, thermophilic bacterial and archaeal

endoglucanases have been shown to be active in 20 % [C2mim][OAc], suggesting that thermophilic prokaryotes may be an important source of enzymes for the development of more robust enzyme cocktails (Datta et al. 2010).

Although free cellulases and cellulosomes employ very different physical mechanisms to break down recalcitrant polysaccharides, when combined these systems display dramatic synergistic enzyme activity on cellulose. Two natural enzyme systems—one produced by fungi and the other by bacteria—break down cellulose faster if used in combination. The resulting process shows promise for less expensive biofuels (Resch et al. 2013).

1.13 Enzyme Engineering

The natural diversity of enzymes could provide a large reservoir that can be further improved by engineering enzymes and strains for increased performance (King et al. 2009). A number of designer enzymes called glycosynthases, including cellulases and hemicellulases, have been engineered by replacing nucleophilic residues resulting in higher yields of different oligosaccharides (Kumar et al. 2008).

1.14 Feruloyl Esterases (Faes)

Feruloyl esterases (Faes) represent a subclass of carboxyl esterases that can release phenolic acids, such as ferulic acids or other cinnamic acids, from esterified polysaccharides, especially xylan and pectin. Feruloyl esterases (Faes) constitute a subclass of carboxyl esterases that specifically hydrolyze the ester linkages between ferulate and polysaccharides in plant cell walls. Recently, a comprehensive set of enzymes essential for decomposing plant cell walls, including feruloyl esterase activity, in a newly described in yak ruminal bacterium, *Cellulosilyticum ruminicola* H1. Strain H1 grew robustly on natural plant fibers, such as corn cob, alfalfa, and ryegrass, as the sole carbon and energy sources, as well as on a variety of polysaccharides, including cellulose, xylan, mannan, and pectin (Li et al. 2011). Therefore, Faes are regarded as the key enzymes to loosen the internal crosslink of plant cell walls by acting as the important accessory enzymes in synergy with (hemi)cellulases in plant cell wall hydrolysis.

1.15 Future Scope

Urgent need of the alternate energy resources increased the novel ideas to achieve success to convert lignocelluloses and other carbohydrates to ethanol. In this connection development of systems biology approaches and integrated, predictive

modeling capabilities for metabolic and regulatory networks of biomass degrading microorganisms is considered to be very important. The characterization of microbes and microbial communities from environments with high rates of ligno-cellulose degradation has to be continued, because microorganisms display varied capacity for carbohydrate degradation.

The following are the thrust area of research already initiated by several scientists

- Designing of optimized cellulosomes by synthesizing hybrid scaffolding molecules that contain cohesins with different binding specificity from different organisms is another recent approach to develop more active cellulose degrading enzymes (Wilson 2009). Synergies between purified cellulases and xylanases from the thermophilic bacterium, *Thermobifida fusca* displayed on designer cellulosomes were found to possess higher activity on wheat straw than the corresponding free enzymes (Morais et al. 2010).
- The recent trend is developing multifunctional chimeras by construction, cloning and sequencing the chimeric gene and its expression followed by purification of chimeric proteins (Nair et al. 2010).
- Brunecky et al. (2013) proposed a novel mechanism of cellulose digestion by the dominant multimodular cellulase CelA of the thermophilic bacterium *Caldicellulosiruptor bescii* and compared its hydrolytic performance with that of a binary mixture containing Cel7A fungal exoglucanase (cellobiohydrolase I from *Trichoderma reesei*) and bacterial *Acidothermus cellulolyticus* Cel5A (E1) endoglucanase. The major finding of the report, that CelA is able to excavate extensive cavities into the surface of the cellulosic substrate, is very interesting and indeed reflects a novel cellulose digestion paradigm.
- There are efforts to develop, create, and investigate artificial cellulosomes with high activity, and systematically evaluate the fundamental principles that underlie their structure, dynamics, and catalytic functions. Ranjan (2014) have developed a polycatalytic system consisting of cellulases covalently linked on the surface of colloidal polymers with a magnetic nanoparticle (MNP) core. MNP provides a convenient handle to separate the complex, while the colloidal polymer would serve as a benign scaffold to attach the enzymes.

Although biomass may ultimately only supply a relatively small amount of the world's energy requirements, it will nevertheless be of immense overall value. In some parts of the world, such as Brazil and other countries of similar climatic conditions, biomass will surely attain wider exploitation and utilization. There may still be some disadvantages when comparing it with coal or oil, but the very fact that it is renewable and they are not must be the spur to further research. In time, biomass will become much more easily and economically used as a source of energy for mankind. Approaches like enzyme engineering, reconstitution of enzyme mixtures and bioprospecting for superior enzymes are gaining importance. The current scenario, however, also warrants the need for research and development of integrated biomass production and conversion systems.

References

Aksel T, Barrick D (2009) Analysis of repeat-protein folding using nearest-neighbor statistical mechanical models. Methods Enzymol 455:95–125

Aksel T, Majumdar A, Barrick D (2011) The contribution of entropy, enthalpy, and hydrophobic desolvation to cooperativity in repeat-protein folding. Structure 19:349–360

Arantes V, Saddler JN (2010) Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels 3:4–15

Banerjee G, Scott-Craig JS, Walton JD (2010) Improving enzymes for biomass conversion: a basic research perspective. Bioenerg. Res. 3:82–92

Bayer EA, Belaich JP, Shoham Y, Lamed R (2004) The cellulosomes: multi-enzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 58:521–554

Bayer EA, Shoham Y, Lamed R (2013) The prokaryotes: lignocellulose-decomposing bacteria and their enzyme systems. In: Rosenberg E (ed) The prokaryotes, 4th edn. Springer, Berlin, pp 216–266

Beeson WT, Phillips CM, Cate JHD, Marletta MA (2012) Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. J Am Chem Soc 134:890–892

Belaich JP, Tardif C, Belaich A, Gaudin C (1997) The cellulolytic system of *Clostridium cellulolyticum*. J Biotechnol 57:3–14

Berrin JG, Czjzek M, Kroon PA, McLauchlan WR, Puigserver A, Williamson G, Juge N (2003) Sub- strate (aglycone) specificity of human cytosolic beta-glucosidase. Biochem J 373:41–48

Betton JM, Jacob JP, Hofnung M, Broome-Smith JK (1997) Creating a bifunctional protein by insertion of beta-lactamase into the maltodextrin-binding protein. Nat Biotechnol 15:1276–1279

Bisaria VS (1991) Bioprocessing of agro-residues to glucose and chemicals. In: Martin AM (ed) Bioconversion of waste materials to industrial products. Elsevier, London, pp 210–213

Blumer-Schuette SE, Kataeva I, Westpheling J, Adams MW, Kelly RM (2008) Extremely thermophilic microorganisms for biomass conversion: status and prospects. Curr Opin Biotechnol 19:210–217

Bouws H, Wattenberg A, Zorn H (2008) Fungal secretomes: nature's toolbox for white biotechnology. Appl Microbiol Biotechnol 80:381–388

Brunecky R, Alahuhta M, Xu Q, Donohoe BS, Crowley MF, Kataeva IA, Yang S-J, Resch MG, Adams MMW, Lunin VV, Michael E, Himmel ME, Yannick J, Bomble YJ (2013) Revealing nature's cellulase diversity: the digestion mechanism of *Caldicellulosiruptor bescii* CelA. Science 342:1513–1516

Cai Z, Xing G, Yan X, Xu H, Tsuruta H, Yagi K, Minami K (1997) Methane and nitrous oxide emissions from rice paddy fields as affected by nitrogen fertilisers and water management. Plant Soil 196:7–14

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:233–238

Cantarel BL, Lombard V, Henrissat B (2012) Complex carbohydrate utilization by the healthy human microbiome. PLoS ONE 7(6):121–131

Cha M, Daehwan C, James GE, Adam MG, Janet W (2013) Metabolic engineering of *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic biomass. Biotechnol Biofuels 6:85–93

Chen XA, Ishida N, Todaka N, Nakamura R, Maruyama J, Takahashi H, Kitamoto K (2010) Promotion of efficient saccharification of crystalline cellulose by *Aspergillus fumigatus* SWO1. Appl Environ Microbiol 76:2556–2561

Cutler TA, Mills BM, Lubin DJ, Chong LT, Loh SN (2009) Effect of interdomain linker length on an antagonistic folding-unfolding equilibrium between two protein domains. J Mol Biol 386:854–868

Dadi AP, Varanasi S, Schall CA (2006) Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. Biotech. Bioeng. 95:904–910

Dassa B, Borovok I, Lamed R, Henrissat B, Coutinho P, Hemme L, Huang Y, Zhou J, Bayer EA (2012) Genome-wide analysis of *Acetivibrio cellulolyticus* provides a blueprint of an elaborate cellulosome system. BMC Genom 13:210–223

Datta S, Holmes B, Park JI, Chen Z, Dibble DC, Hadi M, Blanch HW, Simmons BA, Sapra R (2010) Ionic liquid tolerant hyperthermophilic cellulases for biomass pretreatment and hydrolysis. Green Chem 12:338–345

Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Graham DE, Overbeek R, Snead MA, Keller M, Aujay M, Huber R, Feldman RA, Short JM, Olsen GJ, Swanson RV (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature 392 (6674):353–358

Deka D, Jawed M, Goyal A (2013) Purification and characterization of an alkaline cellulase produced by *Bacillus subtilis* (AS3). Prep Biochem Biotechnol 43:256–270

Den HR, Rose SH, Lynd LR, van Zyl WH (2007) Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab Eng 9(1):87–94

Ding SY, Bayer EA, Steiner D, Shoham Y, Lamed R (1999) A novel cellulosomal scaffoldin from *Acetivibrio cellulolyticus* that contains a family 9 glycosyl hydrolase. J Bacteriol 181:6720–6729

Ding SY, Bayer EA, Steiner D, Shoham Y, Lamed R (2000) A scaffoldin of the *Bacteroides cellulosolvens* cellulosome that contains 11 type II cohesins. J Bacteriol 182:4915–4925

Eriksson T, Borjesson J, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocelluloses. Enz Microb Technol 31:353–364

Fontes CM, Gilbert HJ (2010) Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. Annu Rev Biochem 79:655–681

Forsberg Z, Vaaje-Kolstad G, Westereng B, Bunæs AC, Stenstrøm Y, MacKenzie A, Sørlie M, Horn SJ, Eijsink VGH (2011) Cleavage of cellulose by a CBM33 protein. Prot Sci 20:1479–1483

Fujita Y, Takahashi S, Ueda M, Tanaka A, Okada H, Morikawa Y, Kawaguchi T, Arai M, Fukuda H, Kondo A (2002) Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol 68:5136–5141

Fujita Y, Iro J, Ueda M, Fukuda H, Kondo A (2004) Synergistic saccharification and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol 70(2):1207–1212

Gal L, Pages S, Gaudin C, Belaich A, Reverbel-Leroy C, Tardif C, Belaich JP (1997) Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum*. Appl Environ Microbiol 63:903–909

Gao D, Uppugundla N, Shishir P, Chundawat S, Yu X, Hermanson S, Gowda K, Brumm P, Mead D, Balan V, Dale BE (2011) Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnol Biofuels 4:5–16

Garcia-Alvarez B, Melero R, Dias FM, Prates JA, Fontes CM, Smith SP, Romao MJ, Vazana Y, Barak Y, Unger T, Peleg Y, Shamshoum M, Ben-Yehezkel T, Mazor Y, Shapiro E, Lamed R, Bayer EA (2013) A synthetic biology approach for evaluating the functional contribution of designer cellulosome components to deconstruction of cellulosic substrates. Biotechnol Biofuels 6:182–200

Gladden JM, Allgaier M, Miller CS, Hazen TC, Vander Gheynst JS, Hugenholtz P, Simmons BA, Singer SW (2011) Glycoside hydrolase activities of thermophilic bacterial consortia adapted to switchgrass. Appl Environ Microbiol 77:5804–5812

Hamilton-Brehm SD, Mosher JJ, Vishnivetskaya T, Podar M, Carroll S, Allman S, Phelps TJ, Keller M, Elkins JG (2010) *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from Obsidian Pool, Yellowstone National Park. Appl Environ Microbiol 76:1014–1020

Hehemann J, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G (2010) Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature 464:908–912

Horn SJ, Vaaje-Kolstad G, Westereng B, Eijsink VGH (2012) Novel enzymes for the degradation of cellulose. Biotechnol Biofuels 5:45–59

Howell JA, Stuck JD (1975) Kinetics of solka floc cellulose hydrolysis by *Trichoderma viride* cellulase. Biotechnol Bioeng 17:873–893

Ito J, Kosugi A, Tanaka T, Kuroda K, Shibasaki S, Ogino C, Ueda M, Fukuda H, Doi RH, Kondo A (2009) Regulation of the display ratio of enzymes on the *Saccharomyces cerevisiae* cell surface by the immunoglobulin G and cellulosomal enzyme binding domains. Appl Environ Microbiol 75:4149–4154

Jacobson F, Karkehabadi S, Hansson H, Goedegebuur F, Wallace L, Mitchinson C, Piens K, Stals I, Sandgren M (2013) The crystal structure of the core domain of a cellulose induced protein (Cip1) from *Hypocrea jecorina*, at 1.5 Å resolutions. PLoS ONE 8(9):e70562

Jäger G, Girfoglio M, Dollo F, Rinaldi R, Bongard H, Commandeur U, Fischer R, Spiess AC, Büchs J (2011) How recombinant swollenin from *Kluyveromyces lactis* affects cellulosic substrates and accelerates their hydrolysis. Biotechnol Biofuels 4:33–49

Kang K, Wang S, Lai G, Liu G, Xing M (2013) Characterization of a novel swollenin from *Penicillium oxalicum* in facilitating enzymatic saccharification of cellulose. BMC Biotechnol 13:42–51

Kato S, Haruta S, Cui ZJ, Ishii M, Igarashi Y (2005) Stable coexistence of five bacterial strains as a cellulose-degrading community. Appl Environ Microbiol 71:7099–7106

Kaur G, Kumar S, Satyanarayana T (2004) Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. Bioresour Technol 94:239–243

King BC, Donnelly MK, Bergstrom GC, Walker LP, Gibson DM (2009) An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts. Biotechnol Bioeng 102:1033–1044

Kostylev M, Wilson D (2012) Synergistic interactions in cellulose hydrolysis. Biofuels 3(1):61–70

Kristensen JB, Boijesson J, Brunn MH, Tjerneld F, Jorgensen H (2007) Use of surface active additives in enzymatic hydrolysis of wheat straw lignocelluloses. Enz Micro Technol 40:888–895

Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspective. J Ind Microbiol Biotechnol 35:377–391

Lamed R, Setter E, Bayer EA (1983a) Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. J Bacteriol 156:828–836

Lamed R, Setter E, Kenig R, Bayer EA (1983b) The cellulosome—a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol Bioeng Symp 13:163–181

Li XL, Spanikova S, de Vries RP, Biely P (2007) Identification of genes encoding microbial glucuronoyl esterases. FEBS Lett 581(21):4029–4035

Li J, Cai S, Dong X (2011) Three feruloyl esterases in *Cellulosilyticum ruminicola* H1 act synergistically to hydrolyze esterified polysaccharides. Appl Environ Microbiol 77 (17):6141–6147

Li X, Beeson WT, Phillips CM, Marletta MA, Cate JHD (2012) Structural basis for substrate targeting and catalysis by fungal polysaccharide monooxygenases. Structure 20:1051–1061

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577

Lynd LR, Laser MS, Bransby D, Dale BE, Davidson B, Hamilton R, Himmel ME, Keller M, McMillan JD, Sheehan J (2008) How biotech can transform biofuels. Nat Biotechnol 26:169–172

Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 5(5):500–516

Mansfield MC, Touhy M, Saddler J (1998) The effect of the initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Biores Technol 64:113–119

Martins LF, Kolling D, Camassola M, Dillon AJ, Ramos LP (2008) Comparison of *Penicillium echinulatum* and *Trichoderma reesei* cellulases in relation to their activity against various cellulosic substrates. Bioresour Technol 99:1417–1424

Mayer F, Coughlan MP, Mori Y, Ljungdahl LG (1987) Macromolecular organization of the cellulolytic enzyme complex of *Clostridium thermocellum* as revealed by electron microscopy. Appl Environ Microbiol 53:2785–2792

McMillan JD, Jenning EW, Mohagheghi A, Zuccarello M (2011) Comparative performance of precommercial cellulases hydrolyzing pretreated corn stover. Biotech Biofuels 4:29–46

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cellwall polymers by proteins that induce wall extension. Proc Natl Acad Sci USA 91:6574–6578

Mohanram S, Amat D, Choudhary J, Arora A, Nain L (2013) Novel perspectives for evolving enzyme cocktails for lignocellulose hydrolysis in biorefineries. Sustain Chem Process 1:15–27

Morais S, Barak Y, Caspi J, Hadar Y, Lamed R, Shoham Y, Wilson DB, Bayer EA (2010) Cellulase-xylanase synergy in designer cellulosomes for enhanced degradation of a complex cellulosic substrate. mBio 1:00285–00210

Moser F, Irwin D, Chen SL, Wilson DB (2008) Regulation and characterization of *Thermobifida fusca* carbohydrate-binding module proteins E7 and E8. Biotechnol Bioeng 100:1066–1077

Nair NU, Denard CA, Zhao H (2010) Engineering of enzymes for selective catalysis. Curr Org Chem 14:1870–1882

Nakatani Y, Yamada K, Ogino C, Kondo A (2013) Synergetic effect of yeast cell-surface expression of cellulase and expansin-like protein on direct ethanol production from cellulose. Microb Cell Fact 12:66–73

Ohara H, Karita S, Kimura T, Sakka K, Ohmiya K (2000) Characterization of the cellulolytic complex (cellulosome) from *Ruminococcus albus*. Biosci Biotechnol Biochem 64:254–260

Phillips CM, Beeson WT, Cate JH, Marletta MA (2011) Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. ACS Chem Biol 6:1399–1406

Quinlan RJ, Sweeney MD, Lo Leggio L, Otten H, Poulsen JCN, Johansen KS, Krogh K, Jorgensen CI, Tovborg M, Anthonsen A, Tryfona CP, Dupree WP, Xu F, Davies GJ, Walton PH (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci USA 108:15079–15084

Ranjan KK (2014) Adsorption, diffusion and activity of polycatalytic cellulase-nanoparticle conjugates. Ph.D Dissertations. University of Connecticut

Resch MG, Donohoe BS, Baker JO, Decker SR, Bayer EA, Beckham GT, Himmel ME (2013) Fungal cellulases and complexed cellulosomal enzymes exhibit synergistic mechanisms in cellulose deconstruction. Energy Environ Sci 6:1858–1867

Rincon MT, Ding SY, McCrae SI, Martin JC, Aurilia V, Lamed R, Shoham Y, Bayer EA, Flint HJ (2003) Novel organization and divergent dockerin specificities in the cellulosome system of *Ruminococcus flavefaciens*. J Bacteriol 185:703–713

Rincon MT, Martin JC, Aurilia V, McCrae SI, Rucklidge GJ, Reid MD, Bayer EA, Lamed R, Flint HJ (2004) ScaC, an adaptor protein carrying a novel cohesin that expands the dockerin-binding repertoire of the *Ruminococcus flavefaciens* 17 cellulosome. J Bacteriol 186:2576–2585

Rincon MT, Dassa B, Flint HJ, Travis AJ, Jindou S, Borovok I, Lamed R, Bayer EA, Henrissat B, Coutinho PM, Antonopoulos DA, Berg Miller ME, White BA (2010) Abundance and diversity of dockerin-containing proteins in the fiberdegrading rumen bacterium, *Ruminococcus flavefaciens* FD-1. PLoS ONE 5:e12476

Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssönen E, Bhatia A, Ward M, Penttilä M (2002) Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. Eur J Biochem 269:4202–4211

Schnoes AM, Brown SD, Dodevski I, Babbitt PC (2009) Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. PLoS Comput Biol 5:e1000605

Scott BR, Hill C, Tomashek J, Liu C (2009) Enzymatic hydrolysis of lignocellulosic feedstocks using accessory enzymes. United States Patent Application 2009/0061484, 5 Mar 2009

Sedgwick SG, Smerdon SJ (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Biochem Sci 24:311–316

Shoseyov O, Shani Z, Levy I (2006) Carbohydrate binding modules: biochemical properties and novel applications. Microbiol Mol Biol Rev 70:283–295

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production. Bioresour Technol 83:1–11

Sweeney MD, Xu F (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2:244–263

Taherzadeh MJ, Karimi K (2007) Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. BioResources 2(4):707–738

Taylor LE, Henrissat B, Coutinho PM, Ekborg NA, Hutcheson SW, Weiner RM (2006) Complete cellulase system in the marine bacterium Saccharophagus degradans strain 2-40T. J Bacteriol 188:3849–3861

Turnbaugh PJ, Quince C, Faith JJ, McHardy AC, Yatsunenko T, Niazi F, Affourtit J, Egholm M, Henrissat B, Knight R, Gordon JI (2010) Organismal genetic and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. Proc Natl Acad Sci USA 107:7503–7508

Turner MB, Spear SK, Huddleston JG, Holbrey JD, Rogers RD (2003) Ionic liquid salt-induced inactivation and unfolding of cellulose from *Trichoderma reesei*. Green Chem 5:443–447

Vaaje-Kolstad G, Horn SJ, van Aalten DMF, Synstad B, Eijsinkm VGH (2005) The noncatalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. J Biol Chem 280:28492–28497

Valaskova V, Baldrian P (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* production of extracellular enzymes and characterization of the major cellulases. Microbiol 152:3613–3619

Van RP, Van ZWH (1998) Pretorius IS: engineering yeast for efficient cellulose degradation. Yeast 14:67–76

Vazana Y, Barak Y, Unger T, Peleg Y, Shamshoum M, Ben-Yehezkel T, Mazor Y, Shapiro E, Lamed R, Bayer EA (2013) A synthetic biology approach for evaluating the functional contribution of designer cellulosome components to deconstruction of cellulosic substrates. Biotechnol Biofuels 6:182–200

Wang Y, Tang R, Tao J, Gao G, Wang X, Mu Y, Feng Y (2011) Quantitative investigation of non-hydrolytic disruptive activity on crystalline cellulose and application to recombinant swollenin. Appl Microbiol Biotechnol 91:353–1363

Weber S, Stubner S, Conrad R (2001) Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl Environ Microbiol 67:1318–1327

Wen F, Sun J, Zhao H (2010) Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol 76:1251–1260

Westereng B, Ishida T, Vaaje-Kolstad G, Wu M, Eijsink VGH, Igarashi K, Samejima M, Ståhlberg J, Horn SJ, Sandgren M (2011) The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal dependent oxidative enzyme that cleaves cellulose. PLoS ONE 6(11):e27807

Wetzel SK, Settanni G, Kenig M, Binz HK, Pluckthun A (2008) Folding and unfolding mechanism of highly stable full-consensus ankyrin repeat proteins. J Mol Biol 376:241–257

Whitney SE, Gidley MJ, McQueen-Mason SJ (2000) Probing expansin action using cellulose/hemicellulose composites. Plant J 22:327–334

Wilson DB (2009) Cellulases and biofuels. Curr Opin Biotechnol 20:295–299

Wongwilaiwalina S, Rattanachomsria U, Laothanachareona T, Eurwilaichitra L, Igarashib Y, Champredaa V (2010) Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. Enzyme Microb Tech 47:283–290

Wooley R, Ruth M, Glassner D, Sheejan J (1999) Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development. Biotechnol Prog 15:794–803

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153–157

Xu Q, Gao W, Ding SY, Kenig R, Shoham Y, Bayer EA, Lamed R (2003) The cellulosome system of *Acetivibrio cellulolyticus* includes a novel type of adaptor protein and a cell surface anchoring protein. J Bacteriol 185:4548–4557

Xu Q, Bayer EA, Goldman M, Kenig R, Shoham Y, Lamed R (2004) Architecture of the *Bacteroides cellulosolvens* cellulosome: description of a cell surface anchoring scaffoldin and a family 48 cellulase. J Bacteriol 186:968–977

Yamada R, Hasunuma T, Kondo A (2013) Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnol Adv 31(6):754–763

Yang SJ, Kataeva I, Hamilton-Brehm SD, Engle NL, Tschaplinski TJ, Doeppke C, Davis M, Westpheling J, Adams MW (2009) Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe *Anaerocellum thermophilum* DSM 6725. Appl Environ Microbiol 75:4762–4769

Zambare VP, Zambare A, Muthukumarappan K, Christopher LP (2011) Potential of thermostable cellulases in the bioprocessing of switchgrass to ethanol. BioResources 6:2004–2020

Zweifel ME, Barrick D (2001) Studies of the ankyrin repeats of the Drosophila *melanogaster* Notch receptor. 2. Solution stability and cooperativity of unfolding. Biochemistry 40:14357–14367

Zweifel ME, Leahy DJ, Hughson FM, Barrick D (2003) Structure and stability of the ankyrin domain of the *Drosophila* Notch receptor. Protein Sci 12:2622–2632

Part I
Cellulases

Chapter 2
Cellobiohydrolases: Role, Mechanism, and Recent Developments

Neelamegam Annamalai, Mayavan Veeramuthu Rajeswari and Nallusamy Sivakumar

Abstract Cellobiohydrolases or exoglucanases are produced by various bacteria and fungi with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases, which act at the chains end of cellulose resulting in release of glucose as well as cellobiose. The CBH I and II works processively from reducing and nonreducing ends of the cellulose chain, respectively. The catalytic module of CBHs is the tunnel structure formed by two surface loops that may covers entirety or part of active site evidenced that the mode of action proceeds in a processive manner as cellobiohydrolase progresses along the cellulose chain. CBHs are able to work actively in the crystalline region of cellulose, probably peeling them from the microcrystalline structure of Avicel. Although several assays have been proposed, no specific substrate as well as assay method to measure exoglucanases has been described till date. In recent days, several new approaches such as δ-sequence mediated integration, SCHEMA, and FoldX and a 'consensus' sequence have been developed to improve activity and stability of CBHs.

2.1 Introduction

Considering the plant biomass, the main component from plant cell wall is the cellulose, which is the more abundant carbohydrate constituted by glucopyranose monomers linked by β-1,4 glycosidic bonds with two distinct regions such as

N. Annamalai (✉)
Hawaii Natural Energy Institute, University of Hawaii at Manoa,
1680, East-West Road, Honolulu, HI 96822, USA
e-mail: annabact@gmail.com

M.V. Rajeswari
Centre for Ocean Research, Sathyabama University, Jeppiar Nagar,
Chennai, TN 600119, India

N. Annamalai · N. Sivakumar
Department of Biology, College of Science, Sultan Qaboos University,
PO Box 36, PC 123 Muscat, Oman

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_2

crystalline and amorphous regions (Dashtban et al. 2009). Cellulosic biomass is the largest amount of waste generated through human activities, which is the most attractive substrate for 'biorefinery strategies' to produce high-value products such as fuels, bioplastics and enzymes through fermentation processes (Mazzoli et al. 2012). The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat 2000). In recent days, there is an increasing interest on use of biomass for biofuel production is pointed-out as an important alternative for reduction of energetic and environment problems.

Cellulases are classified into three major categories based on their substrate specificities and hydrolysis mechanism as (i) 1,4 β-D-glucanases or endoglucanases (EC 3.2.1.4), (ii) exoglucanases or cellobiohydrolases (EC 3.2.1.91), and (iii) cellobiase or β-glucosidases (EC 3.2.1.21). The most efficient hydrolysis of cellulose is the result of combined synergistic actions of cellulases, whereby the enzymatic activity of an enzyme mixture is significantly higher than individual enzyme activity (Wood 1992; Irwin et al. 1993; Nidetzky et al. 1994).

2.2 Exoglucanases or Cellobiohydrolases (CBH) (EC 3.2.1.91)

Exoglucanases, also known as cellobiohydrolases (CBHs) that act at the end of cellulose chains and releasing glucose as well as cellobiose as product in a processive fashion (Medve et al. 1998). CBHs are the most-studied exoglucanase, which is about 70 % of the secreted cellulases by cellulolytic fungi belongs to glycoside hydrolases (GH) 6 and 7, as well as 48 families (Teeri 1997). Different CBHs are produced by various bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Aerobic fungal CBHs are belongs to families 6 and 7; aerobic bacterial CBHs belong to families 6 and 48; anaerobic fungal CBHs are in family 48 and anaerobic bacterial CBHs are in family 9 as well as 48. In general, family 7 CBHs originate only from fungi, whereas family 48 CBHs from bacteria (Zhang and Zhang 2013).

2.3 CBH I and II

CBHs mainly release cellobiose from cellulose derivatives, and the presence of cellobiose competitively inhibits the rate of hydrolysis. The fungal CBHs belong to glycosyl hydrolase families 6 and 7 (GH-6 and 7) and act most efficiently on highly ordered crystalline cellulose, hydrolyzing from either reducing or non-reducing end to liberate predominantly cellobiose (C2) with a minor amount of cellotriose (C3) (Boer et al. 2000; Takahashi et al. 2010). There are two types of cellobiohydrolase:

CBH I and II that works processively from the reducing and non-reducing end of the cellulose chain respectively. The cellobiohydrolases Cel6A (a CBH II) and Cel7a (a CBH I) are much studied enzymes of fungal origin and are usually expressed by the ascomycete *Trichoderma reesei*, accounts for 40 % of total protein and 70 % of cellulytic activity in the industrially relevant fungus *Hypocrea jecorina* (*T. reesei*) which is used for industrial-scale production due to its ability to produce more sugars from biomass through hydrolysis (Suominen et al. 1993).

2.4 Structure and Mode of Action of CBHs

The most significant topological feature of CBHs catalytic module is the tunnel structure, formed by two surface loops that may cover the entirety (family 7 CBHs) or part of the active site (family 48 CBHs) (Koivula et al. 2002; Vocadlo and Davies 2008; Zhang and Zhang 2013). The CBH I (TrCBH I) is the major component of the *T. reesei* cellulase system that belongs to the GH-7 family and hydrolyzes the β-1,4-linkages of a cellulose chain from its reducing end (Claeyssens et al. 1990). The catalytic core domain (CCD) structures of TrCBH I suggested that a central structural motif constituted mainly by an antiparallel β-sandwich motif that bears four surface loops, which decorate the concave face of the sandwich forming the cellulose-binding tunnel (Divne et al. 1998; Stahlberg et al. 1996). During enzymatic catalysis, the cellulose chain slides through the substrate-binding tunnel, and every second glycosidic bond is correctly presented to the catalytic apparatus located at the far end of the tunnel to progressively liberate disaccharide units (cellobiose) from cellulose (Divne et al. 1994).

The three-dimensional (3D) structures of two GH-6 family members including CBH of *T. reesei* and that of *Humicola insolens* in complex with glucose, cellooligosaccharide, and a non-hydrolyzable substrate analog (Rouvinen et al. 1990; Varrot et al. 2002). It seems that the significant amino acids associated with catalytic core domain, where the catalytic site is buried inside a tunnel-shaped cavity and an enzyme–cello-oligosaccharide hydrogen bond network. This typical structure suggests that the mode of action proceeds in a processive manner as cellobiohydrolase progresses along the cellulose chain (Boisset et al. 2000; Henrissat 1998; Reverbel-Leroy et al. 1997).

Cellobiohydrolases or exoglucanases is able to work effectively on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri 1997). In general, CBHs are active on the crystalline regions of cellulose; whereas, endoglucanases (1,4-β-glucanases) are typically active on the more soluble amorphous region of the cellulose crystal (Rouvinen et al. 1990; Divne et al. 1998). It seems that there is a high degree of synergy observed between exo- and endoglucanases, which is required for efficient hydrolysis of cellulose crystals (Abdel-Shakour and Roushdy 2009).

2.5 Substrates and Assay Methods

In general, enzymes in cellulase mixture which show relatively high activity on Avicel and little activity on carboxymethyl cellulose (CMC) are identified as exoglucanases (CBHs) (Maki et al. 2009). The processive activity of CBHs on CMC is blocked or inhibited by the occurrence of derivatized glucose residues which leads to severe substrate limitation. Avicel (microcrystalline cellulose or hydrocellulose) is mainly used for measuring exoglucanase activity because of its low degree of polymerization. However, Avicel contains some amorphous cellulose and soluble cellodextrans which can act as substrates for both exo- and endoglucanases. Thus, there is no highly specific substrate to measure exoglucanase activity (Wood and Bhat 1988).

There are several different assays have been proposed to measure exoglucanase (CBH I and CBH II) activity; however, all of these assays have some sort of limitations. CBHs acting from nonreducing end of the substrate can be assayed with chromogenic arylcellobiosides, from which the chromogenic aryl residue, attached to the reducing end of the substrate liberated. However, the processive enzymes acting from the reducing end of the carbohydrate chain have been identified recently, but chromogenic substrates to identify these enzymes are not yet commercially available (Boisset et al. 1998). Measurement of the production of cellobiose directly via high-pressure liquid chromatography (Gum and Brown 1976) or via a cellobiose oxidoreductase assay (Kelleher et al. 1987) is complicated in that the endoglucanases that are present in crude cellulase mixtures also produce cellobiose. The substrate 4-methylumbelliferyl-β-D-lactoside was identified as an effective substrate for assaying CBH I of *T. reesei* as lactose, phenol and 4-methylumbelliferone (a fluorescent signal molecule) are the hydrolysis products (van Tilbeurgh et al. 1985). But, the activity of CBH II was not able to detect thus the resulting activity is not an effective representation of true exoglucanase activity. Alternatively, an assay for quantification of exoglucanase activity using p-nitrophenyl-β-D-cellobioside as substrate to yield cellobiose and *p*-nitrophenol has been developed; but (i) CBH II activity cannot be measured using *p*-nitrophenyl-β-D-cellobioside, (ii) the specific activity of the available purified endoglucanases may not be representative for all existing endoglucanases in the mixture, and (iii) the product ratio from endoglucanase actions may be influenced by the presence of exoglucanases (Deshpande et al. 1984).

A double-antibody sandwich enzyme-linked immunosorbent assay was developed for quantifying CBH I in cellulase complex of *T. reesei*. The antibody configuration resulted in the highest specificity for the assay of CBH I employed a monoclonal antibody to coat wells in polystyrene plates and peroxidase-labeled polyclonal antibody to detect CBH I bound to the immobilized monoclonal antibody. Since there was no direct assay of CBH I activity in the presence of the other enzymes in complex till date, it would be useful for quantifying this enzyme between the ranges of 0.1–0.8 μg/ml (Riske et al. 1990).

2.6 Recent Developments

Recently, the cellulolytic yeast (*Saccharomyces cerevisiae*) with enhanced CBH activity was developed by integrating three types of CBH-encoding genes (cbh1 from *Aspergillus aculeatus*, cbh1, and cbh2 from *T. reesei*) with a strong constitutive promoter Ptpi were sequentially integrated into the *S. cerevisiae* W303-1A chromosome via δ-sequence mediated integration. The three recombinant (W1, W2, and W3) and control strains (W303-1A and AADY) produced ethanol (g/l) from acid-and alkali-pretreated corncob was about 5.92 ± 0.51, 18.60 ± 0.81, 28.20 ± 0.84, 1.40 ± 0.12, and 2.12 ± 0.35, respectively (Hong et al. 2014).

The thermostable CBHs could offer potential benefits in the hydrolysis of pretreated lignocellulosic substrates because the harsh conditions often required by several pretreatments can be harmful for conventional biocatalysts. Heinzelman et al. (2010) investigated an efficient SCHEMA recombination-based approach for screening homologous enzymes to identify stabilizing amino acid sequence blocks which has been used to generate active, thermostable CBH I enzymes from the 390,625 possible chimeras that can be made by swapping eight blocks from five fungal homologs. A total of 16 predicted thermostable chimeras, with an average of 37 mutations relative are more thermostable (>65 °C) than the most stable parent CBH I of thermophilic fungus *Talaromyces emersonii*.

Likewise, Komor et al. (2012) attempted to produce thermostable chimeric fungal CBH I by structure-guided recombination through FoldX and a ‘consensus’ sequence approach to identify individual mutations present in the five homologous parent CBH I enzymes which further stabilize the chimeras. With an alignment of 41 CBH I sequences, effects on ΔG (Folding) was calculated using amino acid frequencies at each candidate position and the mutations chosen using these methods increased the $T(50)$ of the most thermostable chimera by an additional 4.7 °C, to yield a CBH I with $T(50)$ of 72.1 °C, which is 9.2 °C higher than that of native CBH I, from *Talaromyces emersonii*.

2.7 Perspectives

The interest in enzymatic hydrolysis of cellulosic biomass for biofuel production is increasing continuously due to availability and reutilization as cheaper source. CBHs are one among the important enzymes which could produce more sugars such as glucose and cellobiose from crystalline cellulose. (i) It is essential to develop easy and suitable method with specific substrate in order to measure the CBHs activity in the cellulose mixture. (ii) Engineering of CBHs is also needed in order to enhance the specific activity to reduce the use of CBHs and also to increase thermostability and reusability of exoglucanases. These approaches mentioned above to enhance the activity and stability of CBHs would be useful in biorefinery and biofuel industries.

References

Abdel-Shakour EH, Roushdy MM (2009) An investigation for cellulase activity of a novel antibiotic producing *Streptomyces* sp. isolate H-1 from Egyptian mangrove sediment. Acad Arena 1(5):89–98

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Boer H, Teeri TT, Koivula A (2000) Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol Bioeng 5:486–494

Boisset C, Armand S, Drouillard S, Chanzy H, Driguez H, Henrissat B (1998) Structure–function relationships in cellulases: the enzymatic degradation of insoluble cellulose. In: Claeyssens M, Nerinckx W, Piens KM (eds) Carbohydrases from Trichoderma reesei and other microorganisms. Royal Society of Chemistry, London, pp 124–132

Boisset C, Fraschini C, Schulein M, Henrissat B, Chanzy H (2000) Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. Appl Environ Microbiol 66:1444–1452

Claeyssens S, Lavoinne A, Freselragot M, Bois-Joyeux B, Chanez M, Peret J (1990) Metabolic changes in rats fed a low protein-diet during post-weaning growth. Metabol Clin Exp 39:676–681

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues: opportunities and perspectives. Int J Biol Sci 5:578–595

Deshpande MV, Eriksson KE, Pettersson LG (1984) An assay for selective determination of exo-1,4-beta-glucanases in a mixture of cellulolytic enzymes. Anal Biochem 138:481–487

Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Petterson G, Knowles JK, Teeri TT, Jones TA (1994) The 3-dimensional crystal-structure of the catalytic core of cellobiohydrolase-I from *Trichoderma reesei*. Science 265:524–528

Divne C, Stahlberg J, Teeri TT, Alwyn JT (1998) High-resolution crystal structures reveal how a cellulose chain is bound in the 50 angstrom long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J Mol Biol 275:309–325

Gum EK Jr, Brown RD Jr (1976) Structural characteristics of a glycoprotein cellulase, 1,4-β-D-glucan cellobiohydrolase from *Trichoderma viride*. Biochim Biophys Acta 446:371–376

Heinzelman P, Komor R, Kanaan A, Romero P, Yu X, Mohler S, Snow C, Arnold F (2010) Efficient screening of fungal cellobiohydrolase class I enzymes for thermostabilizing sequence blocks by SCHEMA structure-guided recombination. Protein Eng Des Sel 23(11):871–880

Henrissat B (1998) Enzymatic cellulose degradation. Cellul Commun 5:84–90

Hong J, Yang H, Zhang K, Liu C, Zou S, Zhang M (2014) Development of a cellulolytic *Saccharomyces cerevisiae* strain with enhanced cellobiohydrolase activity. World J Microbiol Biotechnol 30:2985–2993

Irwin DC, Spezio M, Walker LP, Wilson DB (1993) Activity studies of eight purified cellulases: specificity, synergism and binding domain effects. Biotechnol Bioeng 42:1002–1013

Kelleher TJ, Montenecourt BS, Eveleigh DE (1987) Cellobiose-quinone oxidoreductase—application in monitoring cellobiohydrolase purification. Appl Microbiol Biotechnol 27:299–305

Koivula A, Ruohonen L, Wohlfahrt G, Reinikainen T, Teeri TT, Piens K, Claeyssens M, Weber M, Vasella A, Becker D, Sinnott ML, Zou J-Y, Kleywegt GJ, Szardenings M, Stahlberg J, Jones TA (2002) The active site of cellobiohydrolase Cel6A from *Trichoderma reesei*: the roles of aspartic acids D221 and D175. J Am Chem Soc 124:10015–10024

Komor RS, Romero PA, Xie CB, Arnold FH (2012) Highly thermostable fungal cellobioh ydrolase I (Cel7A) engineered using predictive methods. Protein Eng Des Sel 25(12):827–833

Takahashi M, Takahashi H, Nakano Y, Konishi T, Terauchi R, Takeda T (2010) Characterization of a cellobiohydrolase (MoCel6A) Produced by *Magnaporthe oryzae*. Appl Environ Miccrobiol 76(19):6583–6590

Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 5(5):500–516

Mazzoli M, Lamberti C, Pessione E (2012) Engineering new metabolic capabilities in bacteria: lessons from recombinant cellulolytic strategies. Trends Biotechnol 30(2):111–119

Medve J, Karlsson J, Lee D, Tjerneld F (1998) Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. Biotechnol Bioeng 59(5):621–634

Nidetzky B, Steiner W, Hayn M, Claeyssens M (1994) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. Biochem J 298:705–710

Reverbel-Leroy C, Page S, Belaich A, Belaich JP, Tardif C (1997) The processive endocellulase CelF, a major component of the *Clostridium cellulolyticum* cellulosome: purification and characterization of the recombinant form. J Bacteriol 179:46–52

Riske FJ, Eveleigh DE, Macmillan JD (1990) Double-antibody sandwich enzyme-linked immunosorbent assay for cellobiohydrolase I. Appl Environ Microbiol 56(11):3261–3265

Rouvinen J, Bergfors T, Teeri TT, Knowles JKC, Jones TA (1990) Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science 249:380–386

Stahlberg J, Divne C, Koivula A, Piens K, Claeyssens M, Teeri TT (1996) Activity studies and crystal structures of catalytically deficient mutants of cellobiohydrolase I from *Trichoderma reesei*. J Mol Biol 264:337–349

Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen H (1993) High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. Mol Gen Genet 241(5–6):523–530

Teeri TT (1997) Crystalline cellulose degradation: new insights into the function of cellobiohydrolases. Trends Biotechnol 15:160–167

van Tilbeurgh H, Pettersson G, Bhikabhai R, De Boeck H, Claeyssens M (1985) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-beta-glucan cellobiohydrolase II. Eur J Biochem 148:329–334

Varrot A, Frandsen TP, Driguez H, Davies GJ (2002) Structure of the *Humicola insolens* cellobiohydrolase Cel6A D416A mutant in complex with a non-hydrolysable substrate analogue, methyl cellobiosyl-4-thio-β-cellobioside at 1.9 Å. Acta Crystallogr D Biol Crystallogr 58:2201–2204

Vocadlo DJ, Davies GJ (2008) Mechanistic insights into glycosidase chemistry. Curr Opin Chem Biol 12:539–555

Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–117

Wood TM (1992) Fungal cellulases. Biochem Soc Trans 20:46–53

Zhang XZ, Zhang YHP (2013) Cellulases: characteristics, sources, production, and applications. In: Yang S-T, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, New York, pp 131–146

Chapter 3
Endo-1,4-β-glucanases: Role, Applications and Recent Developments

Neelamegam Annamalai, Mayavan Veeramuthu Rajeswari and Thangavel Balasubramanian

Abstract The biomass conversion processes are highly dependent on the use of efficient enzymes to degrade polymeric cellulose or hemicellulose into simple saccharides, sugars which can be fermented by microorganisms for the production of valuable fuel and chemicals. The cellulose hydrolysis involves enzymes such as endo-1,4-β-glucanases (EC 3.2.1.4), cellobiohydrolases (or exo-1,4-β-glucanases) (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Endo-β-(1,4)-glucanases (EGs) or β-(1,4)-D-glucan-4-glucanohydrolases (EC 3.2.1.4), which act randomly on soluble and insoluble β-(1,4)-glucan substrates. EGs breakdown cellulose by attacking the amorphous regions to produce more accessible new free chain ends for the action of cellobiohydrolases. The EG-I, II, III, and V, respectively, GH7, 5, 12, and 45 are most common in natural fungal cellulase mixes. EGs play an important role in increasing yield of fruit juices, beer filtration, and oil extraction, as well as improving the nutritive quality of bakery products and animal feed. Reducing sugar assay is the most convenient and reliable method for EG estimation. However, it is very conventional and time consuming. Recently, a specific and sensitive assay methods have been developed using substrate mixture comprises of benzylidene end-blocked 2-chloro-4-nitrophenyl-β-cellotrioside (BzCNPG3) and 4,6-*O*-benzylidene-4-methylumbelliferyl-β-cellotrioside (BzMUG3).

N. Annamalai (✉)
Hawaii Natural Energy Institute, University of Hawaii at Manoa,
1680, East-West Road, Honolulu 96822, HI, USA
e-mail: annabact@gmail.com

M.V. Rajeswari
Centre for Ocean Research, Sathyabama University, Jeppiar Nagar,
Chennai 600119, TN, India

T. Balasubramanian
CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University,
Parangipettai 608502, TN, India

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_3

3.1 Introduction

Lignocellulosic biomass contains morphologically different cellulose, complex structural and compositional hemicellulose, recalcitrant lignin, diverse proteins and lipids and other substances that interact with each other (Sweeney and Xu 2012). Cellulose is a long homogenous linear polymer of β-D-glucosyl units linked by 1,4-β-D-glucosidic bonds. Over the last few years, the growing challenge has been the setup of industrial-scale conversion of cellulosic biomass into fermentable sugars, which can in turn be used as raw material for the production of biofuels and other bio based high value-added products (Adsul et al. 2011; Du et al. 2011; Somerville et al. 2010). Efficient conversion of cellulosic biomass into fuels and other products is a fossil fuel-saving and environment-friendly biotechnology (Lange 2007). The use of enzymes reduces the harsh chemicals and/or heat for the production of fermentable sugars, raising the environmental feasibility of the process.

The primary role of biomass-converting enzymes is to degrade polymeric cellulose or hemicellulose into simple saccharides, sugars, which can be fermented by microorganisms to, or serve as platform molecules for synthesis of valuable fuel or chemicals. As potential industrial catalysts for biomass conversion, enzymes might provide high specificity, low energy or chemical consumption, or low environment pollution (Sweeney and Xu 2012). Cellulases and most hemicellulases belong to a group of enzymes known as glycoside hydrolases (GH), which can degrade cellulose and hemicellulose to constituent hexoses and pentoses. In general, biomass-converting enzymes have to work in concert, to benefit from synergism among their specificity (towards different components and regions of lignocellulose) as well as mitigation of their inhibition (by different lignocellulose components or degradation products) (Sweeney and Xu 2012). The classical scheme for cellulose hydrolysis involves three main categories of enzymes: endo-1,4-β-glucanases (EC 3.2.1.4), cellobiohydrolases (or exo-1,4-β-glucanases) (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The complete degradation of cellulose into glucose is a complex process due to its amorphous/crystalline structure, which requires the cooperative action of endo and exo-β-1,4-glucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). According to CAZy database (http://www.cazy.org), these enzymes are grouped into 15 of 132 glycoside hydrolase (GH) families, i.e., 1, 3, 5–9, 12, 27, 44, 45, 48, 51, 61 and 74 (Wang et al. 2014).

In this respect, the most important enzyme has long been realized to be an endo-β-1-3,1-4-glucanase (EC 3.2.1.73), which hydrolyzes β-1-4 linkages on the reducing side of β-1-3 linkages to produce primarily of oligosaccharides with contiguous β-1-4 linkages that derive from cellulosic regions in the polymer as substrates for an endo-β-1-4-glucanase (Kanauchi and Bamforth 2008).

3.2 Endo-1,4-β-glucanases (EC 3.2.1.4, Endocellulase)

Endo-β-(1,4)-glucanases or β-(1,4)-D-glucan-4-glucanohydrolases (EC 3.2.1.4) act randomly on soluble and insoluble β-(1,4)-glucan substrates. The endo-1,4-β-glucanase (EGase, EC 3.2.1.4) often referred to as cellulase, hydrolyzes 1,4-β-D-glucan chain molecules and plays a role in various physiological processes (Yu et al. 2013). Endoglucanases (EG) are also referred to as carboxymethylcellulases (CMCase), named after the artificial substrate used to measure the enzyme activity. The active sites of endoglucanases typically attain a cleft-like topology for random fashion and bond cleavage can occur anywhere along the chain of the cellulose (Himmel et al. 2010). Endoglucanase breakdown the cellulose by attacking the amorphous regions of the cellulose to produce more accessible new free chain ends for the action of cellobiohydrolases which has been shown by the effect of the enzyme on carboxymethylcellulose and amorphous cellulose (Sandgren et al. 2005).

Degradation of amorphous cellulose can be carried out by endoglucanases (EGs) (EC 3.2.1.4). Unlike cellobiohydrolases (CBH), EG hydrolyzes internal glycosidic bonds in cellulose with a random, on-off fashion which make them well-suited to less orderly or partially shielded cellulose parts, generating new cellulose chain ends for CBH action. A few EGs can act "processively" on crystalline cellulose (Wilson 2008; Li et al. 2010). Also known as EG-I, II, III and V, respectively, GH7, 5, 12 and 45 EG are most common in natural fungal cellulase mixes. EGs genes are found in many prokaryotic and eukaryotic organisms, including bacteria, fungi, slime molds, nematodes, animals and plants (Levy et al. 2002; Libertini et al. 2004). Most cellulolytic fungi and bacteria produce numerous EGs. Although they all act on the same cellulose substrate, they do with different mechanisms ("inverting" for GH6, 9, 45 and 48 EGs; "retaining" for GH5, 7, 12 EGs), which may relate to different EGs' side-activities on hemicellulose in degrading complex lignocellulose (Vlasenko et al. 2010), or synergism between processive and conventional EGs (Wilson 2008). The active sites of most EGs are cleft- or groove-shaped, inside which a cellodextrin or a cellulose segment may be bound and acted on by EG. In addition to the catalytic core, EGs may possess CBMs which is the direct host, but is not a pre-requisite, for EG's action (Sweeney and Xu 2012).

The fungal endoglucanases (EGs) are generally monomers with no or low glycosylation and have an open binding cleft. They mostly have pH optima between 4 and 5 and temperature optima from 50 to 70 °C. A typical cellulolytic fungus secretes EGs at ~20 % wt level in their secretomes (Sipos et al. 2010; Chundawat et al. 2011). In addition, many of the fungi produce multiple EGs. For example, *T. reesei* produces at least 5 EGs (EG I/Cel 7B, EG II/Cel5A, EG III/Cel12A, EG IV/Cel61A and EG V/Cel45A) whereas three EGs were isolated from white-rot fungus *P. chrysosporium* (EG28, EG34 and EG44) (Baldrian and Valaskova 2008; Foreman et al. 2003). In addition, some EGs lack a CBM while some other EGs with CBM have been described. For example, four of five EGs in

T. reesei including EG I, EG II, EG IV and EG V have CBM whereas EG III does not have a CBM (Sandgren et al. 2005).

In plants, EGases are encoded by a large gene family, belonging to the glycosyl hydrolase gene family 9 (GH9) (Henrissat 1991). According to their sequence domain structure, this multigene family can be grouped into three subclasses: A subclass (GH9A), membrane-anchored GH9; B subclass (GH9B), secreted GH9; and C subclass (GH9C), secreted proteins with a carbohydrate binding module, CBM49 (Urbanowicz et al. 2007). Alternatively, the plant EGase gene family can be divided into α, β and γ subfamilies based on phylogenetic analysis (Libertini et al. 2004). In Arabidopsis, the α-subfamily mainly consists of secreted GH9 proteins (including GH9B and GH9C subclasses) except for one GH9A subclass protein. All γ-subfamily members are transmembrane proteins, belonging to GH9A subclass (Yu et al. 2013).

3.3 Criteria for Optimized EG Production

In general, cellulase production accounts for 40 % of cost in bioethanol synthesis (Sateesh et al. 2012). Carbon and nitrogen sources are the important factors for endoglucanase production and synthesis of various cell wall lipids in fungi (Sarria-Alfonso et al. 2013). The effects of different carbon sources (avicel, cellobiose, CMC, xylan, rice straw, bagasse and palm kernel) on EG production by various microorganisms were investigated till date. Among these, rice straw was reported to be a cheaper and potential sole carbon source and yeast extract as the nitrogen source for higher EG production. In addition, a significant increase in enzyme production with an increased fermentation time, which was presumed to be due to rapid hydrolysis of cellulose in the medium.

3.4 Industrial Applications of Endo-β-1,4-Glucanase

Cellulases are used in the textile industry, in detergents, pulp and paper industry, improving digestibility of animal feeds and food industry. They account for a significant fraction of industrial enzyme markets. Obviously, cellulase will be the largest industrial enzyme as compared to the current market size for all industrial enzymes ($ ~2 billion) (Zhang and Zhang 2013). Among the cellulases, endoglucanases play a key role in increasing the yield of fruit juices, beer filtration and oil extraction, as well as improving the nutritive quality of bakery products and animal feed (Bhat 2000). Endo-β-1,4-glucanase has been widely used in industries to hydrolyze cellulosic materials to sugars in the biofuel production (Wilson 2009), to increase the rate of wort filtration and reduce the possibility of β-glucan precipitation in beer and reduce the mash viscosity and turbidity in the brewing industry (Celestino et al. 2006), to bioremediate pulp waste in the paper industry

(Ohmiya et al. 1997), and to increase β-glucan digestibility and improve feed conversion efficiency in the feed industry (Mathlouthi et al. 2002). The endo-1,4-β-glucanase is also used in the poultry and animal feed industry to degrade β-glucan and thus increase the digestibility of feeds rich in barley and also overcome the anti-nutritive effects of 1,3-1,4-β-D-glucan (Choct 2001). Further, endo-1,4-β-glucanase is used for denim finishing and cotton softening in textile industries, cleaning and colour care in the detergent industries and to modify fibre and improves drainage in the paper industries (Cherry and Fidantsef 2003).

Thus, the enormous industrial applications demands endoglucanases with varying pH and temperature optima, stabilities and substrate specificities. Although cellulolytic enzymes of *Trichoderma reesei* have been investigated thoroughly (Miettinen-Oinonen et al. 2004), the amount of cellulase secreted by this fungus is insufficient for effective conversion of cellulose to glucose. Moreover, the industrial demand is increasing day by day especially because of the emergence of second generation-advanced biofuel industries, which require tremendous amounts of various enzymes in their processes (Wilson 2009; Yeoman et al. 2010).

Thermostable endoglucanases/cellulases are more favourable for industrial application due to their high activity and stability at high temperatures to decrease process costs and increase the efficiencies (Yeoman et al. 2010). However, most cellulases are not stable at high temperatures, and a number of thermostable cellulases have been purified or cloned and characterized in recent years, some of them display maximum activities even at 100 °C, such as GH 12 endoglucanase from *Pyrococcus furiosus* (100 °C; Bauer et al. 1999), and endoglucanase CelB from *Thermotoga neapolitana* (106 °C; Bok et al. 1998). As the main microbial source of thermophilic endoglucanases, thermophilic fungi like *Talaromyces emersonii* and *Thermoascus aurantiacus* have been reported to produce cellulases of GH 3, 5 and 7 families with temperature optima of 65–80 °C (Gomes et al. 2000; Grassick et al. 2004).

3.5 Recent Developments on Endo-1,4-β-Glucanase Assay

The endo-1,4-β-glucanase in biological materials and microbial fermentation broths can be specifically measured by the decrease in the viscosity of water soluble, chemically modified cellulose derivatives such as CM-cellulose 7 M (McCleary et al. 2012). The reducing sugar assays method is most conventional and time consuming (Somogyi 1952). The other methods such as measuring hydrolysis rate of substrates such as cello-oligosaccharides, borohydride-reduced cello-oligosaccharides (McCleary et al. 2012) or nitrophenyl-cello-oligosaccharides (Bhat et al. 1990; Claeyssens and Henrissat 1992; Rahman et al. 2002) cannot be used to measure endo-1,4-β-glucanase in the presence β-glucosidase and exo-1,4-β-glucanases. Hence, a specific and sensitive substrate mixture comprises benzylidene end-blocked 2-chloro-4-nitrophenyl-β-cellotrioside (BzCNPG3) was prepared as substrate for the assay of endo-1,4-β-glucanase (cellulase). Hydrolysis by β-glucosidase and exo-1,4-β-glucanase is

prevented by the presence of the benzylidene group on the non-reducing end D-glucosyl residue (McCleary et al. 2014).

In addition to this, novel fluorometric assay for endo-1,4-β-glucanase that is based on the use of 4,6-*O*-benzylidene-4-methylumbelliferyl-β-cellotrioside (BzMUG3) in the presence of an ancillary β-glucosidase was developed for both quantitative and qualitative measurements. The substrate BzMUG3 can be cleaved in the presence of cellulase to generate a protected cello-oligosaccharide fragment and a fluorogenic cello-oligosaccharide which will then be acted on by excess β-glucosidase (EC 3.2.1.21) present in the reagent mixture to liberate 4-methylumbelliferone and 4,6-*O*-benzylidene protecting group prevents the action of β-glucosidase on the parent substrate. The rate of formation of 4-methylumbelliferone is directly proportional to the cellulase activity in a given sample (Mangan et al. 2014).

3.6 Future Prospective

Biomass saccharification is the largest technical and economic obstacle to biorefinery and biofuels production. For the efficient conversion of cellulose from biomass and to glucose for the subsequent production of fuel ethanol, protein engineering of endo-1,4-β-glucanase should be focused to enhance specific activity towards pre-treated biomass using enzyme cocktail and either rational design or directed evolution (Sathitsuksanoh et al. 2010; Zhang 2008), improve the stability for recycling and reduce production costs ($ per kilogram of dry protein) (Zhu et al. 2009). In addition, engineering of cellulolytic enzymes with improved catalytic efficiency and enhanced thermostability is important to commercialize lignocelluloses biorefinery and further improvement on cellulase performance needs the better understanding of hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function and substrate characteristics. The consolidated bioprocessing (CBP) of microorganisms or consortium would make the biomass hydrolysis easier and enhance the productivity. The improved simultaneous saccharification and fermentation (SSF) technology would be useful to reduce end-product inhibition, investment costs and higher yield efficient biofuel production.

References

Adsul MG, Singhvi MS, Gaikaiwari SA, Gokhale DV (2011) Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. Bioresour Technol 102:4304–4312

Baldrian P, Valaskova V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501–521

Bauer MW, Driskill LE, Callen W, Snead MA, Mathur EJ, Kelly RM (1999) An endoglucanase, EglA, from the hyperthermophilic archaeon *Pyrococcus furiosus* hydrolyzes β-1, 4 bonds in mixed-linkage (1–3), (1–4)-β-d-glucans and cellulose. J Bacteriol 181:284–290

Bhat KM, Hay AJ, Claeyssens M, Wood TM (1990) Study of the mode of action and site-specificity of the endo-(1-4)-beta-D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-3H-labelled, reduced and chromogenic cello-oligosaccharides. Biochem J 266:371–378

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Bok JD, Yernool DA, Eveleigh DE (1998) Purification, characterization, and molecular analysis of thermostable cellulases CelA and CelB from *Thermotoga neapolitana*. Appl Environ Microbiol 64:4774–4781

Celestino KRS, Cunha RB, Felix CR (2006) Characterization of a β-glucanase produced by *Rhizopus microsporus var. microsporus*, and its potential for application in the brewing industry. BMC Biochem 7:23

Cherry JR, Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol 14:438–443

Choct M (2001) Enzyme supplementation of poultry diets based on viscous cereals. In: Bedford M, Partridge G (eds) Enzymes in farm animal nutrition. CABI Publishing, Oxon, pp 145–160

Chundawat SPS, Lipton MS, Purvine SO, Uppugundla N, Gao D, Balan V, Dale BE (2011) Proteomics-based compositional analysis of complex cellulase-hemicellulase mixtures. J Proteome Res 10:4365–4372

Claeyssens M, Henrissat B (1992) Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. Protein Sci 1: 1293–1297

Du J, Shao Z, Zhao H (2011) Engineering microbial factories for synthesis of value-added products. J Ind Microbiol Biotechnol 38:873–890

Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, Meerman HJ, Mitchell T, Mitchinson C, Olivares HA, Teunissen PJ, Yao J, Ward M (2003) Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. J Biol Chem 278:31988–31997

Gomes I, Gomes J, Gomes DJ, Steiner W (2000) Simultaneous production of high activities of thermostable endoglucanase and β-glucosidase by the wild thermophilic fungus *Thermoascus aurantiacus*. Appl Microbiol Biotechnol 53:461–468

Grassick A, Murray PG, Thompson R, Collins CM, Byrnes L, Birrane G, Higgins TM, Tuohy MG (2004) Three-dimensional structure of a thermostable native cellobiohydrolase, CBH IB, and molecular characterization of the cel7 gene from the filamentous fungus, *Talaromyces emersonii*. Eur J Biochem 271:4495–4506

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Himmel ME, Xu Q, Luo Y, Ding SY, Lamed R, Bayer EA (2010) Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels 1(2):323–341

Kanauchi M, Bamforth CW (2008) The relevance of different enzymes for the hydrolysis of β-glucans in malting and mashing. J Inst Brew 114(3):224–229

Lange JP (2007) Lignocellulose conversion: an introduction to chemistry, process and economics. Biofuel Bioprod Biorefin 1:39–48

Levy I, Shani Z, Shoseyov O (2002) Modification of polysaccharides and plant cell wall by endo-1,4-beta-glucanase and cellulose-binding domains. Biomol Eng 19:17–30

Li Y, Irwin DC, Wilson DB (2010) Increased crystalline cellulose activity via combinations of amino acid changes in the family 9 catalytic domain and family 3c cellulose binding module of *Thermobifida fusca* Cel9A. Appl Environ Microbiol 76:2582–2588

Libertini E, Li Y, McQueen-Mason SJ (2004) Phylogenetic analysis of the plant endo-beta-1,4-glucanase gene family. J Mol Evol 58:506–515

Mangan D, McCleary BV, Liadova A, Ivory R, McCormack N (2014) Quantitative fluorometric assay for the measurement of endo-1,4- β -glucanase. Carbohydr Res 395:47–51

Mathlouthi N, Mallet S, Saulinier L, Quemener B, Larbier M (2002) Effects of xylanase and beta-glucanase addition on performance, nutrient digestibility and physic-chemical conditions

in the small intestine contents and caecal microflora of broiler chickens fed a wheat and barley-based diet. Anim Res 51:395–406

McCleary BV, McKie V, Draga A (2012) Measurement of endo-1,4-β-glucanase. Methods Enzymol 510:1–17

McCleary BV, Mangan D, Daly R, Fort S, Ivory R, McCormack N (2014) Novel substrates for the measurement of endo-1,4-β-glucanase (endo-cellulase). Carbohydr Res 385:9–17

Miettinen-Oinonen A, Londesborough J, Joutsjoki V, Lantto R, Vehmaanpera J (2004) Three cellulases from *Melanocarpus albomyces* for textile treatment at neutral pH. Enzyme Microb Technol 34:332–341

Ohmiya K, Sakka K, Karita S, Kimura T (1997) Structure of cellulases and their applications. Biotechnol Genet Eng Rev 14:365–414

Rahman M, Bhuiyan SH, Nirasawa S, Kitaoka M, Hayashi KJ (2002) Characterization of an endo-β-1,4-glucanase of *Thermotoga maritima* expressed in *Escherichia coli*. Appl Glycosci 49:487–495

Sandgren M, Stahlberg J, Mitchinson C (2005) Structural and biochemical studies of GH family 12 cellulases: improved thermal stability and ligand complexes. Prog Biophys Mol Biol 89: 246–291

Sarria-Alfonso V, Sierra JS, Morales MA, Rojas IG, Sarmiento NM, Pinales P (2013) Culture media statistical optimization for bio-mass production of a ligninolytic fungus for future rice straw degradation. Indian J Microbiol 53(2):199–207

Sateesh L, Rodhe A, Naseeruddin S, Yadav K, Prasad Y, Rao L (2012) Simultaneous cellulase production, saccharification and detoxification using dilute acid hydrolysate of *S. spontaneum* with *Trichoderma reesei* NCIM 992 and *Aspergillus niger*. Indian J Microbiol 52:258–262

Sathitsuksanoh N, Zhu Z, Ho T-J, Bai M-D, Zhang Y-HP (2010) Bamboo saccharification through cellulose solvent based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings. Bioresour Technol 101:4926–4929

Sipos B, Benko Z, Dienes D, Reczey K, Viikari L, Siika-aho M (2010) Characterisation of specific activities and hydrolytic properties of cell-wall-degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. Appl Biochem Biotechnol 161:347–364

Somerville C, Youngs H, Taylor C, Davis SC, Long SP (2010) Feedstocks for lignocellulosic biofuels. Science 13:790–792

Somogyi MJ (1952) Notes on sugar determination. Biol Chem 195:19–23

Sweeney MD, Xu F (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2:244–263

Urbanowicz BR, Bennett AB, Del Campillo E, Catala C, Hayashi T, Henrissat B, Hofte H, McQueen-Mason SJ, Patterson SE, Shoseyov O, Teeri TT, Rose JKC (2007) Structural organization and a standardized nomenclature for plant endo-1,4-beta-glucanases (cellulases) of glycosyl hydrolase family 9. Plant Physiol 144:1693–1696

Vlasenko E, Schulein M, Cherry J, Xu F (2010) Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour Technol 101:2405–2411

Wang K, Luo H, Bai Y, Shi P, Huang H, Xue X, Yao B (2014) A thermophilic endo-1,4-β-glucanase from *Talaromyces emersonii* CBS394.64 with broad substrate specificity and great application potentials. Appl Microbiol Biotechnol 98:7051–7060

Wilson DB (2008) Three microbial strategies for plant cell wall degradation. Ann N Y Acad Sci 1125:289–297

Wilson DB (2009) Cellulases and biofuels. Curr Opin Biotechnol 20(3):295–299

Yeoman CJ, Han Y, Dodd D, Schroeder CM, Mackie RI, Cann IK (2010) Thermostable enzymes as biocatalysts in the biofuel industry. Adv Appl Microbiol 70:1–55

Yu L, Sun J, Li L (2013) PtrCel9A6, an endo-1,4-β-glucanase, is required for cell wall formation during xylem differentiation in populous. Mol Plant 6(6):1904–1917

Zhang Y-HP (2008) Reviving the carbohydrate economy via multi-product biorefineries. J Ind Microbiol Biotechnol 35:367–375

Zhang XZ, Zhang Y-HP (2013) Cellulases: characteristics, sources, production, and applications. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals and polymers. Wiley, New York, pp 131–146

Zhu Z, Sathitsuksanoh N, Zhang P (2009) Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulase desorption for potential recycling. Analyst 134:2267–2272

Chapter 4
The Role and Applications of β-Glucosidases in Biomass Degradation and Bioconversion

Hanlin Ouyang and Feng Xu

Abstract β-Glucosidases is known as the terminal enzyme in the synergy with other cellulases for biomass degradation. It completes the final step of small oligosaccharides (including cellobiose) conversion into glucose. However, this is only a small part of the roles played by a subgroup of bacterial/fungal β-glucosidases in cellulose biodegradation. To deepen our understanding about the current challenges and limits in biomass conversion and to enlighten the future for the industry, we take one step back and look into a broader range of β-glucosidases that cross the life domains. β-Glucosidases from different subfamilies are compared systematically in their distribution, phylogenetic relationship, structure, in vivo function, mechanism of hydrolysis as well as substrate recognition, and kinetic profile. Protein engineering and application works on the enzymes are discussed based on the knowledge herein.

4.1 Introduction

Biomass industry nowadays is developing and optimizing enzymatic solutions to hydrolyze lignocellulosic plant-based substrates into monomeric sugars, which are eventually fermented to bioethanol. Biomass substrate composition can be simplified as cellulose, hemicellulose, and lignin in general while each component is targeted by different enzymes. Glucan, or cellulose, consists of up to more than 40 % of biomass substrates, and therefore cellulases are major players in the enzymatic hydrolysis of glucan. Cellulases refer to a synergistic collection of three different groups of enzyme: cellobiohydrolase (CBH, exo-β-1,4-glucanase), endo-β-1,4-glucanase (EG), and β-glucosidase (BGL). While the exo- and endoglucanases cleave poly-

H. Ouyang (✉)
Novozymes North America Inc., 77 Perrys Chapel Church Road, PO BOX 576, Franklinton, NC 27525, USA
e-mail: hou@novozymes.com

F. Xu
Novozymes Inc., 1445 Drew Ave, Davis, CA 95618, USA
e-mail: fxu@novozymes.com

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_4

meric cellulose into small oligosaccharides (cellodextrins), BGL is responsible to release glucose (Glc) from these oligosaccharides, especially cellobiose. In addition, different BGLs can have activity on many other glycosides, releasing the nonreducing terminal glycosyl moiety from these substrates, which may be involved in various physiological events (Ketudat Cairns and Esen 2010). The 'Second Generation' (cellulosic) bioethanol industry locks their preference on BGLs from microbes that feed on biomass (Rani et al. 2014; Sørensen et al. 2013; Teugjas and Väljamäe 2013; Tiwari et al. 2013; Singhania et al. 2013; Sweeney and Xu 2012; Krisch et al. 2010; Quinlan et al. 2010; Xu 2004, 2010a, b; Eyzaguirre et al. 2005; Bhatia et al. 2002). The development of viable industrial BGLs has been challenging. Native BGLs produced in many fungal strains occur in low abundance, which needs to be greatly enhanced for the enzymes to become economically viable for industrial application. The BGL's product inhibition by Glc can be significant during the industrial enzymatic cellulose hydrolysis. Besides, biomass-based ethanol producers rely on thermochemical pretreatment of lignocellulosic substrates to improve enzyme access to cellulose and cellulose hydrolysis often occurs at elevated temperature to speed up the process. These two steps create harsh conditions for BGL's thermostability and inhibition resistance while the challenges have propelled active studies on BGL.

4.2 Classification and Distribution

Historically, BGLs have been categorized into three groups based on the substrates: aryl-BGLs, true cellobiases, and broad substrate specificity BGLs. Canonical BGLs that catalyze the hydrolysis of β-glucopyranosides are assigned with the Enzyme Commission (E.C.) number E.C.3.2.1.21. However, given the variety of β-linkage terminal nonreducing glycosides in nature, many E.C. numbers have been assigned to a larger group of enzymes that hydrolyze β-linkage glycosidic bond. For glucan substrates, glucan 1,3-β-glucosidase (3.2.1.58) and glucan 1,4-β-glucosidase (3.2.1.74) remove successively Glc from the nonreducing end with 1,3 and 1,4 β-linkage to the polysaccharide chain. Enzymes with hydrolytic activity on β-glucosides with aglycon moieties also fall into E.C.3.2.1 family. These include glucosylceramidase (3.2.1.45), steryl-β-glucosidase (3.2.1.104), 3α(S)-strictosidine β-glucosidase (3.2.1.105), amygdalin-β-glucosidase (3.2.1.117), prunasin β-glucosidase (3.2.1.118), vicianin β-glucosidase (3.2.1.119), raucaffricine β-glucosidase (3.2.1.125), coniferin β-glucosidase (3.2.1.126), and β-D-glucopyranosyl abscisate β-glucosidase (3.2.1.175). More complicated substrates like thio-linkage, 7-[β-D-apiofuranosyl-(1, 6)-β-D-glucopyranosyloxy]isoflavonoid, β-D-galactopyranose-(1, 4)-α-L-galactopyranose-6-sulfate in porphyran, or even 3-*O*-(*N*-acetyl-β-D-glucosaminyl)-L-serine/threonine in proteins can be hydrolyzed by thioglucosidase (myrosinases) (3.2.1.147), β-apiosyl-β-glucosidase (3.2.1.161), β-porphyranase (3.2.1.178) and protein *O*-GlcNAcase (3.2.1.169) respectively.

E.C. numbering system is a classification based on the chemical reactions which enzymes catalyze. It has little information on structure, mechanism, or phylogenetic

relationship. A sequence-based classification has been developed since 1996. The glycoside hydrolases (GH) are currently classified into 133 families in the Carbohydrate Active enZyme, or CAZy database (www.cazy.org). Each GH family contains proteins that are related by sequence, corollary, folding (based on the hydrophobic cluster similarity). This allows the possibility of useful mechanistic predictions as the motifs, key residues and geometry around the active sites usually share high similarity for a conserved enzymatic mechanism, even when the substrates (thus the E.C numbers) are different. Almost all the BGLs identified so far belong to the GH1, GH3, GH5, GH9, GH30, and GH116 families. Based on the CAZy as of June 2015, the GH1 family includes six BGLs from archaea, 194 from bacteria, 210 from eukaryotes (especially fungi and plants), one from a virus and six from unclassified organisms, along with 42 myrosinases and one glucosylceramidase from eukaryotes. The GH3 family includes one BGL from an archaeon, 118 from bacteria, 141 from eukaryotes (especially fungi and plants), ten from unclassified organisms, and several likely from animal (Gabrisko and Janecek 2015). The GH5 family includes one BGL from a virus, one from plant and four in fungi, while the GH30 family includes one BGL from a fungus, two from animals and one from human. The GH30 family also has two animal glucosylceramidases. The GH9 family so far only covers two BGLs from the bacterium *Vibrio cholera*.

Most of these BGLs are intracellular or cytoplasmic enzymes. However, some of them are membrane associated, such as bacterial *Pyrococcus horikoshii bgl* (GH1) (Matsui et al. 2000) and *Prevotella ruminicola cdx*A (GH3) (Wulff-Strobel and Wilson 1995). Some of them can reside in periplasmic space, such as bacterial *Azospirillum irakense salA, salB* (GH3) (Faure et al. 1999), *Bacillus subtilis bglA* (GH1) (Srivastava et al. 1999), *Erwinia chrysanthemi bglx* (GH3) (Vroemen et al. 1995), *Erwinia herbicola bglA* (GH1) (Marri et al. 1995) and fungal (mold) *Aspergillus kawachii bglA* (GH3) (Iwashita et al. 1999). Some can be extracellular, such as bacterial *Ruminococcus albus bgl* (GH3) (Takano et al. 1992), fungal *Aspergillus niger bgl1*(GH1) (Dan et al. 2000), *Trichoderma reesei* (*Hypocrea jecorina*) *bgl1* (GH3) *bgl2* (GH1) (Barnett et al. 1991; Takashima et al. 1999), *Saccharomycopsis fibuligera bgl1*, *bgl2* (both GH3) (Machida et al. 1988) and barley (*Hordeum vulgare*) bgq60 (GH1) (Leah et al. 1995), *Catharanthus roseus* strictosidine *bgl* (GH1) (Geerlings et al. 2000). The extracellular microbial BGLs are of particular industrial interest because of their production costs (that can be significantly lower than that of the intracellular counterparts).

4.3 In Vivo Function

4.3.1 Bacteria and Fungi

One of the functions for BGL is being a part of the synergistic cellulase system that breaks down cellulose, deployed by a large population of organisms including bacteria, fungi (molds, mushrooms, yeasts), and animals (e.g., termites). BGL is

used for energy/nutrition uptake in metabolism as polysaccharide is degraded into Glc. During the same process, solid surfaces of substrate like plant cell walls are penetrated to allow these organisms to establish pathogenic or symbiotic relationships (Gilbert et al. 2008). There are generally two strategies for how the secreted, cellulose-degrading enzymes work: to form a cell surface-anchored supramolecular complex called cellulosome, or enzymes acting as cell-free entities. The first case can been found with a collection of anaerobic cellulolytic bacteria like *Bacteroides, Clostridia,* and *Ruminococcus* (Bayer et al. 2004) as well as some anaerobic cellulolytic fungi like *Neocallimastix frontalis* (Wilson and Wood 1992) and *Piromyces* (Ali et al. 1995). The second case can be found in most of the aerobic cellulolytic fungi and bacteria. Along with multitudes of EGs, CBHs, and auxiliary enzymes, extracellular BGLs are transported out of the cell and act in synergy with the other cellulases to breakdown plant cell walls. Well-established examples are BGL1 (GH3) and BGL2(GH1) from *T. reesei* and BGL1(GH1) from *A. niger*. Some fungi, such as white rot *Phanerochaete chrysoporium*, may contain both cytoplasmic and extracellular BGLs. Some of these may serve in the metabolism of the organism's own cell wall, while others may play a role in plant cell wall metabolism (Igarashi et al. 2003; Tsukada et al. 2006).

4.3.2 Plants

Plant BGLs have been demonstrated with a wide functional diversity in defense, symbiosis, cell wall catabolism and lignification (monolignol conversion), signaling and secondary metabolism. The wide spectrum of biological activity is supported by a large number of BGLs from different GH families. For instance, 48 GH1 genes are found in *Arabidopsis thaliana* (Xu et al. 2004), while the number in rice is 40 (Opassiri et al. 2006). These GH1 enzymes cover a cluster of classical myrosinases and another cluster of myrosinases and BGLs found in the endoplasmic reticulum (ER) and peroxisome. Interestingly, GH1 enzymes from chloroplast of other plants such as maize, sorghum and wheat are much more closely related to homologues in thermophilic bacteria and archaea than rice and Arabidopsis enzymes (Thorlby et al. 2004). In addition, most plants also encode BGLs from GH families 3 and 5 (Opassiri et al. 2007; Arthan et al. 2006). To defend themselves, plants have developed a wide range of molecules. The first group is cyanogenic β-glycosides, including linamarin from clover and cassava, dhurrin from sorghum and prunasin from cherry. These cyanogenic glycosides can be hydrolyzed by BGL and release their α-hydroxynitrile moiety, which then breakdown either enzymatically or spontaneously to yield cyanide (Poulton 1990; Morant et al. 2008a, b). The second group consists of non-cyanogenic molecules, such as β- and γ-hydroxynitriles (Morant et al. 2008a, b) and isoflavones in legumes (Suzuki et al. 2006), flavonoids (Chuankhayan et al. 2005), coumarins, hydroxaminic acids (e.g., 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) in maize and wheat (Esen 1992; Sue et al. 2006; Nikus et al. 2001), and

saponins (Nisius 1988). These defensive molecules are also stored as β-D-glucosides and unleashed by specific BGLs.

In the symbiosis between the endophytic fungus *Piriformospora indica* and plant *Arabidopsis*, the fungus prevents *Arabidopsis* from overgrowing the roots and ends up triggering a defense response (Sherameti et al. 2008; Nagano et al. 2005). A special BGL in ER, AtBGLU23, is revealed to be critical in this symbiosis (Lipka et al. 2005; Bednarek et al. 2009) by demonstrated activity on scopolin, the most abundant coumarin glucoside in root (Ahn et al. 2010). Upon cell wall disruption, plant defensive BGLs and myrosinases often bind to cytoplasmic aggregating factors, which are believed to localize the otherwise soluble glucosidases at the site and therefore generate the defense compounds on site (Nisius 1988; Esen and Blanchard 2000; Kittur et al. 2007). In addition to the plant–microbe interaction, BGL may also be involved in the plant–insect interaction.

BGLs also play an important role in cell wall metabolism. Several BGLs that hydrolyze cell wall derived oligosaccharides have been identified. One BGL in germinating barley seedlings showed activity toward β-1,3- and β-1,4-linked oligosaccharides (Leah et al. 1995; Hrmova et al. 1998), preferentially mannooligosaccharides, which are found in barley endosperm cell walls (Hrmova et al. 2006). Rice seedling BGLs Os3BGlu7, Os3BGlu8, and Os3BGlu26 demonstrate an increased activity as degree of polymerization (DP) increase from 2 to 6 (Kuntothom et al. 2009), while Os4BGlu12 (Opassiri et al. 2006) is not sensitive to substrate DP, and Os3BGlu6 (Seshadri et al. 2009) is most efficient on disaccharides. Os3BGlu7 and Os3BGlu8 are widely expressed in rice tissues and are thus believed able to remodel cell wall by releasing Glc from oligosaccharides derived from it. Some other BGLs play roles in lignification of secondary cell walls. Two examples are coniferin BGL from lodgepole pine tree xylem found in differentiating region of the xylem (Dhamawardhana et al. 1995) and BGL (AtBGLU45/46) from *Arabidopsis*. The later has been found active in hydrolyzing coniferin, syringin and coumaryl alcohol glucosides to release monolignols (Escamilla-Trevino et al. 2006).

Phytohormone activation in many cases also requires glucosides hydrolysis by BGLs, such as the maize BGL Zm-p60.1 which hydrolyzes and activates cytokinin β-glucosides (Brzobohaty et al. 1993), an extracellular BGL which hydrolyzes abscisic acid glucosyl (Glc) ester transported from roots to leaves (Dietz et al. 2000), the BGL hydrolyzing auxin Glc ester 6-*O*-(4-*O*)-indole-3-ylacetyl-β-D-Glc (Jakubowska and Kawalczyk 2005), and the *Arabidopsis* ER BGL AtBG1 that hydrolyzes abscissic acid Glc ester in response to drought stress (Lee et al. 2006). Mutation in this *Arabidopsis* AtBG1 leads to early germination and defective stomata closing.

BGLs can also play metabolic roles to remove Glc protective group from metabolic intermediates and consequently trigger the biosynthesis of various natural products (phytochemicals) such as monoterpene alkaloids. For instance, one of the downstream products of strictosidine is raucaffricine, a glucoside that could only be further metabolized into ajmaline after deglucosylated by raucaffricine BGL (Barleben et al. 2007).

Plant BGLs can also release volatile terpenols from glycosides as flower and fruit scents (Reuveni et al. 1999) or attractants for parasitoid wasp to attack herbivores parasites (Mattiacci et al. 1995).

4.3.3 Animals

Some insects feeding on plants also developed GH1 enzymes in their larvae stage, such as BGLs from *Tenebrio molitor* and *Spodoptera frugiperda* (Ferrieira et al. 2001; Marana et al. 2001) and myrosinases from cabbage aphid *Brevicoryne brassicae* (Jones et al. 2001). These enzymes have hydrolysis capability on gluco-oligosaccharides and plant cellobiose, gentiobiose and even amygdalin (Marana et al. 2001) and share higher sequence similarity with vertebrate lactase-phloridzin hydrolase (LPH) than with GH1 from plants, indicating their further divergence from the same animal ancestor after their first divergence from plants.

Mammalian cells contain BGLs for metabolism of glycolipids and dietary glucosides. Human acid BGLs releases Glc ceramide; defects in this enzyme lead to glycoceramides accumulation and eventual Gaucher disease (Butters 2007). Human LPH is involved in food digestion and displays activity on β-glucosides, β-galactosides and phloridzin, a polyphenol carrying glycoside found in many fruits. Deficiency of this enzyme ends up with the widespread lactose intolerance (Tribolo et al. 2007). Human acid BGL GBA1 and bile acid BGL GBA2 belong to the GH30, and participate in the metabolism of glycolipids and dietary glucosides (Berrin et al. 2002) (Fig. 4.1).

4.4 Structure

BGLs from various GH families can have amino acid sequences ranging from 420 to 840 units, some comprising signal peptides for secretion or other domains in addition to the catalytic core. The structures of the catalytic core (Fig. 4.2) can be divided into three groups. The first one is a $(\beta/\alpha)_8$-barrel found in GH1, GH5, and GH30. The second one contains an $(\alpha/\alpha)_6$-barrel found in GH9. The last one consists of a $(\beta/\alpha)_8$-barrel and an α/β sandwich in which a 6-stranded β-sheet is flanked by two sets of three α-helices, and is found in GH3. The $(\beta/\alpha)_8$-barrel group features a catalytic domain ranging from 420 to 560 residues in length, depending on the highly variable loop regions on the β-strands at the C terminus (Sanz-Aparicio et al. 1998). Within the catalytic domain, two conserved Glu or Asp from β-strands 4 and 7 respectively are identified as the catalytic acid/base and nucleophilic residue (Ketudat Cairns and Esen 2010; Vallmitjana et al. 2001; Henrissat et al. 1995). Monomers of GH1 enzymes can dimerize or form even higher orders of aggregates. The GH5 BGLs have a $(\beta/\alpha)_8$-barrel core structure as the GH1 BGLs (both belonging to the GH-A clan), and the rice GH5 BGL has a

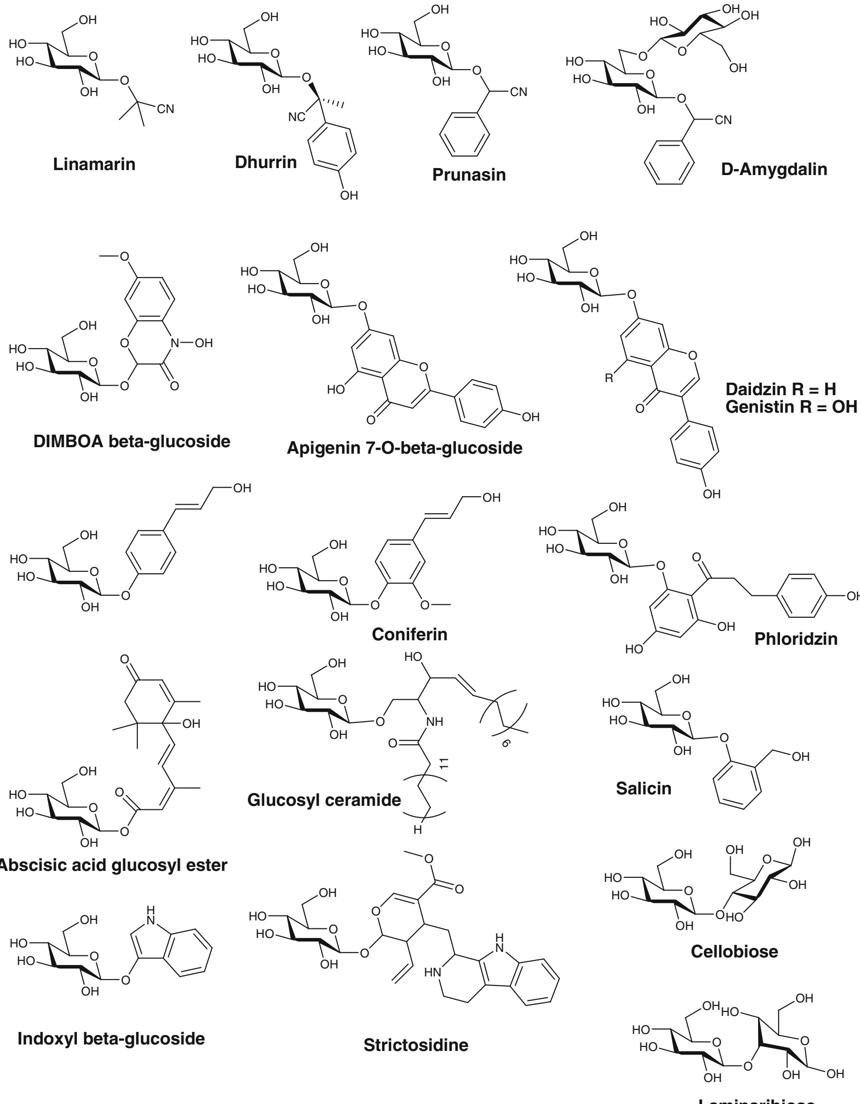


Fig. 4.1 Structures of some BGLs substrates involved in various physiological processes. *1* Plant defense: *1a* cyanogenic glucosides: linamarin (clover/cassava), dhurrin (sorghum), prunasin (cherry) and its precursor D-amygdalin. *1b* noncyanogenic glucosides: 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one 2-*O*-β-glucoside (DIMBOA-Glc, maize/wheat), apigenin 7-*O*-β-glucoside, daidzin, genistin, phloridzin. *2* Plant cell wall metabolism: coniferin, *p*-coumaryl alcohol 4-*O*-β-glucoside. *3* Plant phytohormone activation: abscisic acid glucosyl ester, indoxyl β-glucoside, salicin. *4* Plant secondary metabolism: strictosidine, precursor to a wide range of monoterpene alkaloids. *5* Fungal and bacterial degradation of plant primary cell walls: cellobiose, laminaribiose. *6* Animal (structural, signaling and metabolic functions): glucosylceramide

fascin-like domain (Opassiri et al. 2007). The $(\alpha/\alpha)_6$-barrel group seen in GH9 is dominated by a large number of endoglycosidases and leave only a few BGLs like that from *V. cholera*. So far there is no conclusive identification of the catalytic residues on the BGL from this family. However, the study on another GH9 exo-cellulase has identified a Glu residue on the loop connecting α-helices 12 and 13 as the catalytic acid/base, while a water molecule activated by two conserved, hydrogen bond-forming Asp acts as the nucleophilic residue (Schubot et al. 2004). A similar situation might be expected in BGLs from the same family. For the GH3 enzymes, the interface of the $(\beta/\alpha)_8$-barrel and α/β sandwich harbors the catalytic nucleophilic residue (a conserved Asp) and acid/base, which may be variable in terms of position and identity among different BGLs (Gabrisko and Janecek 2015; Pozzo et al. 2010; Varghese et al. 1999). A fibronectin type III domain is found at the C-termini of *Thermotoga neapolitana* and *Aspergillus aculeatus* BGLs, and the domain's function remains unclear.

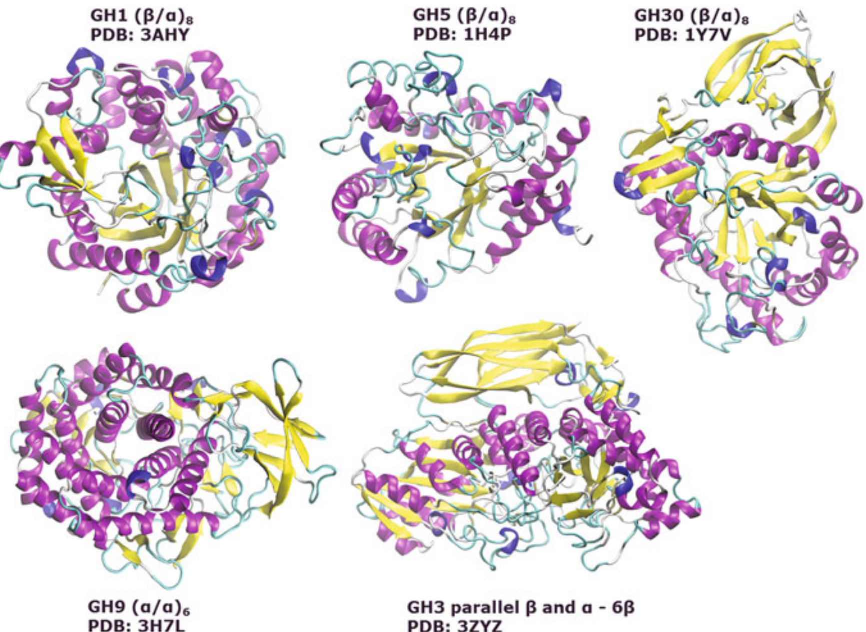


Fig. 4.2 Structures of BGLs from different GH families. GH1: *T. reesei* BGL 2 (PDB: 3AHY), GH3: *T. reesei* BGL 1 (PDB: 3ZYZ), GH5: *Saccharomyces cerevisiae* flavonoid BGL (PDB: 1H4P), GH9: *Vibrio parahaemolyticus* BGL-like protein (PDB: 3H7L), GH30: *Homo sapiens* acid BGL (PDB: 1Y7 V)

4.5 Catalytic Mechanism

All BGLs mentioned so far are exoglycoside hydrolases working on the nonreducing end of a cellodextrin or glucoside substrate. Glucosidases initiate β-glucosidic bond cleavage with a nucleophilic attack on the anomeric carbon of the Glc. This is carried out by either the side chain carboxyl of the conserved catalytic Glu/Asp (Keresztessy et al. 1994), or a water molecule activated by a conserved Asp. The oxygen in the β-glycosidic bond is activated by the conserved proximal catalytic acid while the anomeric carbon and the ring oxygen form an oxocarbenium-like ionic transition state (Fig. 4.3). When the water serves as the nucleophile (as in the case of GH9), the transition moves forward as the aglycone moiety leaves the anomeric carbon in concert with the hydroxyl (from the activated water) displacement on the same carbon to release the Glc (Qi et al. 2008). This leads to the inversion of chirality on the anomeric carbon and thus is referred as the inverting mechanism. With the endogenous nucleophile, the first transition state leads to the leaving of the aglycone moiety as well; however, the β-glycosidic bond on the anomeric carbon is now displaced with the same carboxyl that initiates the nucleophilic attack. This α-linked covalent enzyme-glycone intermediate then undergoes another round of nucleophilic attack on the anomeric carbon. The catalytic acid in the first step gets deprotonated after activating aglycone leaving group and turns into a catalytic base. It then activates a water to attack the anomeric carbon to release the Glc from the enzyme-glycone α-linkage. This retaining mechanism allows the product keep the anomeric chirality. In myrosinases, the conserved catalytic Glu in the second step is replaced by a Gln, and therefore an ascorbic acid is required to fulfill the role of the catalytic base.

Unequivocal evidences of the covalent intermediates have been obtained through incubation of *Agrobacterium* sp. GH1 BGL with 2,4-dinitrophenyl-2-deoxy-2-fluoroglucoside (2F-DNPG) (Withers et al. 1987; Withers and Street 1988). The electronegative property of fluoride destabilizes the transition state upon binding in the active site, 2,4-dinitrophenyl aglycone moiety is a highly reactive leaving group; both favor the formation of a lasting and stable enzyme-fluoroglycone intermediate. The presence of fluoride was firstly supported by ^{19}F-NMR (Withers and Street 1988) and later observed in crystal structures (Hrmova et al. 2001; Chuenchor et al. 2008) and inactivated enzyme pepsin digest (Withers et al. 1990). In a similar approach, [^{3}H] conduritol B epoxide (CBE) forms a trapped, radioactively labeled glycosyl-enzyme intermediate in *Aspergillus wentii* BGL A_3. Radioactivity detected in trypsin-cleaved fragments establishes the catalytic role of the nucleophilic Asp, albeit CBE possesses less specificity than 2F-DNPG and labels nearby residues (Bause and Legler 1980). Nevertheless, the majority of the catalytic residues have been identified through homology, mutagenesis, chemical modification, and crystal structure analyses. For instance, the catalytic acid/base residue and nucleophile residue have been inactivated by mutagenesis, respectively, in the investigation of retaining mechanism. Replacement of the nucleophile with a small non-nucleophilic residue, e.g., Ala and Gly, inactivates the enzyme in attacking the anomeric carbon

(a) Inverting Mechanism

(b) Retaining Mechanism

Fig. 4.3 The inverting and retaining mechanisms of BGLs. The *inverting mechanism* in Scheme A is found in the GH9 BGL where water-anomeric carbon bond is formed in a single step of displacement reaction. However, in the *retaining mechanism* (Scheme B), water does not attack the anomeric carbon in the first displacement step which is done in a similar way by the catalytic nucleophile. This leads to a covalently bonded glycosyl intermediate with inverted anomeric carbon conformation. The following hydrolysis step ends up with an invertion of anomeric carbon conformation for the second time and a retaining glycoside product as a result. *ES* enzyme–substrate complex, *TS* ≠ transition state, *E* + *P* (resting) enzyme and product

(Trimbur et al. 1992). However, it can be rescued by exogenous small nucleophiles, e.g., azide and fluoride, similar to the effect of the activated water in GH9, to form an α-D-glucoside azide, a chirality-inverted product (Wang et al. 1994; Mackenzie et al. 1998). Alternatively, using α-fluoroglucoside as an intermediate analog could circumvent the nucleophilic attack and allow the enzyme to release a regular Glc product after hydrolysis (Ly and Withers 1999). When the catalytic acid/base is replaced by Gly, water cannot be activated for the hydrolysis while small nucleophiles like azide again are able to fulfill this step and yield a β-D-glucoside azide (Wang et al. 1995).

4.6 Substrate–Enzyme Interaction

Compared with the thorough characterization of the catalytic residues and their mechanism in β-glycosidic bond cleavage, less is known about how the interaction between BGLs and their substrates gives rise to the tremendous substrate diversity and how the interaction determines BGLs' substrate specificity. Crystal structures have revealed that the binding pockets are cavities composed of several subsites. The subsite that binds the Glc (glycone) unit is assigned subsite −1, whereas the aglycone moiety binds to the subsites +1, +2, +3 and so on. The substrate specificity of BGL is a combination of both Glc and aglycone specificities (Marana 2006).

Besides glucosides and cellodextrins, BGLs may act on other glycosides or oligosacchardies. Such non-BGL activities are related to the coexistence of other glycosidases in the GH families that BGLs belong to, a likely divergent evolution effect. The same BGL may display different activities toward substrates with different glycosyls or glycones. For instance, *S. frugiperda* BGL hydrolyzes fucosides 40 times more quickly than galactosides (Marana et al. 2001). *Aspergillus oryzae* GH3 BGL binds Glc well, but mannose (2-epimer of Glc) much more weakly, and no allose or galactose (3- and 4-epimer) (Langston et al. 2006). GH1 BGLs might act on β-D-mannosides, β-D-galactosides, β-D-fucosides and β-D-glucuronides, while GH3 BGLs might act on β-D-xylosides and α-L-arabinosides. Hydrogen bonding plays a key role in the BGL-substrate interaction (Fig. 4.4). In a set of studies, the hydrogen bonds involving the glycone hydroxyls and subsite −1 are studied in three different GH1 BGLs: human LPH (Fernandez et al. 1995), *Agrobacterium* sp. Agβglu (Namchuck and Withers 1995) and *S. frugiperda* Sfβglu (Marana et al. 2002). For LPH and Agβglu, replacement of the glycone hydroxyls by hydrogen and/or fluorine is evaluated using enzyme kinetics. In the case of Sfβglu, a combination of site-directed mutagenesis and enzyme kinetics is deployed to determine the energy of isolated interaction between subsite −1 and glycone OH in the glycosylation transition state. The analysis leads to the conclusion that the specificity of glycone is controlled by a hydrogen bond network involving at least five amino acid residues and four substrate hydroxyls. A Glu bridging the glycone hydroxyls 4 and 6 are key in differentiating fucosides, glucosides, and galactosides. In addition, interactions with the hydroxyl 2 are essential to the BGL activity. In another set of studies, one GH3 cellodextrinase and three β-xylosidases from rumen

bacterium *Prevotella bryantii* B_14 are examined for their gylcone specificity by comparing kinetic parameters on different substrates (Dodd et al. 2010). Mutations of each conserved residues in subsite −1 severely decrease the catalytic efficiency without changing the overall structure of the enzyme variants. Similar to GH1 enzymes, replacing the conserved Glu115 (Asp120 in barley glucan β-glucosidase) leads to 1.1-fold increase in k_{cat}/K_m for *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) and 14-fold decrease for *p*NP-β-D-xylopyranoside. Glu115 is therefore believed to contribute to the discrimination between β-xylosides and β-glucosides. In BGL, the conserved Asp forms hydrogen bond bridging the hydroxyls 4 and 6 of the Glc unit, while in β-xylosidase, the conserved Glu forms hydrogen bond with the hydroxyl 4 (Fig. 4.4b).

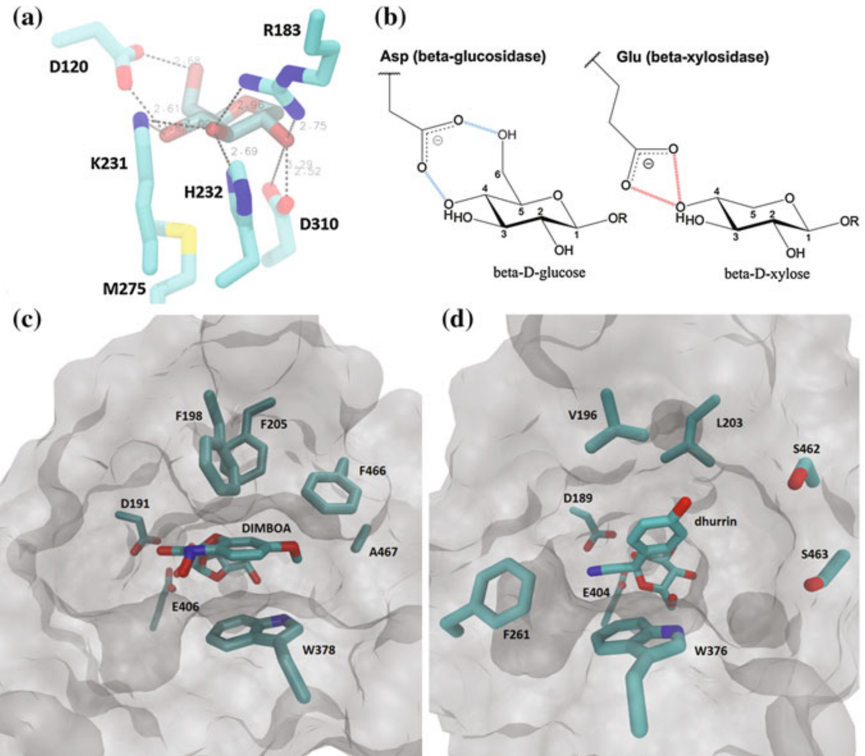


Fig. 4.4 Elements which are important in glycone (Dodd et al. 2010) and aglycone binding subsites (Czjzek et al. 2000; Verdoucq et al. 2004). **a** GH3 active site residues for the β-glucan exohydrolase (ExoI) from barley bound to Glc are shown (PDB: 1EX1). **b** Models for GH3 enzyme's xylosides and glucosides recognition (Dodd et al. 2010). Asp120 from barley ExoI forms hydrogen bond with the hydroxyls 4 and 6 of Glc. Glu115 from *P. bryantii* B14 Xyl3B forms hydrogen bond with the hydroxyl 4 of xylose and may discriminate between Glc and xylose on the basis of steric interactions. **c**, **d** Active site configurations from aglycone side of maize BGL1 (ZMGlu1, **c**) and sorghum dhurrinase 1 (Dhr1, **d**), which are shown for the structures of the ZMGlu1 Glu189Asp mutant in complex with DIMBOA-Glc (PDB: 1E56) and Dhr1 Glu191Asp mutant in complex with dhurrin (PDB: 1V03). Phe261 residue narrows the active site in Dhr1 and is also shown in front of the catalytic nucleophile Glu404

The BGL's interaction with the aglycone part of the substrate may be more diverse or complex, as shown by various structure, mutagenesis and chimera studies. In contrast to the highly conserved amino acid residues at the subsite −1, a different though overlapping set of residues is involved in interacting with the aglycone part of the substrate in BGL. An archetypical case is found in the maize ZmGlu1, a GH1 enzyme, which is active on a wide range of aglycones including DIMBOA. The structure of one of its inactive variant in complex with DIMBOA-Glc, DIMBOA and the inhibitor dhurrin shows that the aglycone is sandwiched by Phe198, Phe205 and Phe466 on one side and Trp378 on the other (Czjzek et al. 2000). In addition, DIMBOA has a hydrophobic contact with Ala467. All these residues, except Trp378, are variable among BGLs. For instance, sorghum dhurrinase SbDhr1 conserves this Trp on the same side of the algycone subsite and use Val196, Leu203 and Ser462 on the other side in replacement of the three Phe residues for a tighter and more polar interaction (Verdoucq et al. 2004). The mutagenesis of the aglycone binding residues in the Zm60.8 isoform of the ZmGlu1 further confirms the importance of these residues (Zouhar et al. 2001). It is thus believed the conserved Trp is required to bind any aglycone and three or four homologous positions on the other side would shape the pocket and therefore define the aglycone specificity. Other factors may contribute to the aglycone binding as well. It is discovered that the conserved Trp may also differentiate substrates like dhurrin and strictosidine via its positional variation (Barleben et al. 2007; Verdoucq et al. 2004; Cicek et al. 2000). Closely related wheat BGL (TaGlu) also hydrolyzes DIMBOA-Glc, although a different set of residues are present in the binding pocket (Sue et al. 2006). In the case of rice BGlu1, two of the three corresponding Phe positions are replaced by Leu and Asn, which makes either no or indirect interaction with oligosaccharide substrates (Chuenchor et al. 2008). Instead, Asn245 plays a key role in binding to the third Glc moiety in the substrate, while the corresponding residues in SbDhr1 and rice Os3BGlu6 appear to block the entrance to their active sites, with no binding of a trimeric substrate (Opassiri et al. 2003; Verdoucq et al. 2004). In general, the GH3 BGLs have fewer restraints on the aglycone part of the substrate than the GH1 BGLs. For instance, *A. oryzae* GH3 BGL has similar activities toward cellobiose, sophorose, laminaribiose, and β-gentiobiose, in which the activated Glc unit (at the subsite −1) is linked to the 'leaving' Glc unit (corresponding to the aglycones of 4-glucosides) via 1,4-, 1,2-, 1,3-, and 1,6-glucosidic bond, respectively (Langston et al. 2006).

The GH1 BGLs have narrow and deep wells-like active sites, whose topology (including space restricted subsites +1 or higher), electrostatic and hydrophobic interaction properties impact the BGLs' substrate specificity and product (Glc) inhibition tolerance (de Giuseppe et al. 2014; Tribolo et al. 2007). The GH3 BGLs have crater-shaped active sites, whose depth, shape, and local amino acids determine the BGLs' substrate specificity. The topological difference is believed to contribute to the difference in substrate reactivity and product inhibition between the GH1 and GH3 BGLs (de Giuseppe et al. 2014).

4.7 Enzymatic Properties

4.7.1 Hydrolase Activity

When the substrate concentration is relatively low, BGLs' main activity is hydrolytic, cleaving cellodextrins or oligosaccharides into smaller carbohydrate molecules such as Glc, or glucosides into carbohydrate and aglycone subunits. Various substrates may be used to assay BGLs' properties. Chromogenic and fluorogenic substrates, exemplified by *p*NP-Glc and 4-methylumbelliferyl-β-D-Glc, are quite reactive and convenient for spectrophotometric detections, which are suitable for microplate-based high-throughput or robotized studies (Perry et al. 2007). Because the chromo- or fluorogenic aglycones are often unnatural, these substrates might not yield data applicable for quantitative or comprehensive understanding of BGLs' active sites, especially the subsites +1, +2, etc. Cellodextrins, other oligosaccharides, and glucosides (such as that in Fig. 4.1), are able to reveal BGLs properties under physiological or real applications conditions. BGLs' action on these substrates may be monitored with a range of detections including HPLC, mass spectrometry, coupled secondary chemical or enzyme reactions for the BGL reaction products (such as a glucose oxidase or hexokinase assay on Glc), activity-overlay electrophoresis (Saqib and Whitney 2006), or thermochemistry (Jeoh et al. 2005).

In general, BGLs' hydrolase activity follows the Michaelis–Menten kinetics. With cellobiose as the substrate, k_{cat}, K_m, and k_{cat}/K_m in the order of 10^1–10^2 s^{-1}, 10^0–10^1 mM, and 10^1–10^2 mM^{-1} s^{-1} are often observed, while with *p*NP-Glc as the substrate, k_{cat}, K_m, and k_{cat}/K_m in the order of 10^2 s^{-1}, 10^0 mM, and 10^2 mM^{-1} s^{-1} are often observed (Ferrara et al. 2014; Uchiyama et al. 2013; Frutuoso and Marana 2013; Sørensen et al. 2013; Teugjas and Väljamäe; 2013; Singhania et al. 2013; Kuntothom et al. 2009; Mendonca and Marana 2008; Eyzaguirre et al. 2005). Significant variations (tenfold or higher) may exist for these parameters, depending on the BGL active sites (Tsukada et al. 2008) and assay conditions (buffer, pH, temperature, enzyme and substrate concentrations), and no significant statistical differences are observed between the GH1 and GH3 BGLs (p = 0.19, 0.26, 0.59, 0.07 for the k_{cat} (cellobiose), K_m (cellobiose), k_{cat} (*p*NP-Glc), K_m (*p*NP-Glc), from two-tail t Tests) (Fig. 4.5).

BGLs' hydrolase activity has an optimal pH range from weakly acidic to neutral, mainly determined by the actions of the catalytic Asp and/or Glu as the nucleophile and general acid/base, but also with contributions from other components of the active site (Tsukada et al. 2008). Most of the BGLs (from the mesophilic organisms) have optimal temperatures at 50–70 °C, but the BGLs from extreme thermophiles can have optimal temperatures close to or above 100 °C (Park et al. 2005).

Many molecules may inhibit or inactivate BGLs. Nonspecific, general protein-denaturing inhibitors include ionic surfactants, heavy metal ions such as Hg^+ and Ag^+, and high concentration salts (including NaCl) which exert the typical Hofmeister (salt-out) effect (on the enzymes' apparent pK_a, not the second-order rate constant) (Bowers et al. 2007). Redox-active metal ions such as Cu^{2+} and Fe^{3+} may

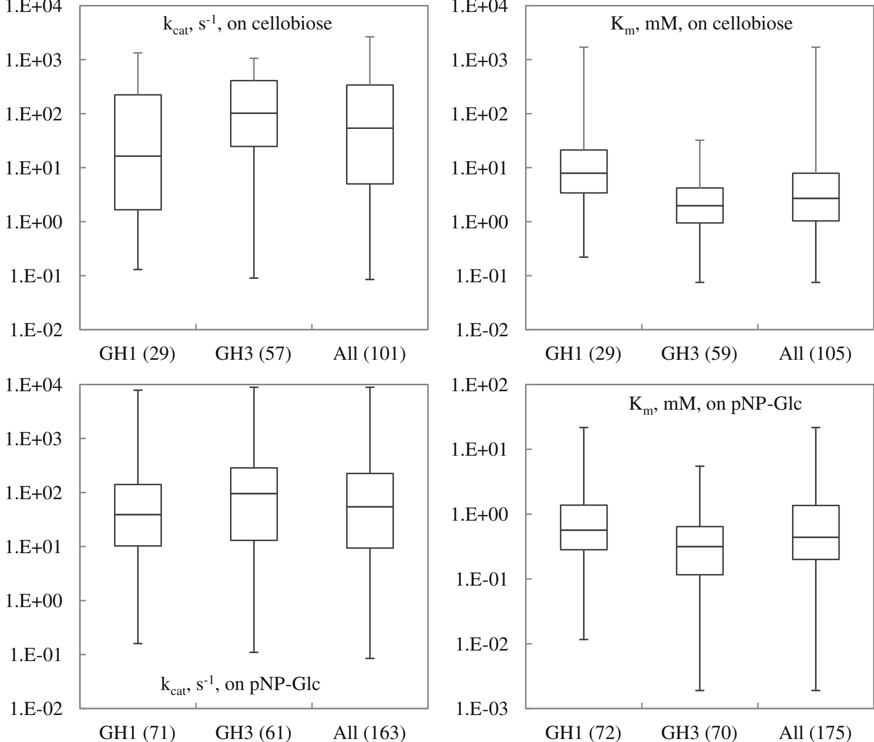


Fig. 4.5 *Box-and-whisker plots* of the k_{cat} and K_m of BGLs, including those from the GH1 and GH3 family, for the hydrolysis of cellobiose or *p*NP-Glc. In the *parentheses* are numbers of BGLs analyzed. Data are from Brenda enzyme database (www.brenda-enzymes.org) and other literature sources

inhibit BGL by oxidation (Tejirian and Xu 2010; Jeng et al. 2011; Sharmila et al. 1998). Tris, a commonly used 'biological' buffer, may also inhibit BGL by binding to the active site (Jeng et al. 2011). BGLs may be deactivated by various protein adsorbents that include soil minerals (Lammirato et al. 2010), oligo/polyphenols (Tejirian and Xu 2011) and lignin, whose effect can become significant in the enzymatic biomass hydrolysis (Haven and Jørgensen 2013; Xu et al. 2007).

Specific BGL inhibitors include its substrate, products, and the transition-state mimics. Because of BGL active sites' topological features, the products (Glc, aglycone, or shortened oligosaccharide), which may be regarded as equivalent to the subunits of a substrate, can bind to certain subsites and impede the enzymatic reaction. Structural analogs of the products or substrates, or even competing substrates with low-reactivity (e.g., fluoroglucosides), can also inhibit BGLs. The transition-state mimics can lock up the active site with their structural features highly similar to that of the activated substrate, and alter the enthalpy–entropy for the binding, hence exerting competitive inhibitions more potent than the other

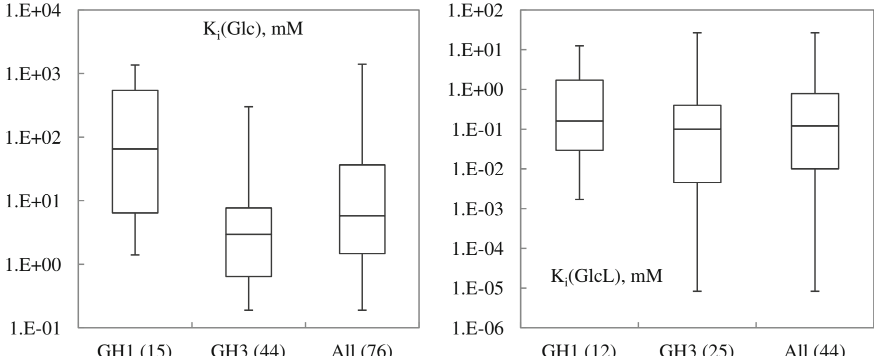


Fig. 4.6 *Box-and-whisker plots* of the K_i of Glc and GlcL on the hydrolysis of *p*NP-Glc by BGLs, including those from the GH1 and GH3 family. In the *parentheses* numbers of BGLs analyzed. Data are from Brenda enzyme database (www.brenda-enzymes.org) and other literature sources

inhibitors. The highly inhibitory transition-state mimics include D-glucono-1,5-lactone (GlcL, the first derivative from Glc oxidation, a side reaction often accompanying cellulose degradation), deoxynojirimycin, and other heteroatom-substituted carbohydrate molecules (Gloster and Davies 2010; Gloster et al. 2007). As inhibitor, Glc and GlcL often show K_i in the order of 10^1–10^2 and 10^{-1}–10^0 mM, respectively, on the *p*NP-Glc hydrolysis by BGL (Uchiyama et al. 2013; Sørensen et al. 2013; Teugjas and Väljamäe; 2013; Mendonca and Marana 2008; Seidle et al. 2005, 2006; Seidle and Huber 2005; Eyzaguirre et al. 2005; Marana et al. 2001). Significant variations (10- to 100-fold or higher) may exist for the parameter, depending on the BGLs, substrates and assay conditions (buffer, pH, temperature, enzyme, and substrate concentrations) (Fig. 4.6). Statistically, the GH1 BGLs appear to have higher K_i for Glc (more tolerance to the Glc inhibition) than that of the GH3 BGLs (p = 0.023), but comparable K_i for GlcL to that of the GH3 BGLs (p = 0.76, from two-tail t Tests).

4.7.2 Glycosyltransferase/Transglycosylase Activity

When the substrate concentration is high, BGLs (except the GH9 ones) may show significant glycosyltransferase or transglycosylase activity to synthesize oligosaccharides or glucosidases, opposing the hydrolase activity (Uchiyama et al. 2013). The (trans)glycosylation activity is allowed by the reversibility of the retaining mechanism, when the water is replaced by a carbohydrate or another acceptor with higher activity for the deacylation of the enzymatic intermediate (donor) (Fig. 4.3b), as in the cases of other retaining glycosidases. The (trans)glycosylation may yield sophorosyl, laminaribiosyl, or even cellobiosyl, products via 1,2-, 1,3-, or 1,4-glucosidic bond, or various glucosides (de Giuseppe et al. 2014).

In general, a BGL's hydrolase activity is controlled by thermodynamic factors and water activity, while its glycosyltransferase activity is controlled by kinetic factors and the glycosyl acceptors, with rates independent on pH (Seidle and Huber 2005). The (trans)glycosylation may be detected directly by the related products formation, or indirectly by the apparent substrate or product inhibition of the hydrolysis, based on the decrease of the hydrolysis products formation (Frutuoso and Marana 2013; Kawai et al. 2004). The (trans)glycosylation may contribute to a BGL's tolerance to or stimulation by Glc, or maintain a BGL's turnover. Many examples of Glc stimulation for the GH1 BGLs, fewer for the GH3 BGLs, have been reported (de Giuseppe et al. 2014).

4.7.3 Non-BGL Activities

BGLs may have significant side activities on non-Glc substrates (Brenda enzyme database www.brenda.org, Uchiyama et al. 2013; Sørensen et al. 2013; Singhania et al. 2013; Kuntothom et al. 2009; Harnipcharnchai et al. 2009; Mendonca and Marana 2008; Tribolo et al. 2007; Kim et al. 2006; Seidle et al. 2005; Park et al. 2005; Eyzaguirre et al. 2005; Opassiri et al. 2004; Berrin et al. 2002; Hrmova et al. 2002; Vallmitjana et al. 2001; Marana et al. 2001; Perry et al. 2007). With *p*NP-β-D-fucopyranoside as substrate, k_{cat}, K_m, and k_{cat}/K_m of 10^1–10^2 s^{-1}, 10^0–10^1 mM, and 10^2 mM^{-1} s^{-1} are often observed for BGL's β-fucosidase activity. With *p*NP-β-D-xylopyranoside as substrate, k_{cat}, K_m, and k_{cat}/K_m of 10^0–10^1 s^{-1}, 10^0 mM, and 10^0–10^3 mM^{-1} s^{-1} are often observed for BGL's β-xylosidase activity. With *p*NP-α-L-arabinofuranoside as substrate, k_{cat}, K_m, and k_{cat}/K_m of 10^0–10^1 s^{-1}, 10^0–10^1 mM, and 10^0–10^2 mM^{-1} s^{-1} are often observed for BGL's α-arabinosidase activity. With *p*NP-β-D-galactopyranoside as substrate, k_{cat}, K_m, and k_{cat}/K_m of 10^0–10^1 s^{-1}, 10^0–10^2 mM, and 10^{-1}–10^1 mM^{-1} s^{-1} are often observed for BGL's β-galactosidase activity. Close range of BGL's activities on these substrates with different glycones indicate that the interaction between the active subsite −1 and the substrate's nonreducing glycone subunits 4- and 6-OH (which are changed in the galactosyl, fucosyl, xylosyl, and arabinosyl subunits of the non-Glc substrates) does not dominate BGL's activity. In GH1, BGLs coexist with β-fucosidase, β-xylosidase, and β-galactosidase, as well as β-mannosidase, β-glucuronidase. In GH3, BGLs coexist with β-xylosidase and α-arabinofuranosidase. Therefore, the side activities might arise from divergent evolution.

4.7.4 Engineering

Recombinant DNA technology has been applied to BGLs. In combination with 3D structure and enzymatic reaction studies, site-directed mutagenesis enables elucidation of the structural–function relationship in BGL, especially the aspects on how

the composition and topology of the active site affect the substrate specificity, pH or temperature profile, or substrate/product inhibition (Jeng et al. 2012; Khan et al. 2011; Liu et al. 2011 Chuenchor et al. 2011; Tsukada et al. 2008; Mendonca and Marana 2008; Chuenchor et al. 2008; Vallmitjana et al. 2001).

BGL variants with improved activity, stability, or specificity have been made by either rational design or directed evolution (Lee et al. 2012; Khan et al. 2011; Hong et al. 2006; Hancock et al. 2005). Engineering the active site (especially the subsites that bind the aglycone of a substrate) has been pursued for the abatement of the product (Glc) inhibition; however, the increase in the Glc tolerance is often accompanied by a decrease in the hydrolase activity, due to the contribution made by the BGL-Glc interaction to both the catalysis and inhibition. Domain swap between mesophilic and thermophilic BGLs may lead to improved thermal or other properties for BGLs originated from mesophiles, such as the chimera constructed from *Agrobacterium tumefaciens* or *Cellvibrio gilvus* and *Thermotoga maritime* (Deog and Kiyoshi 2011; Kim et al. 2006).

Most BGLs have no distinct functional domains other than the catalytic core (although some GH3 BGLs have a C-terminal FnIII domain). The enzymes may be engineered to possess domains with targeted functions, such as the carbohydrate-binding module potentially useful for the enzymatic biomass hydrolysis (Gundllapalli et al. 2007).

The transglycosylase activity of BGLs may be improved by engineering (Frutuoso and Marana 2013; Jeng et al. 2012; Lundemo et al. 2013; Shim et al. 2012; Choi et al. 2008; Feng et al. 2005). For instance, the Trp49 and Trp262 of *A. niger* GH3 BGL are found regulating the preference to the transglycosylase and hydrolase activity, and substitutions of the two Trp result in BGL variants with abated or enhanced relative transglycosylase activity (Seidle et al. 2005, 2006). The regioselectivity of a transglycosylating BGL may also be modulated by site-directed mutagenesis (Choi et al. 2008).

4.8 Application

4.8.1 Synthesis of Oligosaccharides and Glycosides

BGLs have the potential in biosynthesis of functional, performance, or therapeutic oligosaccharides, glycol-conjugates, and glycosides, based on their glycosyltransferase activity. To increase the efficiency or selectivity, it is desirable to engineer BGLs so that their hydrolase activity can be minimized. Engineering on BGL may be carried out by site-directed mutagenesis or directed evolution (Shim et al. 2012). The kinetic and thermodynamic determinants for the transglycosylation and ‘reverse hydrolysis’ (formation of the compounds that would serve as the substrates for the hydrolase activity), respectively, can be explored by the engineering or changes on the reaction conditions (Bhatia et al. 2002). For instance, alterations of an amino acid at the subsite +2 of *T. neapolitana* GH1 BGL are able to enhance the enzyme’s

glycosyltransferase-to-hydrolase activity ratio, enabling the enzyme to catalyze more efficiently the synthesis of alkyl glycosides as attractive surfactants (Lundemo et al. 2013). The alkyl glycosides may also be synthesized by alcohol-tolerant or stimulating BGL in solvents high in alcohol (Karnaouri et al. 2013).

4.8.2 Beverage, Brewing, Food, Feed, and Dietary

Combination of flavor precursors and β-glucosidases is widely used in beverage and food production. BGLs may be added in different stage of production and brewing to act on specific precursors to release the desired aglycones (e.g., terpenols, benzyl alcohols, and phenylethyl alcohols) for flavor enhancement and stability. A combination of α-rhamnosidase and BGL may be applied to debitter citrus fruits by hydrolyzing bitter glucosides such as prunin and naringin, while BGL may be applied to decolorize juices or wines by hydrolyzing various anthocyanins such as malvidin, peonidin, and cyanidin (Eyzaguirre et al. 2005). The astringent diadzin, genistin, or other isoflavonoid glucosides in soybean products may be hydrolyzed by BGL to daidzein, genistein, or other isoflavones with more pleasant taste. BGLs are also used in nutrition enrichment of feeds, foods and beverages to generate vitamins, antioxidants and other dietary molecules from their glycoside precursors. For instance, vitamin B6 is stored predominantly in its glycoside form and can be released by rice BGLs (Opassiri et al. 2003). Feeds for the monogastric farm animals like pigs and chickens are often treated with crude BGLs to improve nutrition availability together with the native enzymes in the animal's small intestine. Aglycones from soybean isoflavone glycosides and phenylthiocarbamide derived from cruciferous vegetable glycosides have antioxidant and anticarcinogenic effect. They are also direct or derivative products from BGL and myrosinase hydrolysis, respectively (Chuankhayan et al. 2007; Esen 2003).

4.8.3 Biomass Conversion

Biomass conversions lay the basis for the bioenergy, biorefinery, and other biomass-sustained industries. The enzymatic hydrolysis of lignocellulose is a key step of the processes, and its development has benefited greatly from the basic biochemical studies on the natural lignocellulose degradation (Payne et al. 2015; Xie et al. 2014; Sweeney and Xu 2012; van den Brink and de Vries 2011; Quinlan et al. 2010; Xu 2004, 2010a, b; McFarland et al. 2007; Teter et al. 2006). The industrial enzymatic biomass conversion can differ significantly from the natural process in various aspects, especially the requirement for high efficiency: the much shortened reaction time (days instead of years), enhanced turnover (to minimize the cost of enzymes), and close to 100 % yield (to maximize the output), need for enzymes to tolerate high levels of end products and insoluble substrates that are

often altered in composition or structure by pretreatments. Cellulolytic microbes secrete consortia of enzymes that can work cooperatively and synergistically, but they need adjustment in industrial applications, in terms of composition and specificity. *T. reesei* is one of the best known cellulolytic microbes and industrial hemicellulases and cellulases producers. Although the filamentous fungus can secrete more than 20 lignocellulose-active enzymes, including at least one BGL (Herpoël-Gimbert et al. 2008), its enzyme consortium cannot effectively hydrolyze various industrial biomass feedstocks (such as dilute acid pretreated corn stover), unless supplemented with exogenous BGLs (such as *A. oryzae* GH3 BGL), as demonstrated by the development of the first generation of the industrial biomass enzymes products (Rani et al. 2014; Sørensen et al. 2013; Teugjas and Väljamäe 2013; Tiwari et al. 2013; Singhania et al. 2013; Krisch et al. 2010; Eyzaguirre et al. 2005; Xu 2004; Bhatia et al. 2002). During an industrial biomass hydrolysis, the CBHs and EGs of *T. reesei* hydrolyze cellulose and produce high levels of cellobiose. The cellobiose cannot be degraded effectively by the native BGL present in the fungus' secretome in low abundance, and thus a severe product inhibition builds on the CBHs and EGs, resulting in an underperformance of the overall enzymatic hydrolysis. The inhibition can be mitigated by increasing the BGL abundance.

Supplementing the BGL activity to an industrial hemicellulase and cellulase consortium by co-expressing the enzyme in the same host (that produces the hemicellulase and cellulase) is more economically viable than producing the enzyme separately. Often, heterologous expression of a non-native BGL is selected to take advantage of the enzyme's superior properties (in comparison with the endogenous BGL). The selection is initiated by multiple rounds of screening of individual BGLs, based on activity or bioinformatics, for either wild-type or engineered variants. The initial screening is followed by synergy study with other hemicellulase and cellulase acting under industrial conditions and by heterologous expression trials. The preferred properties of superior BGLs include high specific activity, high thermal stability, suitable pH profile, low production inhibition, low lignin absorption, low glycosyltransferase side activity, and high expression yield. A BGL's stimulation by Glc or xylose may further improve the enzyme's action in hydrolyzing high concentrations of cellulosic materials (Souza et al. 2010). A BGL's tolerance of, or stimulation by, ethanol or other alcohol may further improve the enzyme's action in the biomass conversion processes where elevated levels of ethanol are present, such as the simultaneous hydrolysis (saccharification) and fermentation (SHF, SSF) or consolidated bioprocessing (CBP) (Karnaouri et al. 2013).

BGLs may be applied in individual, free forms as in the secretomes of *T. reesei* and most of the aerobic cellulolytic fungi. The relative abundance (molar ratio) of BGL in biomass-active enzyme consortia may be roughly adjusted by co-expression or post-production mixing. BGLs engineered with altered properties, including that gained through fused functional domains, such as the carbohydrate-binding-module (Gundllapalli et al. 2007), may be used. The claimed benefits of applying BGL in

free forms include the freedom a BGL has to act close to other enzymes or away from them, pursuing diffused cellobiose (or other oligosaccharides) or avoiding common deactivators.

BGLs may also be applied in associated, part of supramolecular assembly forms as in the cellulosomes of *Clostridium thermocellum* and most of the anaerobic cellulolytic bacteria. The molar ratio of the BGL in biomass-active cellulosomes may be finely adjusted by specific dockerin–cohesin pairing. Non-native or non-cellulosomal BGLs may be incorporated into designer cellulosomes (with selected enzyme composition and molar ratio) with fused dockerins (Kellermann and Rentmeister 2014; Hyeon et al. 2013). Working in the same supramolecular assembly allows BGL to cooperate with other enzymes in close vicinity.

BGLs may also be applied in cell surface display forms. A BGL can be displayed, alone or with other biomass-active enzymes, on the surface of a fermentative but noncellulolytic yeast or bacterium. The displayed BGL and other enzymes can hydrolyze biomass to Glc and other fermentable sugars, which are then fermented by the yeast or bacterium in situ. In the so-called consolidated bioprocessing process, enzyme production and biomass-to-ethanol conversion are done in a single unit process step, by engineered microbes such as the thermophilic *Kluyveromyces marxianus* displaying *A. aculeatus* BGL and *T. reesei* EG (Kellermann and Rentmeister 2014; Yamada et al. 2013; van Zyl et al. 2007).

References

Ahn YO, Shimizu B, Sakata K, Gantulga D, Zhou Z, Bevan DR, Esen A (2010) Scopulin-hydrolyzing β-glucosidases in the roots of Arabidopsis. Plant Cell Physiol 51:131–143

Ali BR, Zhou L, Graves FM, Freedman RB, Black GW, Gilbert HJ, Hazelwood GP (1995) Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and encoded by multigene families. FEMS Microbiol Lett 125:15–22

Arthan D, Kittakoop P, Esen A, Svasti J (2006) Furostanol glycoside 26-*O*-β-glucosidase from the leaves of *Solanum torvum*. Phytochemistry 67:27–33

Barleben L, Panjikar S, Ruppert M, Koepke J, Stockigt J (2007) Molecular architecture of strictosidine glucosidase: the gateway to the biosynthesis of the monoterpenoid indole alkaloid family. Plant Cell 19:2886–2897

Barnett CC, Berka RM, Fowler T (1991) Cloning and amplification of the gene encoding and extracellular β-glycosidase from *Trichoderma reesei*: evidence for improved rates of saccharification of cellulosic substrates. Nat Biotechnol 9:552–567

Bause E, Legler G (1980) Isolation and structure of a tryptic glycopeptide from the active site of β-glucosidase A3 from *Aspergillus wentii*. Biochim Biophys Acta 626:459–465

Bayer EA, Belaich JP, Shoham Y, Lamed R (2004) The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 58:521–554

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323:101–106

Berrin JG, McLauchlan WR, Needs P, Williamson G, Puigserver A, Kroon PA, Juge N (2002) Functional expression of human liver cytosolic β-glucosidase in *Pichia pastoris*. Insights into its role in the metabolism of dietary glucosides. Eur J Biochem 269:249–258

Bhatia Y, Mishra S, Bisaria VS (2002) Microbial β-glucosidases: cloning, properties, and applications. Crit Rev Biotechnol 22:375–407

Bowers EM, Ragland LO, Byers LD (2007) Salt effects on β-glucosidase: pH-profile narrowing. Biochim Biophys Acta 1774:1500–1507

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science 262: 1051–1054

Butters TD (2007) Gaucher disease. Curr Opin Chem Biol 11:412–418

Choi W, Park KM, Jun SY, Park CS, Park KH, Cha J (2008) Modulation of the regioselectivity of a *Thermotoga neapolitana* β-glucosidase by site-directed mutagenesis. J Microbiol Biotechnol 18:901–907

Chuankhayan P, Hua Y, Svasti J, Sakdarat S, Sullivan PA, Ketudat Cairns JR (2005) Purification of an isoflavonoid 7-*O*-bapiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurz. Phytochemistry 66:1880–1889

Chuankhayan P, Rimlumduan T, Svasti J, Ketudat Cairns JR (2007) Hydrolysis of soybean isoflavonoid glycosides by *Dalbergia* β-glucosidases. J Agric Food Chem 55:2407–2412

Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Svasti J, Ketudat Cairns JR (2011) The structural basis of oligosaccharide binding by rice BGlu1 beta-glucosidase. J Struct Biol 173:169–179

Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR, Esen A, Chen CJ, Opassiri R, Svasti J, Ketudat Cairns JR (2008) Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation. J Mol Biol 377:1200–1215

Cicek M, Blanchard D, Bevan DR, Esen A (2000) The aglycone specificity-determining sites are different in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (maize beta-glucosidase) and dhurrinase (sorghum beta-glucosidase). J Biol Chem 275:20002–20011

Czjzek M, Cicek M, Zamboni V, Bevan DR, Henrissat B, Esen A (2000) The mechanism of substrate (aglycone) specificity in β-glucosidases is revealed by crystal structures of mutant maize β-glucosidase-DIMBOA, -DIMBOAGlc, and dhurrin complexes. Proc Natl Acad Sci USA 97:13555–13560

Dan S, Marton T, Dekol M, Bravdo BA, He S, Withers SG, Shoserov O (2000) Cloning, expression, characterization and nucleophile identification of family 3 *Aspergillus niger* β-glycosidase. J Biol Chem 275:4973–4980

Deog KJ, Kiyoshi H (2011) Construction and characterization of novel chimeric β-glucosidases with *Cellvibrio gilvus* (CG) and *Thermotoga maritima* (TM) by overlapping PCR. In: Carpi A (ed) Progress in molecular and environmental bioengineering—from analysis and modeling to technology applications. InTech, Rijeka, pp 633–646

de Giuseppe PO, Souza Tde A, Souza FH, Zanphorlin LM, Machado CB, Ward RJ, Jorge JA, Furriel Rdos P, Murakami MT (2014) Structural basis for glucose tolerance in GH1 β-glucosidases. Acta Crystallogr D Biol Crystallogr 70:1631–1639

Dhamawardhana DP, Ellis BE, Carlson JE (1995) A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. Plant Physiol 107:331–339

Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung W (2000) Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. J Exp Bot 51:937–944

Dodd D, Kiyonari S, Mackie RI, Cann IK (2010) Functional diversity of four glycoside hydrolase family 3 enzymes from the rumen bacterium *Prevotella bryantii* $B_1$4. J Bacteriol 192: 2335–2345

Escamilla-Trevino LL, Chen W, Card ML, Shih MC, Cheng CL, Poulton JE (2006) *Arabidopsis thaliana* β-glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. Phytochemistry 67:1651–1660

Esen A (1992) Purification and partial characterization of maize (*Zea mays* L.) β-glucosidase. Plant Physiol 98:174–182

Esen A (2003) β-Glucosidases. In: Whitaker JR, Voragen AGJ, Wong DWS (eds) Handbook of food enzymology. Marcel Dekker, New York, pp 791–804

Esen A, Blanchard DJ (2000) A specific β-glucosidase-aggregating factor (BGAF) is responsible for the β-glucosidase null phenotype in maize. Plant Physiol 122:563–572

Eyzaguirre J, Hidalgo M, Leschot A (2005) β-Glucosidases from filamentous fungi: properties, structure, and applications. In: Yarema KJ (ed) Handbook of carbohydrate engineering. Taylor & Francis, Boca Raton, pp 645–685

Faure D, Desair J, Keijers V, Bekri MA, Proost P, Henrissat B, Vanderleyden J (1999) Growth of *Azospirillum irakense* KBC 1 on the aryl β-glycosidase salicin requires either Sal A or Sal B. J Bacteriol 181:3003–3009

Feng HY, Drone J, Hoffmann L, Tran V, Tellier C, Rabiller C, Dion M (2005) Converting a β-glycosidase into a β-transglycosidase by directed evolution. J Biol Chem 280:37088–37097

Fernandez P, Canada FJ, Jimenez-Barbero J, Martın-Lomas M (1995) Substrate specificity of small-intestinal lactase: study of the steric effects and hydrogen bonds involved in enzyme-substrate interaction. Carbohydr Res 271:31–42

Ferrara MC, Cobucci-Ponzano B, Carpentieri A, Henrissat B, Rossi M, Amoresano A, Moracci M (2014) The identification and molecular characterization of the first archaeal bifunctional exo-beta-glucosidase/*N*-acetyl-beta-glucosaminidase demonstrate that family GH116 is made of three functionally distinct subfamilies. Biochim Biophys Acta 1840:367–377

Ferrieira AHP, Marana SR, Terra WR, Ferreira C (2001) Purification, molecular cloning, and properties of a β-glycosidase isolated from midgut lumen of *Tenebrio molitor* (Coleoptera) larvae. Insect Biochem Mol Biol 31:1065–1076

Frutuoso MA, Marana SR (2013) A single amino acid residue determines the ratio of hydrolysis to transglycosylation catalyzed by β-glucosidases. Protein Peptide Lett 20:102–106

Gabrisko M, Janecek S (2015) Novel family GH3 β-glucosidases or β-xylosidases of unknown function found in various animal groups, including birds and reptiles. Carbohydr Res 408: v44–50

Geerlings A, Ibanez MML, Memelinks J, Heijden RVD, Verpoorte R (2000) Molecular cloning and analysis of strictosidine β-D-glycosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J Biol Chem 275:3051–3056

Gilbert HJ, Stalbrand H, Brumer H (2008) How the walls come tumbling down: recent structural biochemistry of plant polysaccharide degradation. Curr Opin Plant Biol 11:338–348

Gloster TM, Davies GJ (2010) Glycosidase inhibition: assessing mimicry of the transition state. Org Biomol Chem 8:305–320

Gloster TM, Meloncelli P, Stick RV, Zechel D, Vasella A, Davies GJ (2007) Glycosidase inhibition: an assessment of the binding of 18 putative transition-state mimics. J Am Chem Soc 129:2345–2354

Gundllapalli SB, Pretorius IS, Cordero Otero RR (2007) Effect of the cellulose-binding domain on the catalytic activity of a β-glucosidase from *Saccharomycopsis fibuligera*. J Ind Microbiol Biotechnol 34:413–421

Hancock SM, Corbett K, Fordham-Skelton AP, Gatehouse JA, Davis BG (2005) Developing promiscuous glycosidases for glycoside synthesis: residues W433 and E432 in *Sulfolobus solfataricus* beta-glycosidase are important glucoside- and galactoside-specificity determinants. ChemBioChem 6:866–875

Harnipcharnchai P, Champreda V, Sornlake W, Eurwilaichitr L (2009) A thermotolerant β-glucosidase isolated from an endophytic fungi, *Periconia* sp. with a possible use for biomass conversion to sugars. Prot Express Purif 67:61–69

Haven MØ, Jørgensen H (2013) Adsorption of β-glucosidases in two commercial preparations onto pretreated biomass and lignin. Biotechnol Biofuels 6:165

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc Natl Acad Sci USA 92:7090–7094

Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, Mathis H, Sigoillot JC, Monot F, Asther M (2008) Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 1:18

Hong J, Tamaki H, Kumagai H (2006) Unusual hydrophobic linker region of β-glucosidase (BGLII) from *Thermoascus aurantiacus* is required for hyper-activation by organic solvents. Appl Microbiol Biotechnol 73:80–88

Hrmova M, MacGregor EA, Biely P, Stewart RJ, Fincher GB (1998) Substrate binding and catalytic mechanism of a barley β-D-glucosidase/(1, 4)-β-D-glucan exohydrolase. J Biol Chem 273:11134–11143

Hrmova M, Varghese JN, De Gori R, Smith BJ, Driguez H, Fincher GB (2001) Catalytic mechanisms and reaction intermediates along the hydrolytic pathway of a plant beta-D-glucan glucohydrolase. Structure 9:1005–1016

Hrmova M, De Gori R, Smith BJ, Fairweather JK, Driquez H, Varghese JN, Fincher GB (2002) Structural basis for broad substrate specificity in higher plant β-D-glucan glucohydrolases. Plant Cell 14:1033–1052

Hrmova M, Burton RA, Biely P, Lahnstein J, Fincher GB (2006) Hydrolysis of (1,4)-β-D-mannans in barley *(Hordeum vulgare* L.) is mediated by the concerted action of (1,4)-β-D-mannans endohydrolase and β-D-mannosidase. Biochem J 399:77–90

Hyeon JE, Jeon SD, Ha SO (2013) Cellulosome-based, *Clostridium*-derived multi-functional enzyme complexes for advanced biotechnology tool development: advances and applications. Biotechnol Adv 31:936–944

Igarashi K, Tani T, Kawal R, Samejima M (2003) Family 3 beta-glucosidase from cellulose-degrading culture of the white-rot fungus *Phanerochaete chrysosporium*. J Biosci Bioeng 95:572–576

Iwashita K, Nagahara T, Kimura H, Takano M, Shimoi K, Ito K (1999) The *bgl* gene of *Aspergillus kawachii* encodes both extracellular and wall bound β-glycosidases. Appl Environ Microbiol 65:5546–5553

Jakubowska A, Kawalczyk S (2005) A specific enzyme hydrolyzing 6-*O*(4-*O*)-indole-3-ylacetyl-β-D-glucose in immature kernels of *Zea mays*. J Plant Physiol 162:207–213

Jeng WY, Wang NC, Lin CT, Chang WJ, Liu CI, Wang AHJ (2012) High-resolution structures of *Neotermes koshunensis* β-glucosidase mutants provide insights into the catalytic mechanism and the synthesis of glucoconjugates. Acta Cryst D68:829–838

Jeng WY, Wang NC, Lin MH, Lin CT, Liaw YC, Chang WJ, Liu CI, Liang PH, Wang AH (2011) Structural and functional analysis of three β-glucosidases from bacterium *Clostridium cellulovorans*, fungus *Trichoderma reesei* and termite *Neotermes koshunensis*. J Struct Biol 173:46–56

Jeoh T, Baker JO, Ali MK, Himmel ME, Adney WS (2005) β-D-Glucosidase reaction kinetics from isothermal titration microcalorimetry. Anal Biochem 347:244–253

Jones AME, Bridges M, Bones AM, Cole R, Rossiter JT (2001) Purification and characterisation of a non-plant myrosinase from the cabbage aphid *Brevicoryne brassicae*. Insect Biochem Mol Biol 31:1–5

Karnaouri A, Topakas E, Paschos T, Taouki I, Christakopoulos P (2013) Cloning, expression and characterization of an ethanol tolerant GH3 β-glucosidase from *Myceliophthora thermophile*. PeerJ 1:e46

Kawai R, Igarashi K, Kitaoka M, Ishii T, Samejima M (2004) Kinetics of substrate transglycosylation by glycoside hydrolase family 3 glucan (1 → 3)-beta-glucosidase from the white-rot fungus Phanerochaete chrysosporium. Carbohydr Res 339:2851–2857

Kellermann SJ, Rentmeister A (2014) Current developments in cellulase engineering. ChemBioEng Rev 1:6–13

Keresztessy Z, Kiss L, Hughes MA (1994) Investigation of the active site of the cyanogenic beta-D-glucosidase (linamarase) from *Manihot esculenta* Crantz (cassava). II. Identification of Glu-198 as an active site carboxylate group with acid catalytic function. Arch Biochem Biophys 315:323–330

Ketudat Cairns JR, Esen A (2010) β-Glucosidases. Cell Mol Life Sci 67:3389–3405

Khan S, Pozzo T, Megyeri M, Lindahl S, Sundin A, Turner C, Karlsson EN (2011) Aglycone specificity of *Thermotoga neapolitana* b-glucosidase 1A modified by mutagenesis, leading to increased catalytic efficiency in quercetin-3-glucoside hydrolysis. BMC Biochem 12:11

Kim BJ, Singh SP, Hayashi K (2006) Characteristics of chimeric enzymes constructed between *Thermotoga maritima* and *Agrobacterium tumefaciens* beta-glucosidases: role of C-terminal domain in catalytic activity. Enzyme Microb Technol 38:952–959

Kittur FS, Lalgondar M, Yu HY, Bevan DR, Esen A (2007) Maize β-glucosidase-aggregating factor is a polyspecific jacalin related chimeric lectin, and its lectin domain is responsible for β-glucosidase aggregation. J Biol Chem 282:7299–7311

Krisch J, Takó M, Papp T, Vágvölgyi C (2010) Characteristics and potential use of β-glucosidases from Zygomycetes. In: Vilas AM (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology extremadura. Formatex Research Center, Spain, pp 891–896

Kuntothom T, Luang S, Harvey AJ, Fincher GB, Opassiri R, Hrmova M, Ketudat Cairns JR (2009) Rice family GH1 glycosyl hydrolases with β-D-glucosidase and β-D-mannosidase activities. Arch Biochem Biophys 491:84–95

Lammirato C, Miltner A, Wick LY, Kästner M (2010) Hydrolysis of cellobiose by β-glucosidase in the presence of soil minerals—interactions at solid-liquid interfaces and effects on enzyme activity levels. Soil Biol Biochem 42:2203–2210

Langston J, Sheehy N, Xu F (2006) Substrate specificity of *Aspergillus oryzae* family 3 β-glucosidase. Biochim Biophys Acta Proteins Proteom 1764:972–978

Leah R, Kiegel J, Suendson IB, Mundy J (1995) Biochemical and molecular characterization of a barley seed β-glycosidase. J Biol Chem 22:15789–15796

Lee HL, Chang CK, Jeng WY, Wang AH, Liang PH (2012) Mutations in the substrate entrance region of β-glucosidase from *Trichoderma reesei* improve enzyme activity and thermostability. Protein Eng Des Sel 25:733–740

Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell 126:1109–1120

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–1183

Liu J, Zhang X, Fang Z, Fang W, Peng H, Xiao Y (2011) The 184th residue of β-glucosidase Bgl1B plays an important role in glucose tolerance. J Biosci Bioengineer 112:447–450

Lundemo P, Adlercreutz P, KarlssonImproved EN (2013) Transferase/hydrolase ratio through rational design of a family 1 β-glucosidase from *Thermotoga neapolitana*. Appl Environ Microbiol 79:3400–3405

Ly HD, Withers SG (1999) Mutagenesis of glycosidases. Annu Rev Biochem 68:487–522

Machida M, Ohtsuki I, Fukui S, Yamashita I (1988) Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular β-glycosidases as expressed in *Saccharomyces cerevisiae*. Appl Environ Microbiol 54:3147–3155

Mackenzie LF, Wang Q, Warren RAJ, Withers SG (1998) Glycosynthases: mutant glycosidases for oligosaccharide synthesis. J Am Chem Soc 120:5583–5584

Marana SR (2006) Molecular basis of substrate specificity in family 1 glycoside hydrolases. IUBMB Life 58:63–73

Marana SR, Jacobs-Lorena M, Terra WR, Ferrieira C (2001) Amino acid residues involved in substrate binding and catalysis in an insect digestive β-glycosidase. Biochim Biophys Acta 1545:41–52

Marana SR, Terra WR, Ferreira C (2002) The role of amino acid residues Q39 and E451 in the determination of substrate specificity of the *Spodoptera frugiperda* β-glycosidase. Eur J Biochem 269:3705–3714

Marri L, Valentini S, Venditti D (1995) Cloning and nucleotide sequence of *bglA* from *Erwinia herbicola* and expression of β-glycosidase activity in *Escherichia coli*. FEMS Microbiol Lett 128:135–138

Matsui I, Sakai Y, Matsui E, Kikuchi M, Kawarabayasi Y, Honda K (2000) Novel substrate specificity of a membrane-bound β-glycosidase from the hyperthermophilic archeon *Pyrococcus horikoshii*. FEBS Lett 467:195–200

Mattiacci L, Dicke M, Posthumus MA (1995) Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts hostsearching parasitic wasps. Proc Natl Acad Sci USA 92:2036–2040

McFarland KC, Ding H, Teter S, Vlasenko E, Xu F, Cherry J (2007) Development of improved cellulase mixtures in a single production organism. In: Eggleston G, Vercellotti JR (eds) Industrial application of enzymes on carbohydrate based materials. American Chemical Society & Oxford University Press, New York, pp 19–31

Mendonca LMF, Marana SR (2008) The role in the substrate specificity and catalysis of residues forming the substrate aglycone-binding site of a β-glycosidase. FEBS J 275:2536–2547

Morant AV, Bjarnholt N, Kragh ME, Kjaergaard CH, Jørgensen K, Paquette SM, Piotrowski M, Imberty A, Olsen CE, Møller BL, Bak S (2008a) The beta-glucosidases responsible for bioactivation of hydroxynitrile glucosides in *Lotus japonicus*. Plant Physiol 147:1072–1091

Morant AV, Jørgensen K, Jørgensen C, Paquette SM, Sanchez-Perez R, Møller BL, Bak S (2008b) β-Glucosidases as detonators of plant chemical defense. Phytochemistry 69:1795–1813

Nagano AJ, Matsushima R, Hara-Nishimura I (2005) Activation of an ER-body-localized β-glucosidase via a cytosolic binding partner in damaged tissues of *Arabidopsis thaliana*. Plant Cell Physiol 46:1140–1148

Namchuck NM, Withers SG (1995) Mechanism of *Agrobacterium* β-glucosidase: kinetic analysis of the role of noncovalent enzyme/substrate interactions. Biochemistry 34:16194–16202

Nikus J, Daniel G, Jonsson LM (2001) Subcellular localization of beta-glucosidase in rye, maize and wheat seedlings. Plant Physiol 111:466–472

Nisius A (1988) The stroma centre in Avena plastids: an aggregation of β-glucosidase responsible for the activation of oat-leaf saponins. Planta 173:474–481

Opassiri R, Hua Y, Wara-Aswapati O, Akiyama T, Svasti J, Esen A, Ketudat Cairns JR (2004) β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. Biochem J 379:125–131

Opassiri R, Ketudat Cairns JR, Akiyama T, Wara-Aswapati O, Svasti J, Esen A (2003) Characterization of a rice β-glucosidase highly expressed in flower and germinating shoot. Plant Sci 165:627–638

Opassiri R, Pomthong B, Okoksoong T, Akiyama T, Esen A, Ketudat Cairns JR (2006) Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 b-glucosidase. BMC Plant Biol 6:33

Opassiri R, Pomthong B, Akiyama T, Nakphaichit M, Onkoksoong T, Ketudat Cairns M, Ketudat Cairns JR (2007) A stress-induced rice β-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. Biochem J 408:241–249

Park TH, Choi KW, Park CS, Lee SB, Kang HY, Shon KJ, Park JS, Cha J (2005) Substrate specificity and transglycosylation catalyzed by a thermostable β-glucosidase from marine hyperthermophile *Thermotoga neapolitana*. Appl Microbiol Biotechnol 69:411–422

Payne CM, Knott BC, Mayes HB, Hansson H, Himmel ME, Sandgren M, Ståhlberg J, Beckham GT (2015) Fungal cellulases. Chem Rev 115:1308–1448

Perry JD, Morris KA, James AL, Oliver M, Gould FK (2007) Evaluation of novel chromogenic substrates for the detection of bacterial β-glucosidase. J Appl Microbiol 102:410–415

Poulton JE (1990) Cyanogenesis in plants. Plant Physiol 94:401–405

Pozzo T, Pasten JL, Karlsson EN, Logan DT (2010) Structural and functional analyses of beta-glucosidase 3B from Thermotoga neapolitana: a thermostable three-domain representative of glycoside hydrolase 3. J Mol Biol 397:724–739

Qi M, Jun HS, Forsbert CW (2008) Cel9D, an atypical 1, 4-β-D-glucan glucohydrolase from *Fibrobacter succinogenes*: characteristics, catalytic residues, and synergistic interactions with other cellulases. J Bacteriol 109:1976–1984

Quinlan RJ, Teter S, Xu F (2010) Towards the development of cellulases: approaches, obstacles and outlook. In: Waldron K (ed) Bioalcohol production: biochemical conversion of lignocellulosic biomass. Woodhead Publishing, CRC Press, Boca Raton, pp 178–204

Rani V, Mohanram S, Tiwari R, Nain L, Arora A (2014) Beta-glucosidase: key enzyme in determining efficiency of cellulase and biomass hydrolysis. J Bioprocess Biotechnol 5:197

Reuveni M, Sagi Z, Evnor D, Hetzroni A (1999) β-Glucosidase activity is involved in scent production in Narcissus flowers. Plant Sci 147:19–24

Sanz-Aparicio J, Hermoso JA, Martinez-Ripoll M, Lequerica JL, Polaina J (1998) Crystal structure of beta-glucosidase A from *Bacillus polymyxa*: insights into the catalytic activity in family 1 glycosyl hydrolases. J Mol Biol 275:491–502

Saqib AAN, Whitney PJ (2006) Esculin gel diffusion assay (EGDA): a simple and sensitive method for screening β-glucosidases. Enzyme Microb Technol 39:182–184

Schubot FD, Kataeva IA, Chang J, Shah AK, Ljungdahl LG, Rose JP, Wang BC (2004) Structural basis for the exocellulase activity of the cellobiohydrolase CbhA from *Clostridium thermocellum*. Biochemistry 43:1163–1170

Seidle HF, Allison SJ, George E, Huber RE (2006) Trp-49 of the family 3 beta-glucosidase from *Aspergillus niger* is important for its transglucosidic activity: creation of novel beta-glucosidases with low transglucosidic efficiencies. Arch Biochem Biophys 455:110–118

Seidle HF, Huber RE (2005) Transglucosidic reactions of the *Aspergillus niger* family 3 β-glucosidase: qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. Arch Biochem Biophys 436:254–264

Seidle HF, McKenzie K, Marten I, Shoseyov O, Huber RE (2005) Trp-262 is a key residue for the hydrolytic and transglucosidic reactivity of the *Aspergillus niger* family 3 β-glucosidase: substitution results in enzymes with mainly transglucosidic activity. Arch Biochem Biophys 444:66–75

Seshadri S, Akiyama T, Opassiri R, Kuaprasert B, Ketudat Cairns J (2009) Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosides and (1 → 3)- and (1 → 2)-linked disaccharides. Plant Physiol 151:47–58

Sharmila T, Sreeramulu G, Nand K (1998) Purification and characterization of β-1-4,-glucosidase from *Clostridium papyrosolvens*. Biotechnol Appl Biochem 27:175–179

Sherameti I, Venus Y, Drzewiecki C, Tripathi W, Dan VM, Nitz I, Varma A, Grundler F, Oelmuller R (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thanliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Shim JH, Chen HM, Rich JR, Goddard-Borger ED, Withers SG (2012) Directed evolution of a β-glycosidase from *Agrobacterium* sp. to enhance its glycosynthase activity toward C3-modified donor sugars. Protein Eng Des Sel 25:465–472

Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127:500–507

Sørensen A, Lübeck M, Lübeck PS, Ahring BK (2013) Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomolecules 3:612–631

Souza FHM, Nascimento CV, Rosa JC, Masui DC, Leone FA, Jorge JA, Furriel RPM (2010) Purification and biochemical characterization of a mycelial glucose- and xylose-stimulated β-glucosidase from the thermophilic fungus *Humicola insolens*. J Process Biochem 45: 272–278

Srivastava KK, Verma PK, Srivastava R (1999) A recombinant cellulolytic *Escherichia coli*: cloning of the cellulase gene and characterization of a bifunctional cellulase. Biotechnol Lett 21:293–297

Sue M, Yamazaki K, Yajima S, Nomura T, Matsukawa T, Iwamura H, Miyamoto T (2006) Molecular and structural characterization of hexameric beta-D-glucosidases in wheat and rye. Plant Physiol 141:1237–1247

Suzuki H, Takahasi S, Watanabe R, Fukushima Y, Fujita N, Noguchi A, Yokoyama R, Nishitani K, Nishino T, Nakayama T (2006) An isoflavone conjugate-hydrolyzing

β-glucosidase from the roots of soybean (*Glycine max*) seedlings. J Biol Chem 281:30251–30259

Sweeney MD, Xu F (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2:244–263

Takano M, Moriyama R, Ohmiya K (1992) Structure of a β-glycosidase gene from *Ruminococcus albus* and properties of the translated product. J Ferment Bioeng 73:79–88

Takashima S, Nakamura A, Hidaka M, Masaki H, Uozumi T (1999) Molecular cloning and expression of the novel fungal β-glycosidase genes from *Humicola grisea* and *Trichoderma reesei*. J Biochem 125:728–736

Tejirian A, Xu F (2010) Inhibition of cellulase-catalyzed lignocellulosic hydrolysis by iron and oxidative metal ions and complexes. Appl Environ Microbiol 76:7673–7682

Tejirian A, Xu F (2011) Inhibition of enzymatic cellulolysis by phenolic compounds. Enzyme Microb Technol 48:239–247

Teter S, Xu F, Nedwin GE, Cherry JR (2006) Enzymes for biorefineries. In: Kamm B, Gruber PR, Kamm M (eds) Biorefineries—industrial processes and products: status quo and future directions. Wiley-VCH, Weinheim, pp 357–384

Teugjas H, Väljamäe P (2013) Selecting β-glucosidases to support cellulases in cellulose saccharification. Biotechnol Biofuels 6:105

Thorlby G, Fourier N, Warren G (2004) The SENSITIVE TO FREEZING2 gene, required for freezing tolerance in *Arabidopsis thaliana*, encodes a beta-glucosidase. Plant Cell 16: 2192–2203

Tiwari R, Singh S, Nain PK, Rana S, Sharma A, Pranaw K, Nain L (2013) Harnessing the hydrolytic potential of phytopathogenic fungus *Phoma exigua* ITCC 2049 for saccharification of lignocellulosic biomass. Bioresour Technol 150:228–234

Tribolo S, Berrin J-G, Kroon PA, Czjzek M, Juge N (2007) The structure of human cytoplasmic β-glucosidase unravels substrate aglycone specificity of a family 1 glycoside hydrolase. J Mol Biol 370:964–975

Trimbur DE, Warren RAJ, Withers SG (1992) Region-directed mutagenesis of residues surroundingthe active site nucleophile in β-glucosidase from *Agrobacterium faecalis*. J Biol Chem 267:10248–10251

Tsukada T, Igarashi K, Fushinobu S, Samejima M (2008) Role of subsite +1 residues in pH dependence and catalytic activity of the glycoside hydrolase family 1 β-glucosidase BGL1A from the Basidiomycete *Phanerochaete chrysosporium*. Biotechnol Bioeng 99:1295–1302

Tsukada T, Igarashi K, Yoshida M, Samejima M (2006) Molecular cloning and characterization of two intracellular β-glucosidases belonging to glycoside hydrolase family 1 from the basidiomycete *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 73:807–814

Uchiyama T, Miyazaki K, Yaoi K (2013) Characterization of a novel β-glucosidase from a compost microbial metagenome with strong transglycosylation activity. J Biol Chem 288:18325–18334

Vallmitjana M, Ferrer-Navarro M, Planell R, Abel M, Ausín C, Querol E, Planas A, Pérez-Pons JA (2001) Mechanism of the family 1 beta-glucosidase from *Streptomyces* sp.: catalytic residues and kinetic studies. Biochemistry 40:5975–5982

van den Brink J, de Vries RP (2011) Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 91:1477–1492

van Zyl WH, Lynd LR, den Haan R, McBride JE (2007) Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 108:205–235

Varghese JN, Hrmova M, Fincher GB (1999) Three-dimensional structure of a barley β-D-glucan exohydrolase; a family 3 glycosyl hydrolase. Structure 7:179–190

Verdoucq L, Moriniere J, Bevan DR, Esen A, Vasella A, Henrissat B, Czjzek M (2004) Structural determinants of substrate specificity in family 1 β-glucosidases: novel insights from the crystal structure of sorghum dhurrinase-1, a plant β-glucosidase with strict specificity, in complex with its natural substrate. J Biol Chem 279:31796–31803

Vroemen S, Heldens J, Boyd C, Henrissat B, Keen NT (1995) Cloning and characterization of the *bgxa* genefrom *Erwinia chrysanthemi* D1 that encodes a β-glycosidase/xylosidase enzyme. Mol Gen Genet 246:465–477

Wang Q, Graham RW, Trimbur D, Warren RAJ, Withers SG (1994) Changing enzymic reaction mechanisms by mutagenesis: conversion of a retaining glucosidase to an inverting enzyme. J Am Chem Soc 116:11594–11595

Wang Q, Trimbur D, Graham R, Warren RA, Withers SG (1995) Identification of the acid/base catalyst in *Agrobacterium faecalis* beta-glucosidase by kinetic analysis of mutants. Biochemistry 34:14554–14562

Wilson CA, Wood TM (1992) The anaerobic fungus Neocallimastix frontalis: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose. Appl Microbiol Biotechnol 37:125–129

Withers SG, Street IP, Bird P, Dolphin DH (1987) 2-Deoxy-2-fluoroglucosides: a novel class of mechanism based inhibitors. J Am Chem Soc 109:7530–7531

Withers SG, Street IP (1988) Identification of a covalent α-D-glucopyranosyl enzyme intermediate formed on a β-glucosidase. J Am Chem Soc 110:8551–8553

Withers SG, Warren RAJ, Street IP, Rupitz K, Kempton JB, Aebersold R (1990) Unequivocal demonstration of the involvement of a glutamate residue as a nucleophile in the mechanism of a retaining glycosidase. J Am Chem Soc 112:5887–5889

Wulff-Strobel CR, Wilson DB (1995) Cloning, sequencing, and characterization of a membrane associated *Prevotella ruminicola* B1 4-β-glycosidase with cellodextrinase and cyanoglycosidase activities. J Bacteriol 177:5884–5890

Xie S, Syrenne R, Sun S, Yuan JS (2014) Exploration of natural biomass utilization systems (NBUS) for advanced biofuel—from systems biology to synthetic design. Curr Opin Biotechnol 27:195–203

Xu F (2010a) Enzymatic degradation of lignocellulosic biomass. In: Tao A, Kazlauskas R (eds) Biocatalysis for green chemistry and chemical process development. Wiley, Hoboken, pp 361–390

Xu F (2010) Biomass-converting enzymes and their bioenergy applications. In: Baltz RH, Demain AL, Davies JE (ed-in-chief) Bull AT, Junker B, Katz L, Lynd LR, Masurekar P, Reeves CD, Zhao H (eds) The manual of industrial microbiology and biotechnology, 3rd edn. American Society for Microbiology Press, Washington, DC, pp 495–508

Xu F (2004) Enhancing biomass conversion to fermentable sugars: a progress report of a joint government-industrial project. In: Ohmiya K, Sakka K, Karita S, Kimura T, Sakka M, Onishi Y (eds) Biotechnology of lignocellulose degradation and biomass utilization. Uni Publishers, Tokyo, pp 793–804

Xu F, Ding H, Osborn D, Tejirian A, Brown K, Albano W, Sheehy N, Langston J (2007) Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. J Mol Catal B Enz 51:42–48

Xu Z, Escamilla-Trevino LL, Zeng L, Lalgondar M, Bevan DR, Winkel BSJ, Mohamed A, Cheng C, Shih M, Poulton JE, Esen A (2004) Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1. Plant Mol Biol 55:343–367

Yamada R, Hasunuma T, Kondo A (2013) Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnol Adv 31:754–763

Zouhar J, Vevodova J, Marek J, Damborsky J, Su X-D, Bryzobohaty B (2001) Insights into the functional architecture of the catalytic center of a maize β-glucosidase Zm-p60.1. Plant Physiol 127:973–985

Part II
Hemicellulases

Chapter 5
Role and Applications of Feruloyl Esterases in Biomass Bioconversion

Constantinos Katsimpouras, Io Antonopoulou, Paul Christakopoulos and Evangelos Topakas

Abstract Ferulic acid esterases (FAEs) act synergistically with xylanases to hydrolyze the feruloylated decorations of the hemicellulosic fraction of cell wall material and therefore play a major role in the degradation of plant biomass. In this review, their role in plant biomass degradation, their production, classification, and structural determination are discussed. In addition, the production, physicochemical properties, and molecular biology of the different type of FAEs are presented, giving emphasis in their potential applications utilizing their hydrolytic and synthetic activity. A detailed map of the reaction systems used to date is demonstrated, underpinning the potential of these enzymes as biosynthetic tools in the synthesis of bioactive compounds for use in food and cosmeceutical industries.

5.1 Introduction

The combustion of fossil fuels constitutes a great percentage of our current energy supply, and with depleting crude oil reserves and climate change due to the greenhouse gas (GHG) emissions, it is imperative that we focus on more renewable energy sources such as biofuels (Damásio et al. 2013). Biofuels from lignocellulosic biomass, also referred to as second-generation biofuels, are considered to be the key due to their potential to mitigate GHG emissions compared with conventional fuels, reduce oil dependency, and eradicate public concerns about a trade-off between foods and fuels.

C. Katsimpouras · E. Topakas (✉)
Biotechnology Laboratory, School of Chemical Engineering,
National Technical University of Athens, 15780 Athens, Greece
e-mail: vtopakas@chemeng.ntua.gr; vtopakas@central.ntua.gr

I. Antonopoulou · P. Christakopoulos
Biochemical and Chemical Process Engineering, Division of Sustainable Process
Engineering, Department of Civil, Environmental and Natural Resources Engineering,
Luleå University of Technology, SE-97187 Lulea, Sweden

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_5

Production of fuel ethanol from renewable lignocellulosic materials, such as agricultural residues, forest residues, and energy crops has been extensively studied in the last few decades. Lignocellulosic raw materials, being low-cost and abundant as residual biomass from agricultural and agroindustrial waste, provide an attractive feedstock for the production of ethanol and other value-added products under the biorefinery concept without competing with food and feed industry (Larsen et al. 2012). Through enzymatic hydrolysis processes, commonly known as saccharification, the main components of lignocellulosic biomass can be converted to sugars and subsequently fermented to bioethanol. However, the plant cell wall, developed in the span of million of years of co-evolution as the plants' defense system against pathogens, bottlenecks the aforementioned processes and several improvements should be made in order to produce cellulosic ethanol at competitive costs (Malinovsky et al. 2014).

A possible strategy that is being suggested requires the usage of hemicellulose-degrading enzymes in order to generate more efficient enzyme cocktails for the saccharification of lignocellulosic biomass and reduce catalysts costs. In addition, the cooperative action of enzymes could also lead to less severe, and thus more sustainable and environmentally friendly, pretreatment processes (Couturier et al. 2011).

A set of enzymes that is considered to be a biotechnological key in cell wall hydrolysis and in the extraction of phenolic acids is ferulic acid esterases (FAEs) or feruloyl esterases, as they are also known. FAEs (E.C. 3.1.1.73) represent a subclass of the carboxylic ester hydrolases, which liberate phenolic acids such as ferulic acid (FA) or *p*-coumaric acid and their dimers from naturally occurring hemicelluloses (Fig. 5.1) and pectins, where they mainly occur as esters with L-arabinofuranose-containing polysaccharides, such as L-arabino-D-xylans and

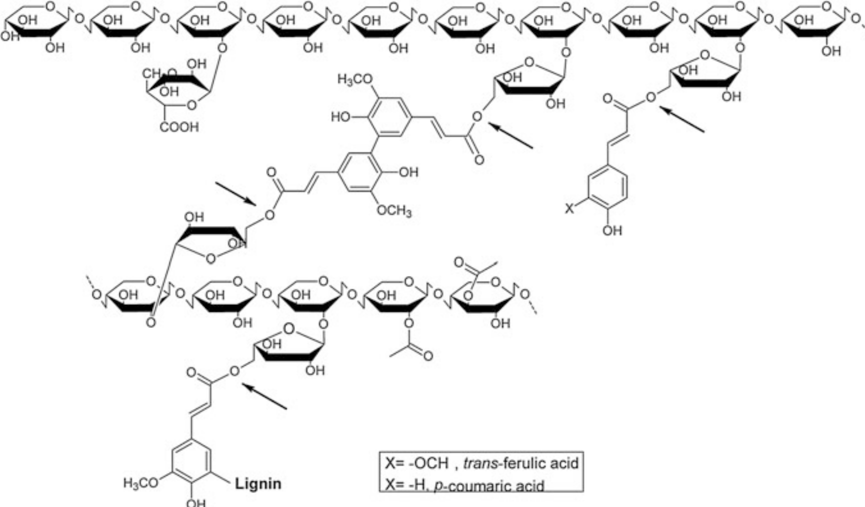


Fig. 5.1 Action of FAEs for the hydrolysis of ester bonds in plant cell wall arabinoxylan, as indicated by *black arrows*. However, most of the characterized FAEs are acting on soluble feruloylated fractions created by the action of endo-β-1,4-xylanases and not directly on the sugar polymer showing strong synergistic interaction

L-arabinans (Topakas et al. 2007). Besides FAEs' role in hydrolytic reactions, they are also applicable for the synthesis of bioactive compounds with antioxidant and/or antimicrobial activity, and other compounds of high industrial interest. The present review is an attempt to elucidate the role and applications of FAEs in lignocellulosic biomass degradation and as biosynthetic tools for the modification of plant derived antioxidants.

5.2 Plant Cell Wall Functionality

Plant cell walls, besides their role as structural support to the plant body, also restrict enzyme access rendering this complex polysaccharide network recalcitrant to biochemical degradation. Cellulose, hemicellulose, lignin, and pectin are the main components of lignocellulosic biomass along with proteins and aromatic compounds at proportions that differ among plant species (de Oliveira et al. 2014). In plants, two major types of cell walls could be distinguished: the primary cell walls, which are composed of cellulose, hemicelluloses, pectins, and proteins, and the secondary cell walls, consisting of cellulose, hemicelluloses, and lignin that are formed after the end of cell expansion (Endler and Persson 2011). Cellulose is a linear natural biopolymer consisting of glucose units linked by β-1,4-glycosidic bonds forming crystalline microfibrils via hydrogen bonding and van der Waals interactions (Horn et al. 2012).

After cellulose, hemicelluloses are the second most abundant polysaccharides on earth with xylans to be their main representative. Hemicelluloses are a group of heterogeneous polysaccharides consisting of a β-1,4-linked backbone of pentoses, hexoses, and sugar acids, which is usually decorated with side branches (Saha 2003). The exact structure and abundance of hemicelluloses diversify widely among different plant species and cell types with xyloglucans occurring mostly in the primary walls of dicots and conifers, while arabinoxylans dominate in commelinid monocots (Scheller and Ulvskov 2010). Through interactions with cellulose, and sometimes lignin, hemicelluloses have the ability to strengthen the cell walls (Endler and Persson 2011).

Pectins are a group of heterogeneous and branched polysaccharides that can be found only in the primary cell wall and consist of a backbone of α-1,4-linked D-galacturonic acid residues that can be methyl-esterified or decorated with acetyl groups. Homogalacturonans, xylogalacturonan, and rhamnogalacturonans (I & II) are the main groups in which pectins could be classified according to their basic structure (Wong 2008). There is also increasing evidence that pectin interacts covalently with hemicelluloses providing structural and functional complexity to the plant cell wall (Caffal and Mohnen 2009).

Cell walls are also fortified by the deposition of the phenylpropanoid polymer lignin, which is one of the main contributors in recalcitrance of cell walls to enzymatic saccharification (Chen and Dixon 2007). The term lignin is employed to describe a group of aromatic and nonsoluble heteropolymers consisting mainly of

phenylpropanoid units derived from the oxidative polymerization of three hydroxycinnamyl alcohols (coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol) derivatives (Vanholme et al. 2010; Vogt 2010). The aforementioned hydroxycinnamyl alcohols are incorporated into the lignin polymer in the form of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units. In gymnosperms G-units are mainly present with small amounts of H-units, while lignin in angiosperm dicots consists of G- and S-units. In grasses and softwoods H-units amounts are higher (Vanholme et al. 2010). In lignocellulosic biomass, lignin is cross-linked with cell wall polymers by ether or ester bonds with FA being the predominant connector as the FA that is etherified to lignin is also esterified to polysaccharides (Iiyama et al. 1990).

Hydroxycinnamic acids such as FA, sinapic acid, and *p*-coumaric acid are components of plant cell wall with the ability to esterify hemicelluloses due to a carboxylic group at the end of their propenyl group. FA plays an important role in the plant cell wall by cross-linking with polysaccharides by ester bonds and with lignin by ether bonds regulating this way the cell wall extensibility and inhibiting pathogen invasion (de Oliveira et al. 2014). Implication of FA and diferulates to function as nucleation sites for the lignification process has also been described (Ralph et al. 1995).

FA is synthesized in the phenylpropanoid pathway from phenylalanine and in nature exists mainly in *E* (*trans*) form since isomerization in *Z* (*cis*) form occurs during extraction due to the light sensitivity of hydroxycinnamates (Faulds and Williamson 1999). In monocotyledons such as grasses and cereals, the arabinofuranoses are esterified by hydroxycinnamates with *trans*-FA, (*E*)-4-hydroxy-3-methoxycinnamic acid to be the most abundant whereas dicots contain ferulated pectic polysaccharides (Mueller-Harvey et al. 1986). FA is esterified at position *O*-5 to α-L-arabinofuranosyl side chains in arabinoxylans (Mueller-Harvey et al. 1986), at position *O*-6 to β-D-galactopyranosyl residues in pectic rhamno-galacturonanans (Colquhoun et al. 1994) and at position *O*-4 to a-D-xylopyranosyl residues in xyloglucans (Ishii et al. 1990). In some dicots such as spinach (Fry 1983) and sugar beet pulp (Colquhoun et al. 1994), feruloylation occurs at position *O*-2 to α-L-arabinofuranosyl residues in arabinans and at position *O*-6 to β-D-galactopyranosyl residues in pectic rhamno-galacturonanans. FA amount varies in the cell walls of different plant materials ranging from 0.1 to 3.8 % (w/w, dry weight). In wheat bran, FA (w/w, dry weight) account for 0.66 %, 3.1 % in maize bran, 1.24 % in wheat straw, 0.9 % in rice endosperm cells, 0.14 % in barley grains, 0.32 % in barley spent grain, 1.4 % in barley hulls, 2.2–3.8 % in oat hulls, and 0.87 % in sugar beet pulp (Smith and Hartley 1983; Saulnier et al. 1995; Benoit et al. 2006; Shibuya 1984; Nordkvisk et al. 1984; Bartolome et al. 1997a, b; Tenkanen et al. 1991; Garleb et al. 1988; Yu et al. 2002a, b; Kroon and Williamson 1996).

Covalent cross-linking between feruloylated polysaccharide chains or between hemicellulose and lignin can be achieved through the formation of ferulate dimers. Dimers of FA can be formed by radical coupling reactions or by photochemical coupling reactions (Ralph et al. 1994; Ishii 1997). Photochemical [2+2]-cyclodimerization (Ford and Hartley 1990) produces cyclodimers, while peroxidase-catalyzed coupling produces several forms of dehydrodimers such as 8-5-, 8-*O*-4-, 5-5-, 8-8-, and 4-*O*-5-coupled dehydrodimers (Bunzel et al. 2005) commonly known

as diferulates. In pectins isolated from sugar beet pulp, 8.8 % of the ferulates were mostly 8-8- and 8-*O*-4-coupled dehydrodimers (Oosterveld et al. 1997).

Fry et al. (2000) suggested that trimers or even larger products contribute highly to cross-linking between polysaccharides in culture maize cells. The first FA dehydrotrimer was isolated from maize bran insoluble fiber and found to be a 5-5/8-*O*-4-coupled dehydrotrimer (Bunzel et al. 2003). Since then, more dehydrotrimers and dehydrotetramers have been identified and characterized (Rouaou et al. 2003; Bunzel et al. 2005, 2006; Funk et al. 2005; Barron et al. 2007; Hemery et al. 2009).

5.3 Role of FAEs in Plant Biomass Degradation and Their Synergistic Action

The complex and extended polysaccharide networks in plant cell walls, due to the covalent cross-linking mediated by FA, raise hurdles in utilizing lignocellulosic biomass for biofuel production, as it contributes to biomass recalcitrance to enzymatic degradation (Wong et al. 2013). In order to surmount these hurdles, along with cellulases and hemicellulases, accessory enzymes such as FAEs should be employed, enhancing this way the fermentable sugars yield from lignocellulosic biomass (Faulds 2010). FAEs action depends highly on the type of xylanases that is being used with, having an effect not only on the amount but also in the form of FA released, with family 11 xylanases being more efficient in the hydrolysis of FA whereas family 10 xylanases present a more synergistic effect on the release of diferulic acid (Faulds et al. 2006). The synergism of a recombinant FAE isolated from a rumen microbial metagenome (RuFae2), in association with GH10 and GH11 endoxylanases was investigated by Wong et al. (2013). The results obtained from the release of FA from several natural substrates such as corn fiber, corn bran, wheat bran, wheat-insoluble arabinoxylan, and switchgrass indicated that the GH10 xylanase showed a greater synergistic effect than that of the GH11 xylanase (Wong et al. 2013). The synergistic action between xylanases and FAEs on the release of FA from feruloylated polysaccharides seems to render the biomass more vulnerable to glycoside hydrolase enzymes (Yu et al. 2003).

Tabka et al. (2006) reported a synergistic effect of a recombinant FAE from *Aspergillus niger* when combined with cellulases and xylanases from *Trichoderma reesei* in the hydrolysis of wheat straw. Significant increase in the release of reducing sugars (34.8 % increase in total reducing sugars) from oat hulls was also a result of synergistic interaction between *A. niger* FAE and *T. reesei* xylanase (Yu et al. 2003). The enzymatic saccharification of pretreated corn stover by a cellobiohydrolase from *T.* reesei was significantly enhanced by the addition of small quantities of an endoxylanase, a FAE, and an acetyl xylan esterase. FAE addition to cellobiohydrolase achieved a 37 % synergistic improvement in glucan conversion to cellobiose, while a substantial increase of 85 % was shown when all enzymes were added (Selig et al. 2008). Supplementation of a commercial mixture of cellulase and β-glucosidase with pectinase and FAE preparations resulted in higher arabinose and xylose yields from

pretreated dried distiller's grains with solubles, a co-product of corn ethanol production (Dien et al. 2008). Kim et al. (2008) reported a 15 % increase in glucose yields and 2–4 times enhancement for xylose yields in the enzymatic hydrolysis of pretreated distiller's grain when cellulases were supplemented with xylanase and FAE activities. Two recombinant FAEs from *Penicillium funiculosum* expressed in *Aspergillus awamori* (FaeA and FaeB) were able to enhance the cellulolytic activity of purified *T. reesei* on pretreated corn stover by releasing 19 and 7 % more cellobiose, respectively (Knoshaug et al. 2008). The synergistic action among cellulases, xylanases, β-glucosidases, and FAEs produced by *T. reesei* and *A. awamori* was investigated for the hydrolysis of steam-pretreated sugarcane bagasse, as reported by Gottschalk et al. (2010). Addition of FAE and xylanase activities, produced by *Aspergillus oryzae*, in a commercial enzyme preparation resulted in increased bioconversion of sugarcane bagasse by 36 % (Braga et al. 2014).

Enzymatic release of FA from agroindustrial waste materials with the use of FAEs raises considerable interest due to its antioxidant and anti-inflammatory properties. FA is considered to alleviate oxidative stress in neurodegenerative disorders such as Alzheimer's disease (Sultana et al. 2005). Furthermore, FA could be employed as a source for the bioconversion into value-added products such as vanillin, which is one of the most used flavoring agents in the food, pharmaceutical, and cosmetic industries (Priefert et al. 2001).

Two FAEs from *Streptomyces* sp. were tested on their ability to release FA from corn bran, wheat bran, and defatted rice bran in combination with xylanase and α-L-arabinofuranosidase activities from *Streptomyces* with the results suggesting that the amount of FA released from biomass increased due to a synergistic effect between these enzymes (Uraji et al. 2014). A recombinant FAE from *Aspergillus usamii* in combination with a recombinant GH 11 xylanase from the same microorganism exhibited a 27 % increase in FA release from destarched wheat bran compared to that of the FAE alone (Gong et al. 2013). The amount of FA released from steam exploded corn stalk, when a FAE from *Aspergillus flavus* (*Af*FaeA) combined with a GH 10 xylanase from *Geobacillus stearothermophilus*, was 13-fold higher than that released by *Af*FaeA alone (Zhang et al. 2013). Wu et al. (2012) reported the characterization of two FAEs of myxobacterial origin (*Sorangium cellulosum*) capable of releasing FA from grass biomass, such as triticale bran, and from wheat bran yielding up to 85 % of total alkali-extractable FA in presence of a *Trichoderma viride* xylanase. A recombinant FAE (*Mt*Fae1a) from the thermophilic fungus *Myceliophthora thermophila* (synomym *Sporotrichum thermophile*) was able to release up to 41 % of the total alkali-extractable FA from wheat bran only with the presence of a xylanase, indicating this way the synergistic interaction between *Mt*Fae1a and M3 *Trichoderma longibrachiatum* xylanase (Topakas et al. 2012). Synergistic interaction between a FAE (Tx-Est1) and a GH11 xylanase (Tx-Xyl11) both from *Thermobacillus xylanilyticus* was also reported by Rakotoarivonina et al. (2011), where esterase's efficiency in releasing FA and diferulic acid from destarched wheat bran was seven- to eightfold higher than that of Tx-Est1 alone. A maximum of 67 % of total FA was released from destarched wheat bran by the combined action of a type C FAE from *Fusarium oxysporum* and a M3 *T. longibrachiatum* xylanase (Moukouli et al. 2008).

5.4 FAE Classification and Structural Determination

FAEs appear to be a very diverse set of enzymes, with little unifying sequence and physical characteristics to link them. In order to categorize and classify these carbohydrate esterases into different types sharing the same substrate specificity and/or the same structure, several attempts have been made starting in 2004. Under this scope, the use of multiple alignments of sequences or domains demonstrating FAE activity, as well as related sequences, helped to construct a neighborhood-joining phylogenic tree (Crepin et al. 2004a). The outcome of this genetic comparison supported substrate specificity data and allowed FAEs to be subclassified into 4 types: A, B, C, and D, a classification that is still used in literature by many researchers. Thus, there does appear to be an evolutionary relationship between FAEs, acetyl xylan esterases, tannases and certain lipases. The substrate specificity was based on their specificity toward mono- and diferulates, on substitutions on the phenolic ring, and on their amino acid sequence identity (Crepin et al. 2004a). An unofficial nomenclature for describing FAEs was followed based on this classification, following both the source of the enzyme and the type of the esterases (e.g., the type-A FAE produced by *F. oxysporum* is termed *Fo*FaeA).

It is extremely common for esterases to act on a broad range of substrates. Type A FAEs exhibit relatively high sequence identity with fungal lipases, however lipase activity is not detected. For example, the Type A FAE from *A. niger* (AnFaeA; Faulds et al. 1997) shows homology with lipases from *Thermomyces lanuginosus* TLL (30 % sequence identity) and *Rhizomucor miehei* (37 % sequence identity). This subclass show preference for the phenolic moiety of the substrate containing methoxy substitutions, especially at *meta*- position(s), as occurs in ferulic and sinapic acids, while type B FAEs shows complementary activity, showing preference to substrates containing one or two hydroxyl substitutions as found in *p*-coumaric or caffeic acid. Type B FAEs, such as *M. thermophila Mt*Fae1a (Topakas et al. 2012), *P. funiculosum Pf*FaeB (Kroon et al. 2000) and *Neurospora crassa* Fae-1 (Crepin et al. 2003a), show high sequence identity with acetyl xylan esterases and are the only type of FAEs that are members of carbohydrate esterase (CE) family 1 of the Carbohydrate Active enZymes (CAZy) database (http://www.cazy.org/CE1.html; Lombard et al. 2013). In contrast to type B esterases, type A FAEs appear to prefer hydrophobic substrates with bulky substituents on the benzene ring (Kroon et al. 1997; Topakas et al. 2005a). One of the famous Type of FAEs, due to their synthetic potential, is type C that exhibit high sequence identity with fungal tannases. Together with type D FAEs, these esterases exhibit broad specificity against synthetic hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic and sinapic acid) showing difference only in the ability to release 5-5′ diFA (Crepin et al. 2004a, b; Vafiadi et al. 2006b). Lastly, Type D FAEs have been found only in bacteria, such as XYLD from *Pseudomonas fluorescens* (reclassified as *Cellvibrio japonicus*) (Ferreira et al. 1993) that show sequence similarity with xylanases (Crepin et al. 2004a). These four different types of FAEs have different evolutionary origin, as found by phylogenetic analysis resulting in five major clades I to V

(Olivares-Hernández et al. 2010). Clade I contains Type A FAEs, such as the characterized FAEs from *A. niger*, *A. awamori* and *A. tubingensis* that belong to Eurotiomycetes, except one FAE sequence from *Laccaria bicolor* belonging to *Agaricomycetes* class of basidiomycetes. Clade II contains Type B FAE sequences from both *Sordariomycetes* and *Eurotiomycetes*; however, the clade does not reflect any phylogenetic structure. Clade IV contains characterized Type B and C FAEs including also putative FAE sequences from four taxonomic classes, with its basis consisted of esterases from *Magnaporthe*, *Pyrenophora*, *Phaeosphaeria*, and *Fusarium*.

Recently, a modification to this classification has been proposed by Benoit et al. (2008), while a descriptor-based computational analysis with pharmacophore modeling provided a different approach for the classification of FAEs (Udatha et al. 2011). According to them, FAEs could be classified into seven subfamilies depending on the phylogenetic analysis of fungal genomes, whereas the descriptor-based classification and structural analysis of experimentally verified and putative FAEs proposed 12 families. FAEs are also members to ESTHER database, a comprehensive and continuously updated database devoted to α/β hydrolase fold proteins that hierarchically classifies more than 30,000 proteins by blocks and Rank families (Lenfant et al. 2013). In this database, FAEs are members of five Rank 1 families, four in Block X (Abhydrolase_6, Antigen85c, Esterase_phb, Tannase) and one in Block L (Lipase_3).

Relatively few studies have been performed to elucidate the functional relationships between sequence and diverse FAEs. To date, the crystal structures of FAEs include a Type A or Lipase_3 (ESTHER) *An*FaeA from *A. niger* (Hermoso et al. 2004; related PDB entries: 1UWC, 1USW, 1UZA, 2BJH, 2IX9, 2HL6, 2IX9, 2HL6), a FAE (Est1E) from *Butyrivibrio proteoclasticus* (PDB_ID: 2WTM, 2WTN; Goldstone et al. 2010) that belongs to Abhydrolase_6 family (ESTHER) and FAE domains, XynY and XynZ (Prates et al. 2001; Schubot et al. 2001 with related PDB entries: 1JJF, 1GKK, 1GKL, 1JT2, 1WB4, 1WB5, 1WB6) of the cellulosomal enzymes included in the cellulosome complex from *Clostridium thermocellum* that belongs to Antigen85c family (ESTHER). Recently, a cinnamoyl esterase (LJ0536) from *Lactobacillus johnsonii* was characterized and its 3D structure was determined that resembles the structure of the Est1E from *B. proteoclasticus* (PDB_IDs: 3PF8, 3PF9, 3PFB, 3PFC, 3QM1, 3S2Z; Lai et al. 2011). Unfortunately, none of the proposed FAE classifications include and categorize LJ0536 FAE or any of its homologs. In 2014, the first crystal structure of a type B FAE was solved, belonging to the tannase family of the ESTHER database from *A. oryzae* (*Ao*FaeB) with the serine and histidine residues of the catalytic triad, a typical motif of serine hydrolases, to be directly connected by a disulfide bond of the neighboring cysteine residues (Suzuki et al. 2014; PDB_ID: 3WMT). As reported also in the first attempt of FAE classification by Crepin et al. (2004a), Type C FAEs show homology with members of tannase family showing broad substrate specificity against four methyl esters of hydroxycinnamic acids. However, only one member of Type C FAE from *Talaromyces stipitatus* (*Ts*FaeC; Vafiadi et al. 2006b) has a substrate specificity profile that corresponds to Type C FAEs, while the rest of them show a specificity

profile of Type B FAEs with weak or no activity against methyl sinapate. Such discrepancy is well documented in literature with typical examples of Type C FAEs with B type mode of action, such as *An*FaeB (Kroon and Williamson 1996), *Ao*FaeB (Suzuki et al. 2014) and *Fo*FaeC (Moukouli et al. 2008) from *Aspergillus* and *Fusarium* species. Based on sequence homology, the structure determination of *Ao*FaeB esterase underpins the elucidation of the first structure of Type C FAEs. These Type B and C FAEs are also members of the tannase family of ESTHER database, where most of them do not exhibit tannase activity, however, Tan410 discovered from a soil metagenomic library exhibits both FAE and tannase activities (Yao et al. 2013). The different Types of fungal and bacterial FAEs that have been structural determined to date, is shown in Fig. 5.2.

The aforementioned FAEs have a common α/β-hydrolase fold that is well-known in literature for the diverse activities of the enzymes that belong to this structural superfamily, such as hydrolases, lipases, dehalogenases and peroxidases (Holmquist 2000). In addition, these enzymes also share a conserved catalytic triad (Ser–His–Asp) composed of a nucleophile (Ser, Cys or Asp), a conserved histidine

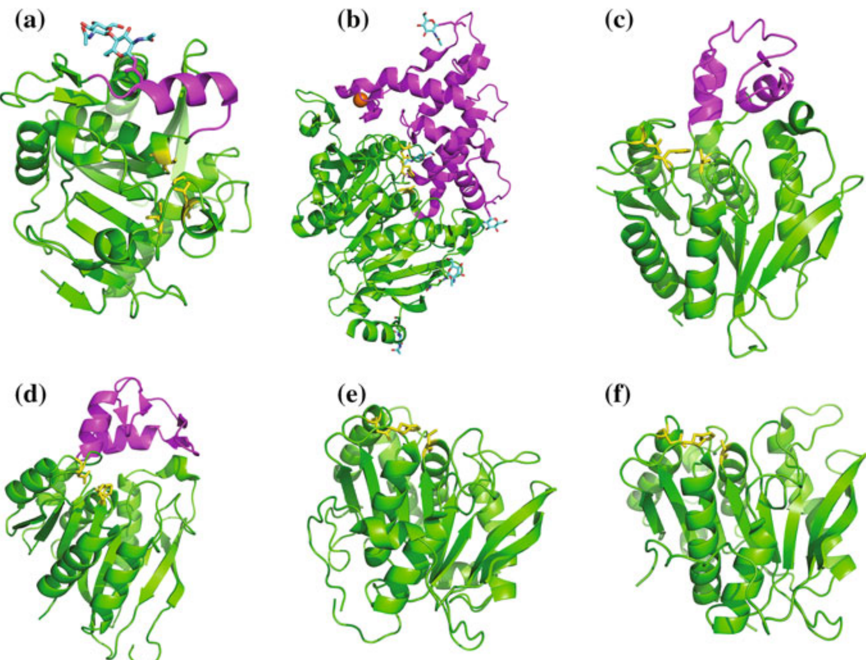


Fig. 5.2 The structures of fungal FAEs, **a** *An*FaeA from *A. niger* (Hermoso et al. 2004), **b** *Ao*FaeB from *A. oryzae* (Suzuki et al. 2014) and bacterial FAEs **c** EstE1 from *B. proteoclasticus* (Goldstone et al. 2010), **d** LJ0536 from *L. johnsonii* (Lai et al. 2011), domains, **e** XynY (Prates et al. 2001) and **f** XynZ (Schubot et al. 2001) of *C. thermocellum* cellulosome complex. The catalytic α/β hydrolase domain (*green*) and the lid domain (*magenta*) are shown. GlcNAc residues of *N*-glucans and the catalytic triad are shown as *cyan* and *yellow sticks*, respectively. A calcium ion is represented as an *orange sphere*. The 3D structures were prepared using PyMOL (http://pymol.org)

and an acidic residue. These residues are sequenced in the order of nucleophile, acidic and histidine residues, with the nucleophile to be located between the fifth β-strand and the following helix of the nucleophile elbow (Nardini and Dijkstra 1999). The tetrahedral oxyanion intermediate formed during catalysis is stabilized by the oxyanion hole that is formed by the contribution of the nucleophilic elbow. The catalytic triad of the Type A FAE *An*FaeA and the active-site cavity is confined by a lid and a loop that confers plasticity to the substrate binding site in analogy with lipases (Fig. 5.2). What is surprising is the fact that FAE's lid exhibits a high ratio of polar residues keeping it in an open conformation that gives the typical preference of catalyzing the hydrolysis of hydrophilic substrates compared to lipases. In addition, the open conformation is further stabilized by a N-glycosylation site (Hermoso et al. 2004). These subtle aminoacid and conformational changes are a result of an evolutionary divergence within the FAE family, as further discussed by Levasseur et al. (2006). A functional shift followed by a duplication event was suggested, which was occurred within the ancestral lipase genes. Such a "neofunctionalization" was a result of positive selection during the functional shift, possibly due to the drastic environmental changes happened by the colonization of land by terrestrial plants, giving the selective advantage to *Euascomycetes* (*Aspergilli*), as hypothesized by the authors (Levasseur et al. 2006). The Type A FAE from *A. niger* together with Est1E from *B. proteoclasticus* exhibit a small lid domain (16 and 46 amino acid residues, respectively) compared with the *Ao*FaeB (159 amino acid residues), as shown by the first solved FAE structure of tannase family (Suzuki et al. 2014). In addition, the lid domain of *Bp*Est1E has a unique fold forming a flexible β-sheet structure around a small hydrophobic core, while on the other hand, FAE domains of *C. thermocellum* do not possess a lid domain (Fig. 5.2). This newly discovered lid underpins the continuing diversity of insertions that decorate the common α/β fold of hydrolases that point toward a dynamic mechanism for binding and release of substrates (Goldstone et al. 2010).

5.5 Production and Physicochemical Characteristics

Since 1987, when FAE activity was detected in *Streptomyces olivochromogenes* cultures grown on wheat bran for the first time (MacKenzie et al. 1987), and the first purified FAE from the same microorganism was reported (Faulds and Williamson 1991), many FAEs have been produced and characterized from fungi and bacteria, and are considered to be a common component of hemicellulose preparations. In the last decade, over 50 FAEs have been produced through various fermentation processes employing different lignocellulose derived carbon sources such as maize bran, corn bran, and destarched wheat bran but with the most interest focused on production through heterologous expression (Table 5.1). An extended table on the microbial production of FAEs is provided by Topakas et al. (2007) that complement the present investigation.

Table 5.1 Microbial production of FAEs both from WT and recombinant sources

Organism	Carbon source/inducer	Cultivation conditions	Assay	FAE activity		References
				(mU/mL)	(mU/mg)	
A. mucronatus YE505 (*E. coli* BL21)	1 mM IPTG	Standard protocol	MFA/EFA	–	9940/7750	Qi et al. (2011)
A. awamori G-2	1 % WB	30 °C, 72 h	1-Naphthyl acetate	394,000	–	Kanauchi et al. (2008)
A. awamori (*E. coli* BL21)	1 mM IPTG	Standard protocol	EFA	–	1850	Fazary et al. (2010)
A. awamori 2B.361 U2/1	3 % WB	V = 300 mL, 30 °C, 200 rpm, 7 days	EFA	170	–	Gottschalk et al. (2013)
A. clavatus NRRL1 (*E. coli* BL21)	0.5 mM IPTG	Standard protocol	MFA/MpCA/MCA	–	–	Damásio et al. (2013)
A. flavipes	1 % WB	28 °C, 6 days	MFA	6820	10,570	Mathew et al. (2005)
	1 % MB	28 °C, 5 days	MFA	33,180	6980	Mathew et al. (2005)
A. flavus CBE332.1 (*P. pastoris* X-33)	Methanol	V = 250 mL, 30 °C, 2 days	CNPF	500	–	Zhang et al. (2013)
A. nidulans (*S. cerevisiae*)	2 % (w/v) Galactose	V = 500 mL, 30 °C, 20 h, 250 rpm	MFA	–	14,900	Shin and Chen (2007)
A. nidulans (*P. pastoris*)	Methanol	30 °C, 3 days	MFA	–	21,700	Debeire et al. (2012), Bauer et al. (2006)
A. niger NRRL3	1 % CB	V = 100 mL, 30 °C, 5 days	MFA/CB	13.9/0	–	Shin and Chen 2006
A. niger CFR 1105	WB	30 °C, 96 h/V = 100 mL, 30 °C, 105 rpm[a]	4NPF	–	12,800/11700[a]	Hegde and Muralikrishna (2009)
A. niger (*S. cerevisae* DY150)	2 % Glycerol	V = 1 L, 30 °C	4NPF	–	8200	Wong et al. (2011)
A. oryzae RIB40 (*P. pastoris* GS115)						Koseki et al. (2009)

(continued)

Table 5.1 (continued)

Organism	Carbon source/inducer	Cultivation conditions	Assay	FAE activity (mU/mL)	FAE activity (mU/mg)	References
AoFaeB	Methanol	Manufacturer's protocol	MCA/M*p*CA	–	3200/30,600	
AoFaeC	Methanol	Manufacturer's protocol	MCA/M*p*CA	–	34,900/14,300	
A. oryzae (*P. pastoris* GS115)	0.5 % (v/v) Methanol	Multicopy Pichia Expression kit manual	MFA	–	58,400	Zeng et al. (2014)
A. tubingensis	1 % DSWB, 1 % Pectin	35 °C, 3 days	DSWB	2440	–	Tai et al. (2014)
A. usamii E001 (*P. pastoris* GS115)	1 % (v/v) Methanol	30 °C, 3 days	MFA	–	4490	Gong et al. (2013)
B. proteoclasticus (*E. coli* BL21)	IPTG	Standard protocol	EFA	–	37,000	Goldstone et al. (2010)
C. ruminicola H1						Li et al. (2011)
FaeI (*E. coli* BL21)	1 mM IPTG	Standard protocol	MFA	–	3780	
FaeII (*E. coli* Rosetta-gami2)	1 mM IPTG	Standard protocol	MFA	–	38,760	
FaeIII (*E. coli* Rosetta)	1 mM IPTG	Standard protocol	MFA	–	56,880	
C. japonicus (*E. coli* BL21)	IPTG	Standard protocol	MFA	–	28	McClendon et al. (2011)
Chaetomium sp. CQ31	4 % CC	V = 250 mL, 40 °C, 200 rpm, 6 days	MFA	2100	–	Yang et al. (2013)
Cotton soil metagenomic library (*E. coli* BL21)	0.5 mM IPTG	V = 50 mL, 37 °C,	pNFA	–	–	Yao et al. (2013)
D. dadantii 3937 (*E. coli* BL21)						Hassan and Hugouvieux-Cotte-Pattat (2011)

(continued)

Table 5.1 (continued)

Organism	Carbon source/ inducer	Cultivation conditions	Assay	FAE activity		References
				(mU/mL)	(mU/mg)	
FaeD	2 mM IPTG	Standard protocol	pNPA	–		
FaeT	2 mM IPTG	Standard protocol	pNPA	–		
F. oxysporumA (*P. pastoris* X-33)	0.5 % (v/v) Methanol	*V* = 150 mL, 30 °C, 6 days	DSWB	–	–	Moukouli et al. (2008)
Fusarium proliferatum NRRL 26517	1 % CB	*V* = 100 mL, 30 °C, 5 days	MFA	33.46	–	Shin and Chen 2006
Fusarium verticillioides NRRL 26518	1 % CB	*V* = 100 mL, 30 °C, 5 days	MFA/CB	19.6/0	–	Shin and Chen 2006
Humicola grisea var. *thermoidea*	2 % BSG/2 % WB	*V* = 100 mL, 45 °C, 10 days	MFA	470/330[b]	–	Mandalari et al. (2008)
JSC-3	1 % WB	28 °C, 9 days	MFA	6320	10,090	Mathew et al. (2005)
	1 % MB	28 °C, 8 days	MFA	7170	36,710	Mathew et al. (2005)
L. johnsonii (*E. coli* BL21)	1 mM IPTG	Standard protocol	Esters	–	–	Kin et al. (2009)
L. plantarum WCFS1 (*E. coli* BL21)	4 mM IPTG	Standard protocol				Esteban-Torres et al. (2013)
M. thermophila (*P. pastoris* X-33)	0.5 % (v/v) Methanol	*V* = 150 mL, 30 °C, 6 days	DSWB	930	–	Topakas et al. (2012)
P. brasilianum IBT 20888	20 % BSG (2.5 g)	30 °C, 196 h, V_{extr} = 10 mL/1 g dry material		1542[c]	–	Panagiotou et al. (2006)
P. funiculosum (*A.awamori*)						Knoshaug et al. (2008)

(continued)

Table 5.1 (continued)

Organism	Carbon source/ inducer	Cultivation conditions	Assay	FAE activity		References
				(mU/mL)	(mU/mg)	
FaeA	Glucoamylase	*V* = 2.8 L, 32 °C, 4–6 days	MFA	–	–	
FaeB	Glucoamylase	*V* = 2.8 L, 32 °C, 4–6 days	MFA	–	–	
Piromyces equi (*T. reesei* RutC30)		30 °C, 120 rpm, 2 days	MpCA	660	20,000	Poidevin et al. (2009)
P. eryngii	SNL medium, 0.4 % Tween 80	24 °C, 13 days, 150 rpm	MFA	–	650	Nieter et al. (2014)
P. sapidus 8266 DSMZ	SNL medium, 0.4 % Tween 80	5 days	pNPB	243	–	Linke et al. (2013)
Rumen microbial metagenome (*E. coli* BL21)	IPTG	Standard protocol	WB	–	10,300	Wong et al. (2013)
Russula virescens			MFA			Wang et al. (2014)
S. cellulosum So ce56 (*E. coli* Tuner cells)						Wu et al. (2012)
ScFAE1	1 mM IPTG	Standard protocol	MFA	–	12,200	
ScFAE2	1 mM IPTG	Standard protocol	MFA	–	19,300	
Streptomyces ambofaciens	1 % (w/v) DSWB		MpCA/MFA			Kheder et al. (2009)
S. cinnamoneus (*E. coli* BL21)		FAE activity was expressed on a dry weight basis				Uraji et al. (2014)

(continued)

Table 5.1 (continued)

Organism	Carbon source/ inducer	Cultivation conditions	Assay	FAE activity		References
				(mU/mL)	(mU/mg)	
R18	IPTG	Standard protocol	MFA	–	23.07	
R43	IPTG	Standard protocol	MFA	–	19.8	
Streptomyces C-248	1 % DSWB	*V* = 1 L, 37 °C, 3 days	DSWB	80	300	Faulds et al. (2006)
Streptomyces S_{10}	1.5 % DSWB	*V* = 20 mL, 30 °C, 96 h	DSWB	2.0	15.45	Mukherjee et al. (2007)
T. stipitatus	2 % BSG/2 % WB	*V* = 100 mL, 37 °C, 10 days	MFA	140/90[b]	–	Mandalari et al. (2008)
Thermoanaerobacter tengcongencis (*E. coli*)	0.1 mM IPTG	Standard protocol	MFA/MpCA	–	456/426	Abokitse et al. (2010)
T. xylanilyticus (*E. coli*)	IPTG	Standard protocol	MFA	–	25410	Rakotoarivonina et al. (2011)

Literature is cited after 2005, for earlier data see Topakas et al. (2007) for a review

WB wheat bran, *MB* maize bran, *DSWB* destarched wheat bran, *BSG* brewer's spent grain, *CB* corn bran, *CC* corn cobs, *IPTG* isopropyl β-D-1-thiogalactopyranoside, *MFA* methyl ferulate, *MpCA* methyl *p*-coumarate, *EFA* ethyl ferulate, *MCA* methyl caffeate, *4NPF* 4-nitrophenyl ferulate, *pNFA* *p*-nitrophenyl ferulate, *pNPA* *p*-nitrophenyl acetate, *pNPB* *p*-nitrophenyl butyrate, *CNPF* 2-chloro-4-nitrophenyl ferulate, *SNL* standard nutrition liquid

[a]ssf/smf

[b]BSG/WB

[c]FAE activity was expressed on a dry weight basis

FAE production through native secretory pathways of microorganisms is mainly performed with two types of fermentation. Submerged fermentation (SmF), where the microorganism is in contact with the liquid growth medium, and solid-state fermentation (SSF), where the microorganism grows on solid substrates in the near absence of water. SSF main advantages over SmF include higher yields, simpler technology with rare operational problems, and resemblance to microorganism's natural habitat (Katsimpouras et al. 2014). Among *Aspergillus* species, *A. flavipes* (Mathew et al. 2005), *A. niger* (Johnson et al. 1989) and *A. awamori* (Kanauchi et al. 2008) were reported as very efficient FAE producers under SmF in the presence of lignocellulosic materials such as wheat bran and destarched wheat bran while *P. brasilianum* was the more active producer under SSF conditions on brewer's spent grain (Panagiotou et al. 2006).

The choice of the optimal substrate stand to be crucial for the homologous esterase expression as its role is not only to serve as a carbon and energy source but also to provide the necessary inducing compounds for the microorganism. Lignocellulosic carbon sources that contain high amounts of esterified FA such as wheat bran (Mathew et al. 2005; Kanauchi et al. 2008; Mandalari et al. 2008; Hegde and Muralikrishna 2009; Gottschalk et al. 2013), corn bran (Shin and Chen 2006), corn cobs (Yang et al. 2013), destarched wheat bran (Faulds et al. 2006; Mukherjee et al. 2007; Kheder et al. 2009; Tai et al. 2014), maize bran (Mathew et al. 2005) and brewer's spent grain (Panagiotou et al. 2006; Mandalari et al. 2008), have been successfully employed for FAEs production. Furthermore, in many cases the effect of removal of FA from these materials was investigated with the results revealing that deesterified carbon sources lead to a decreased production of FAE compared to untreated materials even when the media were supplemented with free FA (Topakas et al. 2003a, b, c; Topakas and Christakopoulos 2004; Shin and Chen 2006). In contrast to these results, production of FAE was enhanced with the addition of free FA but also FA's breakdown products and other aromatic compounds could induce FAE production as well (Faulds et al. 1997; Faulds and Williamson 1999; de Vries and Visser 1999; de Vries et al. 2002).

FAEs' wide range of potential applications necessitates the efficient production of novel recombinant enzymes, and the availability of more and more genome sequences for several microorganisms constitutes a powerful tool facilitating their discovery. Native enzyme mixtures production poses some difficulties due to their high production cost, as there is a need for special culturing and induction conditions (Lambertz et al. 2014). In order to overcome this obstacle, gene cloning and heterologous expression have been employed, producing enzymes with superior properties.

To date, host microorganisms such as bacteria and yeasts have been used for FAEs expression. The most widely used bacterial expression host is *Escherichia coli* and more specifically the strain BL21, which has the advantage of being deficient in the *Ion* and *ompT* proteases. As shown in Table 5.1, *E. coli* BL21 cells were employed in recombinant FAEs expression from several microorganisms such as *Streptomyces cinnamoneus*, *Thermobacillus xylanilyticus*, *C. japonicus*, *L. plantarum*, *Aspergillus clavatus*, *Cellulosilyticum ruminicola*, *Anaeromyces*

mucronatus, *Dickeya dadantii*, *Thermoanaerobacter tengcongencis*, *A. awamori*, *B. proteoclasticus* and *L. johnsonii*. Li et al. (2011) used *E. coli* Rosetta-gami 2 and Rosetta for the expression of two FAEs (FaeII and FaeIII, respectively) from *C. ruminicola* as these particular strains enable the enhanced formation of disulfide bonds in the cytoplasm and facilitate the expression of genes that encode rare *E. coli* codons. Furthermore, *Sc*FAE1 and *Sc*FAE2 from *S. cellulosum* were overexpressed using *E. coli* Tuner cells, which enable precise control of protein expression with IPTG (Wu et al. 2012).

Besides bacterial expression systems, yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* constitute also attractive host organisms for heterologous production of eukaryotic proteins exhibiting several advantages such as high growth rate and protein titers using low-cost growth media (Várnai et al. 2014). Yeasts are also capable of protein folding and posttranslational modifications such as glycosylation, with *P. pastoris* having the advantage over *S. cerevisae* of not hyperglycosylating secreted proteins (Grinna and Tschopp 1989). In addition, *P. pastoris* secretes low levels of native proteins simplifying the purification process of the recombinant protein (Barr et al. 1992). Moukouli et al. (2008) described first the successful cloning and expression of a type C FAE from *F. oxysporum* in *P. pastoris* X-33 employing a bioinformatic-assisted functional screening in order to discover and analyze the enzyme. FAEs were also cloned and expressed in *P. pastoris* from different *Aspergillus* species such as *A. oryzae* (Koseki et al. 2009; Zeng et al. 2014), *A. flavus* (Zhang et al. 2013), *Aspergillus nidulans* (Bauer et al. 2006; Debeire et al. 2012) and *A. usamii* (Gong et al. 2013) and from *M. thermophila* (Topakas et al. 2012).

A type B recombinant FAE was cloned from the genomic DNA of *A. nidulans* and expressed in *S. cerevisiae* exhibiting about 86 % of total FAE activity in the culture medium indicating an efficient protein secretion (Shin and Chen 2007). Wong et al. (2011) cloned a FAE gene from *A. niger* into *S. cerevisae* achieving an efficient secretion and a yield of about 2 mg/L.

Recently, there are reports describing the production, purification, and characterization of FAEs from basidiomycetes. The limited knowledge on FAEs from basidiomycetes could be explained due to their high laccase activity as it interferes with determination of FA (Nieter et al. 2014). *Schizophyllum commune* and *Pleurotus sapidus* were the only basidiomycetes, which were associated with FAE activity (MacKenzie and Bilous 1988; Linke et al. 2013). However, Haase-Aschoff et al. (2013) came up with a new efficient analytical method, using multiple substrates, for FAEs detection circumventing substrate and FA polymerization. After screening 41 basidiomycete strains, more than half of them exhibited FAE activity with different substrate specificity. *Pleurotus eryngii* was cultivated in standard nutrition liquid supplemented with Tween 80 as inducer and reached maximum FAE activity on the 13th day (Nieter et al. 2014).

Physical characteristics such as molecular weight, isoelectric point, and optimum hydrolytic reaction conditions differ significantly among purified FAEs (Table 5.2). FAEs exhibit a wide range of molecular weights and isoelectric points from 11 to 210 kDa and from 3.0 to 9.9 respectively. The extracellular FAE from

Table 5.2 Physicochemical properties of purified FAEs known to date

Microorganism	Enzyme	Gene	Data bank	FAE type	MW (kDa)	pH_{opt}	T_{opt} (°C)	p*I*	References
A. awamori	FE			–	112			3.7	Wang et al. (2004)
	CE			–	75			4.2	Wang et al. (2004)
	–			–	35	5.0	45	3.8	Koseki et al. (1998)
	AwFAEA	*AwfaeA*	AB032760	A	37	5.0[1]			Koseki et al. (2005)
				–	78	5.0[12]	40[12]		Kanauchi et al. (2008)
	AwFaeA	*AwfaeA*	AB032760	A	35	5.5[13]	55[13]	4.2	Fazary et al. (2010)
A. clavatus	AcFAE			D	31	7[1]	30[1]		Damásio et al. (2013)
A. flavus	*Af*FaeA			A	40	6.0[2]	58[2]		Zhang et al. (2013)
A. mucronatus	Fae1A	*fae1A*		–	37	7.2	37		Qi et al. (2011)
A. nidulans	AnidFAE		AN5267.2	–	28	6.1[2]	37[2]		Debeire et al. (2012), Bauer et al. (2006)
			AN1772.2	B	130	7.0[2]	45[2]		Shin and Chen (2007)
A. niger	FAE-I			B	63[a]			3.0	Faulds and Williamson (1993)
	FAE-II			A	29			3.6	Faulds and Williamson (1993)
	FAE-III or AnFaeA	*faeA*	Y09330	A	36	5.0[2]	55[2]	3.3	Faulds et al. (1997)
	CinnAE or AnFaeB	*faeB*	AJ309807	B[b]	75.8[a]	6.0[3]	50[3]	4.8	Kroon and Williamson (1996)
	CE			–	120				McCrae et al. (1994)
	FAE-1				50	9.0[14]	40[14]		Hegde and Muralikrishna (2009)
	FAE-2				55	6.0[14]	40–50[14]		Hegde and Muralikrishna (2009)

(continued)

Table 5.2 (continued)

Microorganism	Enzyme	Gene	Data bank	FAE type	MW (kDa)	pH_{opt}	T_{opt} (°C)	p*I*	References
	–	*faeA*	XP_001393337		31	6–7[14]	50[14]		Wong et al. (2011)
A. oryzae	FAE			–	30	4.5–6.0[4]		3.6	Tenkanen et al. (1991)
	AoFaeB	*AofaeB*	XP_001818628	B	61	6.0[1]			Koseki et al. (2009)
	AoFaeC	*AofaeC*	XP_001819091	C	75	6.0[1]			Koseki et al. (2009)
	AoFaeA	*AofaeA*		A	37	5.0[2]	50[2]		Zeng et al. (2014)
A. pullulans	–			B	210	6.7[5]	60[5]	6.5	Rumbold et al. (2003)
A. tubingensis	FaeA	*faeA*	Y09331	–	36				Zwane et al. (2014)
A. usamii E001	AuFaeA	*AufaeA*		A	36	5.0[2]	45[2]		Gong et al. (2013)
Butyrivibrio fibrisolvents	–	*cinI* or *cinA*	U44893	–					Barbe and Dubourdieu (1998)
	–	*cinII* or *cinB*	U64802	–					De Vries et al. (1997)
B. proteoclasticus	Est1E	*est1E*			31.6				Goldstone et al. (2010)
C. ruminicola	FaeI	*faeI*		–	58	6–7[2]	40[2]		Li et al. (2011)
	FaeII	*faeII*		–	32	8[2]	35[2]		Li et al. (2011)
	FaeIII	*faeIII*		–	46	9[2]	40[2]		Li et al. (2011)
C. japonicus	XLYD or CjXYLD	*xynD*	X58956	D	59				Ferreira et al. (1993)
C. japonicus	Fee1B	*fee1B*		D	61	6.5[2]	35–40[2]		McClendon et al. (2011)
Chaetomium sp.	–			B	30.2	7.5[2]	60[2]		Yang et al. (2013)

(continued)

Table 5.2 (continued)

Microorganism	Enzyme	Gene	Data bank	FAE type	MW (kDa)	pH_{opt}	T_{opt} (°C)	p*I*	References
Chrysosporium lucknowense	FaeA1		JF826027	A	29	6–7[2]	45[2]	5.5	Kühnel et al. (2012)
	FaeA2		JF826028	A	36	7.5[2]	40[2]	5.2	Kühnel et al. (2012)
	FaeB2		JF826029	B	33	7[2]	45[2]	6.0	Kühnel et al. (2012)
C. stercorarium	–			C or D	33	8.0[2]	65[2]		Donaghy et al. (2000)
C. thermocellum	XynZ	*XynZ*	M22624	–	45	4–7[6]	50–60[6]	5.8	Blum et al. (2000)
	–	*XynY*	X83269	–					Blum et al. (2000)
D. dadantii	FaeT	*faeT*			35				Hassan and Hugouvieux-Cotte-Pattat (2011)
	FaeD	*faeD*			31, 35				Hassan and Hugouvieux-Cotte-Pattat (2011)
F. oxysporum	FoFAE-I or FoFaeB			B	31	7.0[7]	55[7]	>9.5	Topakas et al. (2003a, b, c)
	FAE-II or FoFaeA			A	27	7.0[8]	45[8]	9.9	Topakas et al. (2003a, b, c)
F. proliferatum	FAE			B	31	6.5–7.5[2]	50[2]		Shin and Chen (2006)
Lactobacillus acidophilus[c]	–			–	36	5.6[9]	37[9]		Topakas et al. (2004)
L. johnsonii	Lj0536		WP_004898050.1		31	7.8	20		Kin et al. (2009)

(continued)

Table 5.2 (continued)

Microorganism	Enzyme	Gene	Data bank	FAE type	MW (kDa)	pH_{opt}	T_{opt} (°C)	p*I*	References
	Lj1228		WP_011162057.1		31	6.7	30		Kin et al. (2009)
L. plantarum	Lp_0796	*lp_0796*		–	28	7[15]	30–37[15]		Esteban-Torres et al. (2013)
M. thermophila	FoFaeC-12213	*Foxg-12213.2*		C	62	6.0[2]	65[2]	6.8	Moukouli et al. (2008)
M. thermophila	*Mt*Fae1a	*fae1a*	96,478	B	39	7.0[2]	50[2]		Topakas et al. (2012)
Neocallimastix MC-2	pCAE			–	11[a]	7.2[10]		4.7	Borneman et al. (1993)
	FAE-I			–	69				Borneman et al. (1992)
	FAE-II			–	24				Borneman et al. (1992)
N. crassa	Fae-1	*Fae-1*	AJ293029	B	35	6.0[2]	55[2]		Crepin et al. (2003a)
	NcFaeD-3.544	*faeD-3.544*		D	32				Crepin et al. (2004a, b)
P. equi	EstA	*estA*	AF164516	D	55	6.7[11]	50–60[11]		Fillingham et al. (1999)
		estA	AF16516		30	6.5[7]	50–60[7]		Poidevin et al. (2009)
P. eryngii	PeFaeA			A	67	5.0[2]	50[2]	5.2	Nieter et al. (2014)
Penicillium expansum	–		possible B	57.5	5.6[2]	37[2]			Donaghy and McKay (1997)
P. funiculosum	FAEB or PfFaeB	*faeB*	AJ291496	B	53			6.0	Kroon et al. (2000)
	FaeA	*faeA*	AJ312296						Knoshaug et al. (2008)
Penicillium pinophilum	pCAE/FAE			–	57	6.0[2]	55[2]	4.6	Castanares et al. (1992)
P. sapidus				A	55	6.0[2]	50[2]	5.7	Linke et al. (2013)
Rumen microbial metagenome	RuFae2			C	29	7	50	8.5	Wong et al. (2013)

(continued)

Table 5.2 (continued)

Microorganism	Enzyme	Gene	Data bank	FAE type	MW (kDa)	pH_{opt}	T_{opt} (°C)	p*I*	References
R. virescens				–	62	5.0[2]	50[2]		Wang et al. (2014)
Soil metagenomic library	Tan410	*tan410*		–	55	7[17]	35[17]	4.7	Yao et al. (2013)
S. cinnamoneus	R18		AB921569	D	38	7.5[13]	50[13]		Uraji et al. (2014)
	R43		AB921570	D	52	7[13]	40[13]		Uraji et al. (2014)
S. cellulosum	ScFAE1	*sce1896*		D	35	7[2]		5.07	Wu et al. (2012)
	ScFAE2	*sce8927*		D	34	6–8[2]		4.67	Wu et al. (2012)
S. thermophile	StFAEA or StFaeB			B	33[a]	6.0[2]	55–60[2]	3.5	Topakas et al. (2004)
	StFaeC			C	23[a]	6.0[2]	55[2]	<3.5	Topakas et al. (2005a, b)
S. olivochromogenes	FAE			–	29	5.5[2]	30[2]	7.9	Faulds and Williamson (1991)
T. stipitatus	TsFaeA			A	35			5.3	Garcia-Conesa et al. (2004)
	TsFaeB			B	35			3.5	Garcia-Conesa et al. (2004)
	TsFaeC	*faeC*	AJ505939	C	66	6–7[3]	60[3]	4.6	Garcia-Conesa et al. (2004), Crepin et al. (2003b)
T. tengcongensis	TtFAE			A	33	8.0[16]			Abokitse et al. (2010)
T. xylanilyticus	Tx-Est1	*Tx-est1*		–	37.4	8.5[2]	65[2]		Rakotoarivonina et al. (2011)

Numbers in superscripts indicate the substrates used for pH and temperature optimum: 1. α-naphthylbutyrate; 2. methyl ferulate (MFA); 3. methyl caffeate (MCA); 4. wheat straw (WS); 5. 4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (NPh-5-Fe-Ara*f*); 6. *O*-{5-*O*-(*trans*-feruloyl)-[*O*-*β*-D-xylopyranosyl-(1 → 2)]-*O*-α-L-arabinofuranosyl-[1 → 3]}-*O*-*β*-D-xylopyranosyl-(1 → 4)-D-xylopyranose (FAXXX); 7. methyl *p*-coumarate (M*p*CA); 8. methyl sinapate (MSA); 9. [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose (FAA); 10. *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1 → 3)-*O*-*β*-D-xylopyranosyl-(1 → 4)-D-xylopyranose (PAXX); 11. *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabino-furanosyl]-(1 → 3)-*O*-*β*-D-xylopyranosyl-(1 → 4)-D-xylopyranose (FAXX); 12. 1-naphthyl acetate; 13. Ethyl ferulate (EFA); 14. 4-nitrophenyl ferulate (4NPF); 15. *p*-nitrophenyl butyrate; 16. *p*NP caprylate; 17. *p*-nitrophenyl ferulate

[a]Dimeric proteins (molecular weight estimated with SDS-PAGE electrophoresis)

[b]Phylogenetic analysis of *An*FaeB indicated that this enzyme belongs to the type C subclass (Crepin et al. 2004a)

[c]Typical human intestinal bacterium

Aureobasidium pullulans with an apparent molecular weight of 210 kDa, is the largest single-subunit esterase of its kind due to an extensive Asp-linked glycosylation reaching a degree of 48 % (Rumbold et al. 2003). Optimum hydrolytic conditions also vary with pH ranging from 4.0 to 9.0 and temperature from 20 to 65 °C depending on the source microorganism. Comparing the physicochemical characteristics and conditions for optimal activity of purified FAEs shown in Table 5.2, there is no pattern indicating a correlation among them.

5.6 Applications of FAEs

During the last decade, FAEs have gained increased interest acquiring considerable role in biotechnological processes. Except for their role in biofuel industry as accessory enzymes for the degradation of lignocellulose, FAEs' potential applications cover a broad spectrum, including the pulp and paper industry as bleaching agents, the cosmetic and pharmaceutical industry for the synthesis of bioactive compounds with antioxidant and/or antimicrobial activity or for the production of flavor and fragrance precursors, the food industry as food additives or as feed additives in animal feeds (Fig. 5.3).

5.6.1 *Exploiting the Hydrolytic Activity*

The utilization of the FAEs' hydrolytic activity lies on the release of FA from hemicellulose present in plant cells walls. In ruminal digestion, FAEs are important as they deesterify dietary fibers releasing hydroxycinnamates but are also take part in colonic fermentation where their activities in gut ruminal microorganisms

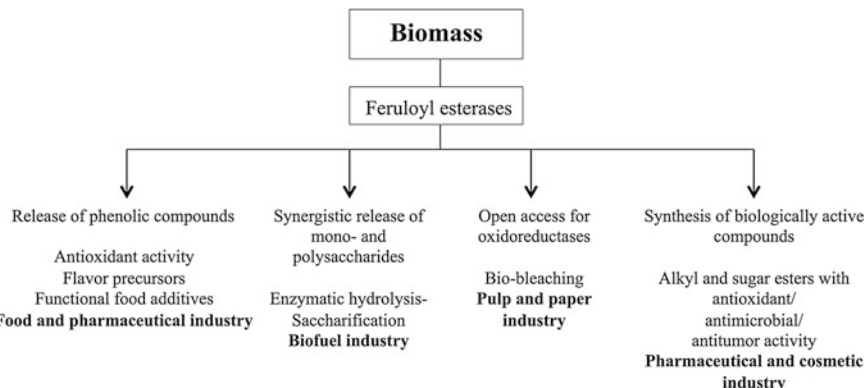


Fig. 5.3 Potential applications of FAEs in Industrial Biotechnology

enhance the breakdown of ester bonds in hydroxycinnamates (Esteban-Torres et al. 2013). This is why FAEs are used for the pretreatment of food and animal feeds or even as food/feed additives, as they allow the in situ improved digestibility of hemicellulose. In animal feed industry the advantages of their use include improved feed utilization, control of body weight gain and higher milk yield in cattle and sheep (Howard et al. 2003). In food industry, FAE preparations are also widely used in the bakery industry along with glucanases and oxidases in order to solubilize arabinoxylan fractions of the dough resulting to increased bread volume and improved quality (Butt et al. 2008). Other applications of FAEs include the synthesis of oligosaccharides that can be utilized as functional food additives and their use for juice clarification. On the other part, FA is one of the major antioxidant additives in food and beverages such as beer (Benoit et al. 2008). The pretreatment of agricultural by-products with FAEs could offer an economic alternative for the production of FA for these purposes. Being also a flavor precursor, FA can be further converted to valuable compounds such as vanillin and vanillic acid. The biotransformation of FA has been investigated using different processes and filamentous fungi (Benoit et al. 2008; Walton et al. 2000). Apart from its use for flavoring, vanillin is also a fundamental constituent for the synthesis of pharmaceuticals and is used extensively in the perfume and metal plating industries, while its herbicidal activity is useful as ripening agent for the achievement of higher yields of sucrose in sugar canes (Fazary and Ju 2008).

In paper pulp processing, environment-friendly approaches include the replacement of chemicals with enzymes, resulting to the reduction of water pollution and associated clean-up costs (Koseki et al. 2009). Xylanases and laccases are mainly used for enzymatic delignification and biobleaching, respectively, leading to the reduction or elimination of chlorine-based chemicals (Valls et al. 2010; Thakur et al. 2012). The use of FAEs along with other accessory enzymes might enhance this process by removing substitutions and linkages between polymers, resulting to the detachment of hemicellulose walls and the release of lignin fragments. A recombinant FAEA from *A. niger* has been used for the first time in 2003 in combination with laccase and xylanase for the efficient delignification of wheat straw pulp, which yielded 78 % (Record et al. 2003). In addition, it has been shown to boost delignification in flax pulp resulting in very low kappa number and pulp brightness (Sigoillot et al. 2005). The commercial lipase A "Amano" with significant FAE and other accessory activities, when tested for its bleachability in kraft pulps, offered the first evidence that accessory enzymes from a commercial preparation such as FAE and arabinofuranosidase can result to direct bleaching effect (Nguyen et al. 2008). Other minor applications of the FAEs' hydrolytic activity in paper industry is the removal of fine particles from the pulp facilitating water removal and the enhancement of chemical and mechanical paper-pulping methods allowing the easier solubilization of lignin–carbohydrate complexes (Fazary and Ju 2008). A prerequisite for their use is that enzyme preparations are free of cellulases, since cellulose degradation would result in a reduction in the quality of the pulp.

5.6.2 FAEs as Biosynthetic Tools of Plant Derived Antioxidants

Ester-linked substituents (ferulic, *p*-coumaric, caffeic, sinapinic acid) on plant cell wall polysaccharides have widespread potential due to their antimicrobial, photoprotectant, antitumour and antioxidant activities (Faulds 2010), while some have long been used as food preservatives in order to inhibit microbial growth (Fazary and Ju 2008). However, their application in oil-based food and cosmetics industry is limited due to their relatively low solubility in aprotic media. The direct esterification of phenolic, including hydroxycinnamic, acids or the transesterification of their esters with fatty alcohols or sugars has gained increased interest as it targets to the production of biologically active compounds with improved properties (Fig. 5.4). The synthesized phenolic fatty esters are generally more lipophilic and allow their application in oil-based processes; on the other hand, the phenolic acid sugar esters are more hydrophilic nonionic biosurfactants and have clear antitumor activity, so the potential to be used in order to formulate antimicrobial, antiviral and/or anti-inflammatory agents is promising (Fazary and Ju 2008). Enzymatic transesterification offers an alternative to the poor selectivity of chemical synthesis. It is a one-step process resulting to the synthesis of one product instead of a mixture of esters thus there is no unwanted side reactions causing darkening or the formation of odors and no need of by-product and catalyst residues removal. Complementary to the attractive environment-friendly configurations of the process, the use of enzymes derived from thermophilic fungi allows the implementation of lower temperature (50–60 °C) than that of the chemical process (160 °C) resulting to lower operating costs. Enzyme-catalyzed synthesis has been widely studied in lipases including hydroxycinnamate (trans)esterification in nonconventional media (organic solvents, ionic liquid mixtures). Lipases are known to be more stable than FAEs in such reaction media, display high activity at low water

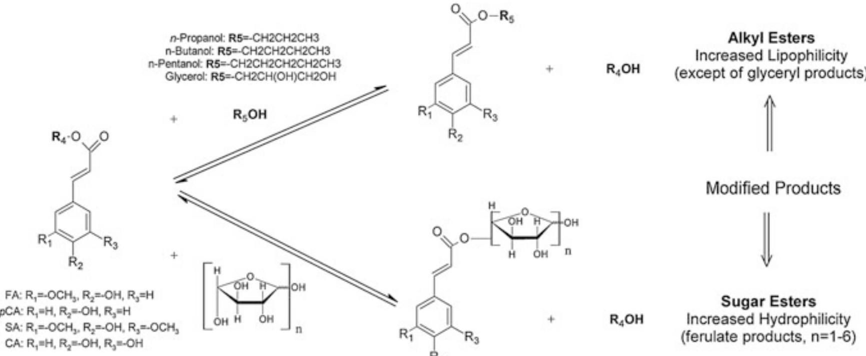


Fig. 5.4 Modification of hydroxycinnamic acids by FAEs in nonconventional reaction media. *FA* ferulic acid, *pCA p*-coumaric acid, *SA* sinapic acid, *CA* caffeic acid

content and give high yields (Zeuner et al. 2011). On the contrary, FAEs exhibit higher substrate specificity constituting them a potential biosynthetic tool with high efficiency.

As with lipases, a reaction medium with low or non-water content is essential in order to boost the FAEs' synthetic activity to the detriment of the hydrolytic one. The first synthetic reaction catalyzed by FAE was achieved in a water-in-oil microemulsion system for the synthesis of 1-pentyl-ferulate (Giuliani et al. 2001). Since then, detergentless microemulsions, which are ternary systems consisted of a hydrocarbon, a short-chained alcohol and water, have mostly been employed for the synthesis of various alkyl and sugar esters (Table 5.3). They are low water content media representing thermodynamically stable dispersions of aqueous microdroplets in the hydrocarbon solvent. The spherical droplets are stabilized by alcohol molecules adsorbed at their surface, while the enclosed in water enzyme is protected from contacting the outer organic phase and from denaturation due to a water-rich layer (Khmelnitsky et al. 1988). An important advantage of these mixtures as reaction systems is that they allow the separation of reaction products and enzyme reuse, while the solubility of relatively polar phenolic acids is high owing to the presence of large amount of polar alcohol. In these media, transesterification reactions using activated esters of FA as acyl donors have been proven to be faster and more efficient compared to the direct esterifications (Olsson et al. 2011). Another approach for efficient transesterification is the use of ionic liquid mixtures (ILs), which comprise organic salts that remain liquid under ambient temperature (Table 5.4). The interest in ILs as reaction media is mainly urged by their lack of vapor pressure as they have the potential to replace volatile organic solvents resulting to the development of greener processes. A major advantage of using ILs is their tailorability as they exhibit increased selectivity and ability to solubilize both polar and nonpolar substrates and products, as well as they can adjust other physicochemical properties such as density, viscosity and solvating power through alternating the cation and anion type to meet the needs of each system (Zeuner et al. 2011). The lack of volatility together with their chemical and thermal stability allow simple recycling and reuse therefore could reduce significantly the cost of the process with respect to organic-based ones. When referring to transesterification with saccharides, the advantage of using ILs, as compared to organic solvents is the increased substrate solubility. Furthermore higher yields in shorter reaction times including regioselectivity may also be achieved in enzymatic acylation of saccharides in ILs compared to organic solvents. Most of the research has used second-generation ILs, which may be too expensive for commercial applications. The availability of advanced ionic liquids- greener, inexpensive and biodegradable- increases the likelihood that they will find commercial use in biocatalysis (Gorke et al. 2010). Although synthesis in organic solvents has been widely studied in lipases, their use in FAEs has been rarely reported in literature due to stability restraints (Table 5.5). An important advantage is that they allow the implementation of one phase reaction and the separation of reaction products, although they do not consist a green approach for transesterification.

Table 5.3 Enzymatic synthesis of hydroxycinnamic acid esters in detergentless microemulsions reported in the literature

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	T (°C)	References
Synthesis of aliphatic esters						
Methyl ferulate	1-Butanol	StFaeC	*n*-Hexane:1-butanol: MES-NaOH pH 6.0 (53.4:43.4:3.2 v/v/v)	Up to 20 % (120)	35	Topakas et al. (2005a, b)
Methyl sinapate				Up to 10 % (∼144)		
Methyl *p*-coumarate				Up to 8 % (∼144)		
Methyl caffeate				Up to 5.5 % (∼144)		
Methyl ferulate	1-Butanol	StFae-A	*n*-Hexane:1-butanol: buffer (47.2:50.8:2.0 v/v/v)	Up to 8 % (144)	35	Topakas et al. (2004)
Methyl *p*-coumarate				Up to 50 % (144)		
Methyl caffeate				Up to 25 % (144)		
Methyl *p*-coumarate	1-Butanol	FoFae-I	*n*-Hexane/1-butanol/MES-NaOH pH 6.0 (47.2:50.8:2.0 v/v/v)	Up to 70 % (144)	35	Topakas et al. (2003a)
Methyl caffeate				Up to 22 % (144)		
Methyl ferulate				Up to 13 % (144)		
Methyl sinapinate				Up to 1 % (144)		

(continued)

Table 5.3 (continued)

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	*T* (°C)	References
p-hydroxyphenylacetic acid	1-Propanol	FoFae-II	*n*-Hexane/1-propanol/MES-NaOH pH 6.6 (47.2:50.8:2.0 v/v/v)	75 (224)	30	Topakas et al. (2003b)
p-Hydroxyl-phenylpropionic acid				70 (224)		
Methyl sinapate	1-Butanol	AnFaeA^{+1}	*n*-Hexane:1- or 2-butanol: MES-NaOH pH 6.0 (47.2: 50.8: 2.0 v/v/v)	56 % (120)	35	Vafiadi et al. (2008a)
				78 %[a] (120)		
Methyl ferulate		AnFaeA		42 % (120)		
Methyl *p*-coumarate				2 % (120)		
Methyl sinapate	2-Butanol			9 % (120)		
Methyl ferulate	1-Butanol	Ultraflo L^{+1}	*n*-Hexane/1-butanol/water (47.2:50.8:2.0 v/v/v)	97 % (144)	37	Vafiadi et al. (2008b)
		Ultraflo		3.6 % (144)		
Methyl ferulate	1-Butanol	Depol 740L^{+1}	*n*-Hexane/1-butanol/water (47.2:50.8:2.0 v/v/v)	87 % (144)	37	Vafiadi et al. (2008b)
		Depol 740L		2.6 % (144)		
Methyl ferulate	1-Butanol	Depol 670L^{+}	*n*-Hexane/1-butanol/water (47.2:50.8:2.0 v/v/v)	5 % (144)	37	Vafiadi et al. (2008b)
Synthesis of sugar esters						
Methyl ferulate	L-Arabinose	StFaeC	*n*-Hexane:*t*-butanol:piperazine- HCl pH 6.0 (53.4:43.4:3.2 v/v/v)	40 % (160)	35	Topakas et al. (2005b)
Methyl ferulate				Up to 50 %[a] (120)		Vafiadi et al. (2005)

(continued)

Table 5.3 (continued)

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	*T* (°C)	References
Ethyl ferulate				6.3 % (–)		
n-Propyl ferulate				3.8 % (–)		
n-Butyl ferulate				3.4 % (–)		
Isopropyl ferulate				3.3 % (–)		
2-Butyl ferulate				2.7 % (–)		
Isobutyl ferulate				4.2 % (–)		
Methyl ferulate	D-Arabinose			45 % (–)		
	D-Glucose			n.q. (120)		Vafiadi et al. (2007a)
	D-Xylose			n.q. (120)		Vafiadi et al. (2005)
	D-Mannose			n.q. (120)		
	D-Fructose			n.q. (120)		
	D-Galactose			n.q. (120)		
	D-Ribose			n.q. (120)		
	*p*NP-arabinofuranoside			n.q. (120)		
				n.q. (120)		
	*p*NP-anabinopyranoside			n.q. (–)		
				n.q. (–)		
	L-Arabinotriose			n.q. (–)	37	Vafiadi et al. (2007b)

(continued)

Table 5.3 (continued)

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	T (°C)	References
	L-Arabinotetraose		*n*-Hexane:2-methyl-2-propanol: piperazine HCl pH 6.0 (47.2:50.8:2.0 v/v/v)	n.q. (–)		
Methyl ferulate	L-Arabinopentaose			24 %[a] (96)		
Ethyl ferulate	L-Arabinohexaose			3 % (96)		
Propyl ferulate	L-Arabinose			4 % (96)	35	Vafiadi et al. (2006a)
Methyl ferulate	L-Arabinose	TsFaeC	*n*-Hexane: *t*-butanol: MOPS-NaOH pH 6.0 (53.4:43.4:3.2 v/v/v)	21.2 %[a] (96)	40	Vafiadi et al. (2006b)
Ethyl ferulate				1.4 % (96)		
Ferulic acid	D-Arabinose	Multifect P 3000	*n*-Hexane/1-butanol or 2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	11.3–36.7 % (144)	35	Couto et al. (2010)
	D-Galactose			5.6–8.6 % (144)		
	D-Xylose			12.1–30.8 % (144)		
Ferulic acid	D-Arabinose	Ceremix	*n*-Hexane/1-butanol or 2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	15.3–32.5 % (144)	35	Couto et al. (2010)
Ferulic acid	D-Galactose	RP-1	*n*-Hexane/1-butanol or 2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	10.2–19.8 % (144)	35	Couto et al. (2010)
	D-Xylose			4.4–16.3 % (144)		

(continued)

Table 5.3 (continued)

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	*T* (°C)	References
Ferulic acid	D-Arabinose	Depol 670	*n*-Hexane/1-butanol or 2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	7.2–17.7 % (144)	35	Couto et al. (2010)
	D-Galactose			3.4–15.8 % (144)		
	D-Xylose			20.9–26.5 % (144)		
Ferulic acid	D-Arabinose	Flavourzyme	*n*-Hexane/1-butanol or 2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	21.9–36.7 % (144)	35	Couto et al. (2010)
	D-Galactose			36.2–41.9 % (144)		
	D-Xylose			20.1–21.7 % (144)		
Ferulic acid	Raffinose	Depol 740L	*n*-Hexane/2-butanone/MES-NaOH pH 6.0 (51:46:3 v/v/v)	11.9 %[a] (168)	35	Couto et al. (2011)
	Xylobiose			9.4 % (144)		
	Arabinose			7.9 % (144)		
	Galactobiose			5.4 % (144)		

(continued)

Table 5.3 (continued)

Hydroxycinnamate	Acyl donor	Enzyme	Solvent system	Yield (h)	*T* (°C)	References
	Sucrose			13.2 % (–)		
	Lactose			4.4 % (–)		
	XOS			2.8 % (–)		
	FOS		*n*-Hexane/1,4-dioxane/MES-NaOH pH 6.0 (51:46:3 v/v/v)	9.6 % (–)		
	Raffinose			3.1 % (144)		
	Xylobiose			4.2 % (144)		
	Galactobiose			26.8 % (144)		

[a]After optimization of reaction conditions; +1: Immobilized with CLEAs methodology; n.q.: detected but not quantified; *St*FaeC: FAE type C from *S. thermophile* ATCC 34628; *St*FaeA: FAE type B from *S. thermophile* ATCC 34628; *Fo*Fae-I/*Fo*Fae-II: FAE from *F. oxysporum* F3; *An*FaeA: FAE type A from *A. niger*; Ultraflo L/ Depol 740L: multienzymatic preparation from *H. insolens*; Depol 670L: multienzymatic preparation from *T. reesei*; *Ts*FaeC: FAE type C from *T. stipitatus*; Multifect P 3000: multienzymatic preparation from *Bacillus amyloliquefaciens*; Ceremix: multienzymatic preparation from *Bacillus* spp.; RP-1: multienzymatic preparation from *Bacillus subtilis*; Depol 670L: multienzymatic preparation from *T. reesei*; Flavourzyme: multienzymatic preparation from *A. oryzae*; Depol 740L: multienzymatic preparation from *H. insolens*. Systems with conversion <1 % were not included

Table 5.4 Enzymatic synthesis of hydroxycinnamic acid esters in ILs reported in the literature

Hydroxycinnamate	Acceptor	Enzyme	Ionic liquid system	Yield (h)	T (°C)	References
Sinapic acid	Glycerol	AnFaeA	$[C_2OHmim][PF_6]$ (85%), MOPS-NaOH pH 6.0 (15%)	72.5%[a] (24)	50	Vafiadi et al. (2009)
			$[C_5OHmim][PF_6]$ (85%), MOPS-NaOH pH 6.0 (15%)	76.5%[a] (24)		
Methyl sinapate		AnFaeA[+1] AnFaeA	$[BMIm][PF_6]$ (85%), MOPS pH 6.0 (15%)	13% (0.5)	40	Zeuner et al. (2011)
			[C2OHMIm][BF4] (85%), MOPS pH 6.0 (15%)	21% (0.5)		
			$[C_5OHmim][PF_6]$ (85%), MOPS-NaOH pH 6.0 (15%)	72.5%[a] (24)	50	Vafiadi et al. (2009)
			$[C_2OHmim][PF_6]$ (94 %), MOPS-NaOH pH 6.0 (6%)	Up to 2.5% (24)		
			$[C_5OHmim][PF_6]$ (94%), MOPS-NaOH pH 6.0 (6%)	Up to 7% (24)		
Sinapic acid	Glycerol	AndFaeC	$[BMIm][PF_6]$ (85%), MOPS pH 6.0 (15%)	1.1% (0.5)	40	Zeuner et al. (2011)
Sinapic acid	Glycerol	Ultraflo L	$[BMIm][PF_6]$ (85%), MOPS pH 6.0 (15%)	1% (0.5)	40	Zeuner et al. (2011)
Ferulic acid	Raffinose	Depol 740L	$[BMIm][PF_6]$ (96%), MES-NaOH pH 6.0 (3%)	2% (144)	35	Couto et al. (2011)
	D-xylose			4.2% (144)		
	D-Arabinose			2.8% (144)		
	D-Galactose			1% (144)		

[a]After optimization of reaction conditions; +1: Immobilized with CLEAs methodology; *An*FaeA: FAE type A from *A. niger*; *An*FaeC: FAE type C from *Aspergillus nidulans*; Ultraflo L/ Depol 740L: multienzymatic preparation from *H. insolens*. Systems with conversion <1 % were not included

Table 5.5 Enzymatic synthesis of hydroxycinnamic acid esters in organic solvents reported in the literature

Hydroxycinnamate		Enzyme	Solvent system	Yield (h)	T (°C)	References
Ferulic acid	Glycerol	FAE-PL	Glycerol/ DMSO/acetate buffer pH 4.0 or 5.0 (85:5:10 v/v/v)	81%[a] (–)	50	Tsuchiyama et al. (2006)
Sinapinic acid				n.q. (0.25)		
3,4-Dimethoxy-cinnamic acid				n.q. (0.25)		
p-Coumaric acid				n.q. (0.25)		
Methyl ferulate				n.q. (0.25)		
Ethyl ferulate				n.q. (0.25)		
Methyl sinapate				n.q. (0.25)		
Methyl 3,4-dimethoxycinnamate				n.q. (0.25)		
	Arabinose			n.q. (0.5)		
	Fructose			n.q. (0.5)		
Ferulic acid	Galactose			n.q. (0.5)		
	Glucose			n.q. (0.5)		
	Xylose			n.q. (0.5)		
	Arabinose			n.q. (0.03)		
	Fructose			n.q. (0.03)		
Ethyl ferulate	Galactose			n.q. (0.03)		
	Glucose			n.q. (0.03)		
	Xylose			n.q. (0.03)		
Methyl ferulate	1-Butanol	Depol 740L^{+2}	1-Butanol/MOPS pH 6.0 (92.5:7.5 v/v)	Up to 90 %[a] (∼192)	37	Thorn et al. 2011
		Depol 740L		Up to 40 % (∼192)		

[a]After optimization of reaction conditions; +2: Immobilized in mesoporous silica MPS-9D; n.q.: detected but not quantified; FAE-PL: FAE from *A. niger* (purified from the commercial preparation Pectinase PL "Amano"); Depol 740L: multienzymatic preparation from *H. insolens*. Systems with conversion <1 % were not included

Novel FAEs purified from various filamentous fungi such as *F. oxysporum*, *S. thermophile,* and *A. niger* have been used for the synthesis of alkyl ferulates (1-propyl ferulate, 1-butyl ferulate, 1-pentyl-ferulate, 1-glyceryl ferulate) and feruloylated-arabino-oligosaccharides (with up to six arabinose units) showing regioselectivity for the primary hydroxyl group of the nonreducing arabinofuranose ring (Table 5.3). In detergentless microemulsions, *Fo*FaeI from *F. oxysporum* has achieved the synthesis of various *1*-butyl hydroxycinnamates exhibiting highest yield on 1-butyl coumarate (up to 70 %), while *Fo*FaeII has esterified *p*-hydroxyphenylacetic and *p*-hydroxyl-phenylpropionic acid with 1-propanol (70–75 % yield) (Topakas et al. 2003a, b). The thermophilic *St*FaeC from *S. thermophile* has synthesized 1-butyl hydroxycinnamates but also a variety of hydroxycinnamic acid sugar esters, including the feruloylation of both L- and D-arabinose, showing no chiral selectivity. The synthesized D-enantiomer successfully inhibited the growth of *Mycobacterium bovis* BCG, indicating that FAEs are novel chemoenzymatic tools for the synthesis of new drug (Vafiadi et al. 2007a). Multienzymatic preparations containing FAE activity such as Ultraflo L and Depol 740L from *Humicola insolens* have shown high yields up to 97 % in the transesterification of methyl ferulate to 1-butyl ferulate when immobilized with Crossed Linked Enzyme Aggregates (CLEAs) methodology (Vafiadi et al. 2008b), while immobilized Depol 740L in mesoporous silica MPS-90 supported significantly higher butyl ferulate yield up to 90 % comparing to the free enzyme (Thörn et al. 2011). Direct esterification of FA with monomer sugars or oligosaccharides has been achieved by FAE multienzymatic preparations with relatively lower yields (up to 40 %; Couto et al. 2010).

The modification of sinapic acid, which is the second strongest antioxidant in nature after FA, has also been achieved. *An*FaeA transesterified methyl sinapate to 1- and 2-butyl sinapate, while *St*FaeC and *Fo*Fae-I have successfully synthesized 1-butyl sinapate in detergentless microemulsions (Topakas et al. 2003a, b; Vafiadi et al. 2008a). Although, immobilized *An*FaeA with CLEAs methodology resulted to 22 % lower conversion of methyl sinapate than that of the free enzyme. ILs have been used for the synthesis of glyceryl-sinapate from sinapic acid and methyl sinapate, where free and CLEAs *An*FaeA also resulted to 76.5 and 72.5 % conversion yield of sinapic acid, respectively (Vafiadi et al. 2009; Table 5.4). Butyl esters of sinapic acid exhibit higher antioxidant activity against Low Density Lipoprotein (LDL) oxidation comparing to their free acid or 1-butyl ferulate, showing that sinapate esters are more promising antioxidants in vitro (Vafiadi et al. 2008a). On the contrary, sinapoylated glycerol has been shown to be a slower antioxidant, when compared to free sinapic acid in LDL oxidation (Tsuchiyama et al. 2006). It has not been established yet why hydroxycinnamate glycerols are poorer antioxidants compared to their starting acids, although they are more water-soluble than FA thus have a wider range of applications in water-based industries, such as food, beverage and cosmetic industries. Sinapic acid and its derivatives have the potential to be used in antiaging skin cosmetics, sun screen and agents to improve DNA repair. They have also been implicated in cosmetic preparations for the treatment of skin and dietary diseases and stimulation of hair growth ailments (Faulds 2010).

5.7 Conclusions

A clean technology for the sustainable production of biofuels and fine chemicals is anticipated, utilizing the agricultural and agroindustrial residual biomass. The use of enzymes is essential for the production and bioconversion of bioactive compounds from waste residues resulting in a competitive price of biofuels through the biorefinery concept. FAEs will have a profound role in utilizing waste biomass for the production of energy and high-added value compounds. Since their discovery in 1990s, novel FAEs have been isolated and characterized showing different variations in physical characteristics that result in a diverse specificity for the hydrolysis of model and natural substrates. In addition, many attempts have been made to elucidate the functional relationships between sequence-diverse enzymes in respect to their corresponding structure; however, the limited number of crystal structures available complicates this mission. In this review, FAEs are shown to be involved not only in the degradation of residual plant biomass, but also as tools for the synthesis of novel bioactive components for use in health and cosmeceutical industries. The improvements in molecular biology and process engineering permit the overproduction of FAEs in different expression hosts, opening the way for their commercial production that is prerequisite for many industrial applications.

Acknowledgments Paul Christakopoulos and Io Antonopoulou would like to thank the EU Framework 7 project OPTIBIOCAT for the financial support.

References

Abokitse K, Wu M, Bergeron H, Grosse S, Lau PCK (2010) Thermostable feruloyl esterase for the bioproduction of ferulic acid from triticale bran. Appl Microbiol Biotechnol 87:195–203

Barbe C, Dubourdieu D (1998) Characterisation and purification of a cinnamate esterase from *Aspergillus niger* industrial pectinase preparation. J Sci Food Agric 78:471–478

Barr KA, Hopkins SA, Sreekrishna K (1992) Protocol for efficient secretion of HAS developed from *Pichia pastoris*. Pharm Eng 12:48–51

Barron C, Surget A, Rouau X (2007) Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. J Cereal Sci 45:88–96

Bartolome B, Faulds CB, Kroon PA, Waldron K, Gilbert HJ, Hazlewood G, Williamson G (1997a) An *Aspergillus niger* esterase (ferulic acid esterase III) and a recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (XylD) released a 5-5′ dihydrodimer (diferulic acid) from barley and wheat cell walls. Appl Environ Microbiol 63:208–212

Bartolome B, Faulds CB, Williamson G (1997b) Enzymic release of ferulic acid from barley spent grain. J Cereal Sci 25:285–288

Bauer S, Vasu P, Persson S, Mort AJ, Somerville CR (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. Proc Natl Acad Sci USA 103:11417–11422

Benoit I, Navarro D, Marnet N, Rakotomanomana N, Lesage-Meessen L, Sigoillot JC, Asther M (2006) Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-products. Carbohydr Res 314:1820–1827

Benoit I, Danchin EGJ, Bleinchodt RJ, de Vries RP (2008) Biotechnological applications and potential fungal feruloyl esterases based on prevalence, classification and biochemical diversity. Biotechnol Lett 30:387–396

Blum DL, Kataeva IA, Li X-L, Ljungdahl LG (2000) Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. J Bacteriol 182:1346–1351

Borneman WS, Ljungdahl LG, Hartley RD, Akin DE (1992) Purification and partial characterization of two feruloyl esterases from the anaerobic fungus Neocallimastix strain MC-2. Appl Environ Microbiol 58:3762–3766

Borneman WS, Ljungdahl LG, Hartley RD, Akin DE (1993) Feruloyl and p-coumaroyl esterases from the anaerobic fungus Neocallimastix strain MC-2: properties and functions in plant cell wall degradation. In: Coughland MP, Hazlewood GP (eds) Hemicellulose and hemicellulases. Portland Press, London and Chapel Hill, pp 85–102

Braga CMP, da Silva Delabona P, da Silva Lima DJ, Paixão DAA, da Cruz Pradella JG, Farinas CS (2014) Addition of feruloyl esterase and xylanase produced on-site improves sugarcane bagasse hydrolysis. Biores Technol 170:316–324

Bunzel M, Ralph J, Funk C, Steinhart H (2003) Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. Eur Food Res Technol 217:128–133

Bunzel M, Ralph J, Funk C, Steinhart H (2005) Structural elucidation of new ferulic acid-containing phenolic dimers and trimmers isolated from maize bran. Tetrahedron Lett 46:5845–5850

Bunzel M, Ralph J, Brüning P, Steinhart H (2006) Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. J Agric Food Chem 54:6408–6418

Butt MS, Tahir-Nadeem M, Ahmad Z, Sultan MT (2008) Xylanases and their applications in baking industry. Food Technol Biotechnol 46:22–31

Caffal KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344:1879–1900

Castanares A, McCrae SI, Wood TM (1992) Purification and properties of a feruloyl/ρ -coumaroyl esterase from the fungus *Penicillium pinophilum*. Enzyme Microb Technol 14:875–884

Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. Nat Biotechnol 25:759–761

Colquhoun IJ, Ralet MC, Thibault JF, Faulds CB, Williamson G (1994) Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. Carbohydr Res 263:243–256

Couto J, Karboune S, Mathew R (2010) Regioselective synthesis of feruloylated glycosides using the feruloyl esterase expressed in selected commercial multi-enzymatic preparations as biocatalysts. Biocatal Biotransform 28:235–244

Couto J, St-Louis R, Karboune S (2011) Optimization of feruloyl esterase-catalyzed synthesis of feruloylated oligosaccharides by response face methodology. J Mol Catal B Enzym 73:53–62

Couturier M, Haon M, Coutinho PM, Henrissat B, Lesage-Meesen L, Berrin JG (2011) *Podospora anserina* hemicellulases potentiate the *Trichoderma reesei* secretome for saccharification of lignocellulosic biomass. Appl Env Microbiol 77:237–246

Crepin VF, Faulds CB, Connerton IF (2003a) A non-modular type B feruloyl esterase from *Neurospora crassa* exhibits concentration-dependent substrate inhibition. Biochem J 370: 417–427

Crepin VF, Faulds CB, Connerton IF (2003b) Production and characterization of the *Talaromyces stipitatus* feruloyl esterase FAEC in *Pichia pastoris*: identification of the nucleophilic serine. Protein Expr Purif 29:176–184

Crepin VF, Faulds CB, Connerton IF (2004a) Functional classification of the microbial feruloyl esterases. Appl Microbiol Biotechnol 63:647–652

Crepin VF, Faulds CB, Connerton IF (2004b) Identification of a type-D feruloyl esterase from *Neurospora crassa*. Appl Microbiol Biotechnol 63:567–570

Damásio ARL, Braga CMP, Brenelli LB, Citadini AP, Mandelli F, Cota J, de Almeida RF, Salvador VH, Paixao DAA, Segato F, Mercadante AZ, de Oliveira Neto M, do Santos WD, Squina FM (2013) Biomass-to-bio-products application of feruloyl esterase from *Aspergillus clavatus*. Appl Microbiol Biotechnol 97:6759–6767

Debeire P, Khoune P, Jeltsch JM, Phalip V (2012) Product patterns of a feruloyl esterase from *Aspergillus nidulans* on large feruloyl-arabino-xylo-oligosaccharides from wheat bran. Bioresour Technol 119:425–428

de Oliveira DM, Finger-Texeira A, Mota TR, Salvador VH, Moreira-Vilar FC, Molinari HBC, Mitchell RAC, Marchiosi R, Ferrarese-Filho O, dos Santos WD (2014) Ferulic acid: a key component in grass lignocellulose recalcitrance to hydrolysis. Plant Biotechnol J 13: 1224–1232

de Vries RP, Visser J (1999) Regulation of the feruloyl esterase (fae A) gene from *Aspergillus niger*. Appl Environ Microbiol 65:5500–5503

de Vries RP, Michelsen B, Poulsen CH, Kroon PA, van den Heuvel RH, Faulds CB, Williamson G, van den Hombergh JP, Visser J (1997) The faeA genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in degradation of complex cell wall polysaccharides. Appl Environ Microbiol 63:4638–4644

de Vries RP, van Kuyk PA, Kester HCM, Visser J (2002) The *Aspergillus niger* faeB gene encodes a second feruloyl esterase involved in pectin and xylan degradation and is specifically induced in the presence of aromatic compounds. Biochem J 363:377–386

Dien BS, Ximenes EA, O'Bryan PJ, Moniruzzaman M, Li X-L, Balan V, Dale B, Cotta MA (2008) Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distiller's grains and their conversion to ethanol. Biores Technol 99:5216–5225

Donaghy JA, Bronnenmeier K, Soto-Kelly PF, McKay AM (2000) Purification and characterization of an extracellular feruloyl esterase from the thermophilic anaerobe *Clostridium stercorarium*. J Appl Microbiol 88:458–466

Endler A, Persson S (2011) Cellulose synthases and synthesis in *Arabidopsis*. Mol Plant 4: 199–211

Esteban-Torres M, Reverón I, Mancheño JM, de las Rivas B, Muñoz R (2013) Characterization of a feruloyl esterase from *Lactobacillus plantarum*. Appl Environ Microbiol 79:5130–5136

Faulds CB (2010) What can feruloyl esterases do for us? Phytochem Rev 9:121–132

Faulds CB, Williamson G (1991) The purification and characterization of 4-hydroxy-3-methoxycinnamic (ferulic) acid esterase from *Streptomyces olivochromogenes*. J Gen Microbiol 137:2339–2345

Faulds C, Williamson G (1993) Ferulic acid esterase from *Aspergillus niger*: purification and partial characterization of two forms from a commercial source of pectinase. Biotechnol Appl Biochem 17:349–359

Faulds CB, Williamson G (1999) Review: the role of hydroxycinnamates in the plant cell wall. J Sci Food Agric 79:393–395

Faulds CB, Mandalari G, Lo Curto RB, Bisignano G, Christakopoulos P, Waldron KW (2006) Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan. Appl Microbiol Biotechnol 71:622–629

Faulds CB, de Vries RP, Kroon PA, Visser J, Williamson G (1997) Influence of ferulic acid on the production of feruloyl esterases by *Aspergillus niger*. FEMS Microbiol Lett 157:239–244

Fazary AE, Ju YH (2008) The large-scale use of feruloyl esterases in industry. Biotechnol Mol Biol Rev 3:95–110

Fazary AE, Hamad HA, Lee JC, Koskei T, Lee CK, Ju YH (2010) Expression of feruloyl esterase from *Aspergillus awamori* in *Escherichia coli*: characterization and crystal studies of the recombinant enzyme. Int J Biol Macromol 46:440–444

Ferreira LMA, Wood TM, Williamson G, Faulds CB, Hazlewood G, Gilbert HJ (1993) A modular esterase from *Pseudomonas fluorescens* subsp. cellulosa contains a non-catalytic binding domain. Biochem J 294:349–355

Fillingham IJ, Kroon PA, Williamson G, Gilbert HJ, Hazlewood GP (1999) A modular cinnamoyl ester hydrolase from the anaerobic fungus *Piromyces equi* acts synergistically with xylanase and is part of a multiprotein cellulose-binding cellulase-hemicellulase complex. Biochem J 343 Pt 1:215–224

Ford CW, Hartley RD (1990) Cyclodimers of p-coumaric and ferulic acids in the cell walls of tropical grasses. J Sci Food Agric 50:29–43

Fry SC (1983) Feruloylated pectins from the primary cell wall: their structure and possible functions. Planta 157:111–123

Fry SC, Willis SC, Paterson AEJ (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211:679–692

Funk C, Ralph J, Steinhart H, Bunzel M (2005) Isolation and structural characterization of 8-O-4/8-O4- and 8-8/8-O4-coupled dehydrotriferulic acids from maize bran. Phytochemistry 66:363–371

Garcia-Conesa M-T, Crepin VF, Goldson AJ, Williamson G, Cummings NJ, Connerton IF, Faulds CB, Kroon PA (2004) The feruloyl esterase system of *Talaromyces stipitatus*: production of three discrete feruloyl esterases, including a novel enzyme, TsFaeC, with a broad substrate specificity. J Biotechnol 108:227–241

Garleb KA, Fahey GC Jr, Lewis SM, Kerley MS, Montgomery L (1988) Chemical composition and digestibility of fibre fractions of certain by-product feedstuffs fed to ruminants. J Anim Sci 66:2650–2662

Giuliani S, Piana C, Setti L, Hochkoeppler A, Pifferi PG, Williamson PG, Faulds CB (2001) Synthesis of pentylferulate by a feruloyl esterase from *Aspergillus niger* using water-in-oil microemulsions. Biotechnol Lett 23:325–330

Goldstone DC, Villas-Bôas SG, Till M, Kelly WJ, Attwood GT, Arcus VL (2010) Structural and functional characterization of a promiscuous feruloyl esterase (Est1E) from the rumen bacterium *Butyrivibrio proteoclasticus*. Proteins 78:1457–1469

Gong YY, Yin X, Zhang HM, Wu MC, Tang CD, Wang JQ, Pang QF (2013) Cloning, expression of a feruloyl esterase from *Aspergillus usamii* E001 and its applicability in generating ferulic acid from wheat bran. J Ind Microbiol Biotechnol 40:1433–1441

Gorke J, Srienc F, Kazlauskas R (2010) Towards advances ionic liquids. Polar enzyme-friendly solvents for biocatalysis. Biotechnol Bioproc Eng 15:40–53

Gottschalk LMF, Oliveira RA, da Silva Bon EP (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 51:72–78

Gottschalk LMF, de Sousa Paredes R, Teixeira RSS, da Silva ASA, da Silva Bon EP (2013) Efficient production of lignocellulolytic enzymes xylanase, β-xylosidase, ferulic acid esterase and β-glucosidase by the mutant strain *Aspergillus awamori* 2B.361 U2/1. Braz J Microbiol 44:569–576

Grinna LS, Tschopp JF (1989) Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. Yeast 5:107–115

Haase-Aschoff P, Linke D, Berger RG (2013) Detection of feruloyl- and cinnamoyl esterases from basidiomycetes in the presence of interfering laccase. Bioresour Technol 130:231–238

Hassan S, Hugouvieux-Cotte-Pattat N (2011) Identification of two feruloyl esterases in *Dickeya dadantii* 3937 and induction of the major feruloyl esterase and of pectate lyases by ferulic acid. J Bacteriol 193:963–970

Hegde S, Muralikrishna G (2009) Isolation and partial characterization of alkaline feruloyl esterases from *Aspergillus niger* CFR 1105 grown on wheat bran. World J Microbiol Biotechnol 25:1963–1969

Hemery Y, Lullien-Pellerin V, Rouau X, Abecassis J, Samson MF, Åman P, von Reding W, Spoerndli C, Barron C (2009) Biochemical markers: efficient tools for the assessment of wheat grain tissue proportions in milling fractions. J Cereal Sci 49:55–64

Hermoso JA, Sanz-Aparicio J, Molina R, Juge N, Gonzalez R, Faulds CB (2004) The crystal structure of feruloyl esterase A from *Aspergillus niger* suggests evolutive functional convergence in feruloyl esterase family. J Mol Biol 338:495–506

Holmquist M (2000) Alpha/beta-hydrolase fold enzymes: structures, functions and mechanisms. Curr Protein Pept Sci 1:209–235

Horn SJ, Vaaje-Kolstad G, Westereng B, Eijink VGH (2012) Novel enzymes for the degradation of cellulose. Biotechnol Biofuels 5:45

Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol 2:602–619

Lai K-K, Stogios PJ, Vu C, Xu X, Cui H, Molloy S, Savchenko A, Yakunin A, Gonzalez CF (2011) An inserted α/β subdomain shapes the catalytic pocket of *Lactobacillus johnsonii* cinnamoyl esterase. PLoS ONE 6(8):e23269

Iiyama K, Lam TBT, Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry 29:733–737

Ishii T (1997) Structure and functions of feruloylated polysaccharides. Plant Sci 127:111–127

Ishii T, Hiroi T, Thomas JR (1990) Feruloylated xyloglucan and *p*-coumaroyl arabinoxylan oligosaccharides from bamboo shoot cell-walls. Phytochemistry 29:1999–2003

Johnson KG, Silva MC, MacKenzie CR, Schneider H, Fontana JD (1989) Microbial degradation of hemicellulosic materials. Appl Biochem Biotechnol 20–21:245–258

Kanauchi M, Watanabe S, Tsukada T, Atta K, Kakuta T, Koizumi T (2008) Purification and characteristics of feruloyl esterase from *Aspergillus awamori* G-2 strain. J Food Sci 73: 458–463

Katsimpouras C, Christakopoulos P, Topakas E (2014) Fermentation and enzymes. In: Varzakas T, Tzia C (eds) Food engineering handbook. CRC Press, Boca Raton, pp 495–497

Kheder F, Delaunay S, Abo-Chameh G, Paris C, Muniglia L, Girardin M (2009) Production and biochemical characterization of a type B ferulic acid esterase from *Streptomyces ambofaciens*. Can J Microbiol 55:729–738

Khmelnitsky YL, Hilhorst R, Veeger C (1988) Detergentless microemulsions as media for enzymatic reactions. Eur J Biochem 176:265–271

Kim Y, Hendrickson R, Mosier NS, Ladisch MR, Bals B, Balan V, Dale BE (2008) Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distiller's grains at high-solids loadings. Biores Technol 99:5206–5215

Kin KL, Lorca GL, Gonzalez CF (2009) Biochemical properties of two cinnamoyl esterases purified from a *Lactobacillus johnsonii* strain isolated from stool samples of diabetes-resistant rats. Appl Environ Microbiol 75:5018–5024

Knoshaug EP, Selig MJ, Baker JO, Decker SR, Himmel ME, Adney WS (2008) Heterologous expression of two ferulic acid esterases from *Penicillium funiculosum*. Appl Biochem Biotechnol 146:79–87

Koseki T, Furuse S, Iwano K, Matsuzawa H (1998) Purification and characterization of a feruloylesterase from *Aspergillus awamori*. Biosci Biotechnol Biochem 62:2032–2034

Koseki T, Fushinobu S, Shirakawa AH, Komai M (2009) Occurrence, properties and application of feruloyl esterases. Appl Microbiol Biotechnol 84:803–810

Koseki T, Takahashi K, Fushinobu S, Iefuji H, Iwano K, Hashizume K, Matsuzawa H (2005) Mutational analysis of a feruloyl esterase from Aspergillus awamori involved in substrate discrimination and pH dependence. Biochim Biophys Acta—Gen Subj 1722:200–208

Kroon PA, Williamson G (1996) Release of ferulic acid from sugar-beet pulp by using arabinanase, arabinofuranosidase and an esterase from *Aspergillus niger*. Biotechnol Appl Biochem 23:263–267

Kroon PA, Faulds CB, Brezillon C, Williamson G (1997) Methyl phenylalkanoates as substrates to probe the active sites of esterases. Eur J Biochem 248:245–251

Kroon PA, Williamson G, Fish NM, Archer DB, Belshaw NJ (2000) A modular esterase from *Penicillium funiculosum* which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module. Eur J Biochem 267:6740–6752

Kühnel S, Pouvreau L, Appeldoorn MM, Hinz SWA, Schols HA, Gruppen H (2012) The ferulic acid esterases of *Chrysosporium lucknowense* C1: purification, characterization and their potential application in biorefinery. Enzyme Microb Tech 50:77–85

Lambertz C, Garvey M, Klinger J, Heesel D, Klose H, Fischer R, Commandeur U (2014) Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol Biofuels 7:135

Larsen J, Østergaard Haven M, Thirup L (2012) Inbicon makes lignocellulosic ethanol a commercial reality. Biomass Bioenergy 46:36–45

Lenfant N, Hotelier T, Velluet E, Bourne Y, Marchot P, Chatonnet A (2013) ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Res 41:D423–D429

Levasseur A, Gouret P, Lesage-Meessen L, Asther M, Asther M, Record E, Pontarotti P (2006) Tracking the connection between evolutionary and functional shifts using the fungal lipase/feruloyl esterase A family. BMC Evol Biol 6:92

Li J, Cai S, Luo Y, Dong X (2011) Three feruloyl esterases in *Cellulosilyticum ruminicola* H1 act synergistically to hydrolyze esterified polysaccharides. Appl Environ Microbiol 77:6141–6147

Linke D, Matthes R, Nimtz M, Zorn H, Bunzel M, Berger RG (2013) An esterase from the basidiomycete *Pleurotus sapidus* hydrolyzes feruloylated saccharides. Appl Microbiol Biotechnol 97:7241–7251

Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B (2013) The carbohydrate-active enzymes database (CAZy) in 2013. Nucl Acids Res 42:D490–D495

MacKenzie CR, Bilous D (1988) Ferulic acid esterase activity from *Schizophyllum commune*. Appl Environ Microbiol 54:1170–1173

Mackenzie CR, Bilous D, Schneider H, Johnson KG (1987) Induction of cellulolytic and xylanolytic enzyme systems in *Streptomyces* spp. Appl Environ Microbiol 53:2835–2839

Malinovsky FG, Fangel JU, Willats WGT (2014) The role of the cell wall in plant immunity. Front Plant Sci 5:1–12

Mandalari G, Bisignano G, Lo Curto RB, Waldron KW, Faulds CB (2008) Production of feruloyl esterases and xylanases by *Talaromyces stipitatus* and *Humicola grisea* var. *thermoidea* on industrial food processing by-products. Biores Technol 99:5130–5133

Mathew S, Abraham TE (2005) Studies on the production of feruloyl esterase from cereal brans and sugar cane bagasse by microbial fermentation. Enzyme Microb Technol 36:565–570

McClendon SD, Shin HD, Chen RR (2011) Novel bacterial ferulic acid esterase from *Cellvibrio japonicus* and its application in ferulic acid release and xylan hydrolysis. Biotechnol Lett 33:47–54

McCrae SI, Leith KM, Gordon AH, Wood TM (1994) Xylan-degrading enzyme system produced by the fungus *Aspergillus awamori*: isolation and characterization of a feruloyl esterase and a p-coumaroyl esterase. Enzyme Microb Technol 16:826–834

Moukouli M, Topakas E, Christakopoulos P (2008) Cloning, characterization and functional expression of an alkalitolerant type C feruloyl esterase from *Fusarium oxysporum*. Appl Microbiol Biotechnol 79:245–254

Mueller-Harvey I, Hartley RD, Harris J, Curzon EH (1986) Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydr Res 148:71–85

Mukherjee G, Singh RK, Mitra A, Sen SK (2007) Ferulic acid esterase production by *Streptomyces* sp. Biores Technol 98:211–213

Nardini M, Dijkstra BW (1999) Alpha/beta hydrolase fold enzymes: the family keeps growing. Curr Opin Struct Biol 9:732–737

Nguyen D, Zhang X, Jiang ZH, Audet A, Paice MG, Renaud S, Tsang A (2008) Bleaching of kraft pulp by a commercial lipase: accessory enzymes degrade hexenuronic acids. Enz Microbial Technol 43:130–136

Nieter A, Haase-Aschoff P, Linke D, Nimtz M, Berger RG (2014) A halotolerant type A feruloyl esterase from *Pleurotus eryngii*. Fungal Biol 118:348–357

Nordkvisk E, Salomonsson AC, Amar P (1984) Distribution of insoluble bound phenolic acids in barley grain. J Sci Food Agric 35:657–661

Olivares- Hernández R, Sunner H, Frisvad JC, Olsson L, Nielsen J, Panagiotou G (2010) Combining substrate specificity analysis with support vector classifiers reveals feruloyl esterase as a phylogenetically informative protein group. PLoS ONE 5:e12781

Olsson L, Panagiotou G, Christakopoulos P, Topakas E, Olivares R (2011) Nutraceutical compounds: feruloyl esterases as biosynthetic tools. Encycl Biotechnol Agric Food 1:448–453

Oosterveld A, Grabber JH, Beldman G, Ralph J, Voragen AGJ (1997) Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. Carbohydr Res 300: 179–181

Panagiotou G, Granouillet P, Olsson L (2006) Production and partial characterization of arabinoxylan-degrading enzymes by *Penicillium brasilianum* under solid-state fermentation. Appl Microbiol Biotechnol 72:1117–1124

Poidevin L, Levasseur A, Paës G, Navarro D, Heiss-Blanquet S, Asther M, Record E (2009) Heterologous production of the *Piromyces equi* cinnamoyl esterase in *Trichoderma reesei* for biotechnological applications. Lett Appl Microbiol 49:673–678

Prates JAM, Tarbouriech N, Charnock SJ, Fontes CMGA, Ferreira LMA, Davies GJ (2001) The structure of the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum* provides insights into substrate recognition. Structure 9:1183–1190

Priefert H, Rabenhorst J, Steinbüchel A (2001) Biotechnological production of vanillin. Appl Microbiol Biotechnol 56:296–314

Qi M, Wang P, Selinger LB, Yanke LJ, Forster RJ, McAllister TA (2011) Isolation and characterization of a ferulic acid esterase (Fae1A) from the rumen fungus *Anaeromyces mucronatus*. J Appl Microbiol 110:1341–1350

Rakotoarivonina H, Hermant B, Chabbert B, Touzel JP, Remond C (2011) A thermostable feruloyl-esterase from the hemicellulolytic bacterium *Thermobacillus xylanilyticus* releases phenolic acids from non-pretreated plant cell walls. Appl Microbiol Biotechnol 90:541–552

Ralph J, Quideau S, Grabber JH, Hatfield RD (1994) Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. J Chem Soc Perkin Trans 1:3485–3498

Ralph J, Grabber J, Hatfield RD (1995) Lignin-ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr Res 275:167–178

Record E, Asther M, Sigoillot C, Pages S, Punt PJ, Delattre M, Haon M, van den Hondel CAMJJ, Sigoillot JC, Lesage-Meessen L, Asther M (2003) Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching application. Appl Microbiol Biotechnol 62:349–355

Rouaou X, Cheynier V, Surget A, Gloux D, Barron C, Meudec E, Louis-Montero J, Criton M (2003) A dehydrotrimer of ferulic acid from maize bran. Phytochemistry 63:899–903

Rumbold K, Biely P, Mastihubova M, Gudelj M, Gubitz G, Robra K-H, Prior BA (2003) Purification and properties of a feruloyl esterase involved in lignocellulose degradation by *Aureobasidium pullulans*. Appl Environ Microbiol 69:5622–5626

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291

Saulnier L, Vigouroux L, Thibault JF (1995) Isolation and partial characterization of feruloylated oligosaccharides from maize bran. Carbohydr Res 272:241–253

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289

Schubot FD, Kataeva IA, Blum DL, Shah AK, Ljungdahl LG, Rose JP, Wand BC (2001) Structural basis for the substrate specificity of the feruloyl esterase domain of the cellulosomal xylanase Z from *Clostridium thermocellum*. Biochemistry 40:12524–12532

Selig MJ, Knoshaug EP, Adney WS, Himmel ME, Decker SR (2008) Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Biores Technol 99:4997–5005

Shibuya N (1984) Phenolic acids and their carbohydrate esters in rice endosperm cell walls. Phytochemistry 23:2233–2237

Shin HD, Chen RR (2006) Production and characterization of a type B feruloyl esterase from *Fusarium proliferatum* NRRL 26517. Enzyme Microb Technol 38:478–485

Shin HD, Chen RR (2007) A type B feruloyl esterase from *Aspergillus nidulans* with broad pH applicability. Appl Microbiol Biotechnol 73:1323–1330

Sigoillot C, Camareto S, Vidal T, Record E, Asther M, Boada MP, Martinze MJ, Sigoillot JC, Asther M, Colom JF, Martinez AT (2005) Comparison of different fungal enzymes from bleaching high-quality paper pulps. J Biotechnol 115:333–343

Smith MM, Hartley RD (1983) Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr Res 118:65–80

Sultana R, Ravagna A, Mohmmad-Abdul H, Calabrese V, Butterfield DA (2005) Ferulic acid ethyl ester protects neurons against amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: relationship to antioxidant activity. J Neurochem 92:749–758

Suzuki K, Hori A, Kawamoto K, Thangudu RR, Ishida T, Igarashi K, Samejima M, Yamada C, Arakawa T, Wakagi T, Koseki T, Fushinobu S (2014) Crystal structure of a feruloyl esterase belonging to the tannase family: a disulfide bond near a catalytic triad. Proteins 82:2857–2867

Tabka MG, Herpoël-Gimbert I, Monod F, Asther M, Sigoillot JC (2006) Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulose xylanase and feruloyl esterase treatment. Enz Microbial Technol 39:897–902

Tai ES, Hsieh PC, Sheu SC (2014) Effect of polygalacturonase and feruloyl esterase from *Aspergillus tubingensis* on demucilage and quality of coffee beans. Process Biochem 49: 1274–1280

Tenkanen M, Schuseil J, Puls J, Poutanen K (1991) Production, purification and characterisation of an esterase liberating phenolic acids from lignocellulosics. J Biotechnol 18:69–84

Thakur VV, Jain RK, Mathur RM (2012) Studies on xylanase and laccase enzymatic prebleaching to reduce chlorine-based chemicals during CEH and ECF bleaching. Bioresources 7: 2220–2235

Thörn C, Gustafsson H, Olsson L (2011) Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield. J Mol Catal B Enzym 72:57–64

Topakas E, Christakopoulos P (2004) Production and partial characterization of alkaline feruloyl esterases by *Fusarium oxysporum* during submerged batch cultivation. World J Microb Biot 20:245–250

Topakas E, Stamatis H, Mastihubova M, Biely P, Kekos D, Macris BJ, Christakopoulos P (2003a) Purification and characterization of a *Fusarium oxysporum* feruloyl esterase (FoFAE-I) catalysing transesterification of phenolic acid esters. Enz Microbial Technol 33:729–737

Topakas E, Stamatis H, Biely P, Kekos D, Macris BJ, Christakopoulos P (2003b) Purification and characterization of a feruloyl esterase from *Fusarium oxysporum* catalyzing esterification of phenolic acids in ternary water-organic solvent mixtures. J Biotechnol 102:33–44

Topakas E, Kalogeris E, Kekos D, Macris BJ, Christakopoulos P (2003c) Production and partial characterization of feruloyl esterase by *Sporotrichum thermophile* under solid-state fermentation. Process Biochem 38:1539–1543

Topakas E, Stamatis H, Biely P, Christakopoulos P (2004) Purification and characterization of a type B feruloyl esterase (StFae-A) from the thermophilic fungus *Sporotrichum thermophile*. Appl Microbiol Biotechnol 63:686–690

Topakas E, Christakopoulos P, Faulds CB (2005a) Comparison of mesophilic and thermophilic feruloyl esterases: characterization of their substrate specificity for methyl phenylalkanoates. J Biotechnol 115:355–366

Topakas E, Vafiadi C, Stamatis H, Christakopoulos P (2005b) *Sporotrichum thermophile* type C feruloyl esterase (StFaeC): purification, characterization and its use for phenolic acid (sugar) ester synthesis. Enz Microbial Technol 36:729–736

Topakas E, Vafiadi C, Christakopoulos P (2007) Microbial production, characterization and applications of feruloyl esterases. Proc Biochem 42:497–509

Topakas E, Moukouli M, Dimarogona M, Christakopoulos P (2012) Expression, characterization and structural modelling of a feruloyl esterase from the thermophilic fungus *Myceliophthora thermophila*. Appl Microbiol Biotechnol 94:399–411

Tsuchiyama M, Sakamoto T, Fujita T, Murata S, Kawasaki H (2006) Esterification of ferulic acid with polyols using a ferulic acid esterase from *Aspergillus niger*. Biochim Biophys Acta 1760:1071–1079

Udatha DBRKG, Kouskoumvekaki I, Olsson L, Panagiotou G (2011) The interplay of descriptor-based computational analysis with pharmacophore modeling builds the basis for a novel classification scheme for feruloyl esterases. Biotechnol Adv 29:94–110

Uraji M, Arima J, Inoue Y, Harazono K, Hatanaka T (2014) Application of two newly identified and characterized feruloyl esterases from *Streptomyces* sp. in the enzymatic production of ferulic acid from agricultural biomass. PLoS ONE 9(8):e104584

Vafiadi C, Topakas E, Wong KKY, Suckling ID, Christakopoulos P (2005) Mapping the hydrolytic and synthetic selectivity of a type C feruloyl esterase (StFaeC) from *Sporotrichum thermophile* using alkyl ferulates. Tetrahydron: Asymmetry 16:373–379

Vafiadi C, Topakas E, Christakopoulos P (2006a) Regioselective esterase-catalyzed feruloylation of L-arabinodiose. Carbohydr Res 341:1992–1997

Vafiadi C, Topakas E, Christakopoulos P, Faulds CB (2006b) The feruloyl esterase system of *Talaromyces stipitatus*: determining the hydrolytic and synthetic specificity of TsFaeC. J Biotechnol 125:210–221

Vafiadi C, Topakas E, Alderwick LJ, Besra GS, Christakopoulos P (2007a) Chemoenzymatic synthesis of feruloyl-*D*-arabinose as a potential anti-mycobacterial agent. Biotechnol Lett 29:1771–1774

Vafiadi C, Topakas E, Bakx EJ, Schols HA, Christakopoulos P (2007b) Structural characterization of ESI-MS of feruloylated arabino-oligosaccharides synthesized by chemoenzymatic esterification. Molecules 12:1367–1375

Vafiadi C, Topakas E, Alissandratos A, Faulds CB, Christakopoulos P (2008a) Enzymatic synthesis of butyl hydroxycinnamates and their inhibitory effect on LDL-oxidation. J Biotechnol 133:497–504

Vafiadi C, Topakas E, Christakopoulos P (2008b) Preparation of multipurpose cross-linked enzyme aggregates and their application to production of alkyl ferulates. J Mol Catal B Enzym 54:35–41

Vafiadi C, Topakas E, Nahmias VR, Faulds CB, Christakopoulos P (2009) Feruloyl esterase-catalyzed synthesis of glycerol sinapate using ionic liquid mixtures. J Biotechnol 139:124–129

Valls C, Colom JF, Baffert C, Gimbert I, Roncero MB (2010) Comparing the efficiency of the laccase-NHA and laccase-HBT systems in eucalyptus pulp bleaching. Biochem Eng J 49: 401–407

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol 153:895–905

Várnai A, Tang C, Bengtsson O, Atterton A, Mathiesen G, Eijsink GH (2014) Expression of endoglucanases in *Pichia pastoris* under control of the GAP promoter. Microb Cell Fact 13:57

Vogt T (2010) Phenylpropanoid Biosynthesis. Mol Plant 3:2–20

Walton NJ, Narbad A, Faulds C, Williamson G (2000) Novel approaches to the biosynthesis of vanillin. Curr Opin Biotechnol 11:490–496

Wang X, Geng X, Egashira Y, Sanada H (2004) Purification and characterization of a feruloyl esterase from the intestinal bacterium *Lactobacillus acidophilus*. Appl Environ Microbiol 70:2367–2372

Wang L, Zhang R, Ma Z, Wang H, Ng T (2014) A feruloyl esterase (FAE) characterized by relatively high thermostability from the edible mushroom *Russula virescens*. Appl Biochem Biotechnol 172:993–1003

Wong D (2008) Enzymatic deconstruction of backbone structures of the ramified regions in pectins. Protein J 27:30–42

Wong DWS, Chan VJ, Batt SB, Sarath G, Liao H (2011) Engineering *Saccharomyces cerevisiae* to produce feruloyl esterase for the release of ferulic acid from switchgrass. J Ind Microbiol Biotechnol 38:1961–1967

Wong DWS, Chan VJ, Liao H, Zidwick MJ (2013) Cloning of a novel feruloyl esterase gene from rumen microbial metagenome and enzyme characterization in synergism with endoxylanases. J Ind Microbiol Biotechnol 40:287–295

Wu M, Abokitse K, Grosse S, Leisch H, Lau PCK (2012) New feruloyl esterases to access phenolic acids from grass biomass. Appl Biochem Biotechnol 168:129–143

Yang SQ, Tang L, Yan QJ, Zhou P, Xu HB, Jiang ZQ, Zhang P (2013) Biochemical characteristics and gene cloning of a novel thermostable feruloyl esterase from *Chaetomium* sp. J Mol Catal B Enzym 97:328–336

Yao J, Chen QL, Shen AX, Cao W, Liu YH (2013) A novel feruloyl esterase from a soil metagenomic library with tannase activity. J Mol Catal B Enzym 95:55–61

Yu P, Maenz DD, McKinnon JJ, Racz VJ, Christensen DA (2002a) Release of ferulic acid from oat hulls by *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase. J Agric Food Chem 50:1625–1630

Yu P, McKinnon JJ, Maenz DD, Racz VJ, Christensen DA (2002b) The interactive effects of enriched sources of *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase on the quantitative release of hydroxycinnamic acids from oat hulls. Can J Anim Sci 82:251–257

Yu P, McKinnon JJ, Maenz DD, Olkowski AA, Racz VJ, Christensen DA (2003) Enzymic release of reducing sugars from oat hulls by cellulase, as influenced by *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase. J Agric Food Chem 51:218–223

Zeng Y, Yin X, Wu MC, Yu T, Feng F, Zhu TD, Pang QF (2014) Expression of a novel feruloyl esterase from *Aspergillus oryzae* in *Pichia pastoris* with esterification activity. J Mol Catal B Enzym 110:140–146

Zeuner B, Stahlberg T, van Buu ON, Kurov-Kruse AJ, Riisager A, Meyer AS (2011) Dependency of the hydrogen bonding capacity of the solvent anion on the thermal stability of feruloyl esterases in ionic liquid systems. Green Chem 13:1550–1557

Zhang SB, Zhai HC, Wang L, Yu GH (2013) Expression, purification and characterization of a feruloyl esterase A from *Aspergillus flavus*. Prot Expr Pur 92:36–40

Zwane EN, Rose SH, Van Zyl WH, Rumbold K, Viljoen-Bloom M (2014) Overexpression of *Aspergillus tubingensis* faeA in protease-deficient *Aspergillus niger* enables ferulic acid production from plant material. J Ind Microbiol Biotechnol 41:1027–1034

Chapter 6
Endo-β-1,4-xylanase: An Overview of Recent Developments

Alexandre Gomes Rodrigues

Abstract This chapter presents the main important aspects about the enzyme endo-beta-1,4-xylanase. It starts with the biological grounds for the use of the enzyme. Moving on, the advancements in the production process and optimisation are substantially described, along with the microorganisms and a broad range of molecular techniques used to obtain best performance enzyme. Finally, it delineates the most common applications related to xylanase, such as in biobleaching, baking, feedstock and the enzymatic role in juice and ink industries, among others. Furthermore, the material brings updated research performed during the last few years combined with essential established facts along the time.

6.1 Introduction

Since ancient times the mankind has made used of enzymes to produce goods as wine, beer and bread. The gained knowledge in fields as chemistry, biology, engineering and biotechnology have enabled more precise and efficient processes to obtain and apply such biomolecules not only in the existing products but also to create new ones, exploring fully the potential of a material and expanding its roles.

In this regard, plants serve us in many aspects, with their fundamental biological and microbiological functions, and have been increasingly exploited to provide valuable biomaterials, which are employed to boost advancements in daily life with better and suitable products, while reducing the damage caused to the environment thereby making our lives more comfortable. Hemicelluloses are the second most present polysaccharide in plant cell wall and are made up of important polymers as xylan (Schaedel et al. 2010). Xylanases are enzymes secreted by microorganisms, mostly fungi and bacteria are used in a broad range of important manufacturing pathways in the papermaking industry as well as in the food industry and in the

A.G. Rodrigues (✉)
Institute of Pharmacy, Martin-Luther University Halle-Wittenberg, Halle, Germany
e-mail: alexgrpharmazie@gmail.com

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_6

production of chemicals such as xylitol, in addition to their natural function. Due to its key role the xylanases have reached an outstanding market position. The estimative was that the market in which the enzyme is present represents about 200 million dollars (Sonia et al. 2005). In 2005, xylanases along with cellulases and pectinases sliced 20 % of the global industrial enzyme market (Polizeli et al. 2005).

The innumerous applications of xylanases have raised interest and attracted many researches and resources towards its whole production chain (Wong et al. 1988; Coughlan and Hazlewood 1993; Beg et al. 2001; Harris and Ramalingam 2010; Juturu and Wu 2012). Xylanase transforms water insoluble hemicellulose into soluble form, which binds water in the dough, therefore decreasing the dough firmness, increasing volume and creating finer and more uniform crumbs (Butt et al. 2008). The enzymes break down xylan into oligosaccharides, lowering the viscosity, resulting in more appropriate processed food and drink, as dough in baking, in animal feed to increase digestibility, wood pulp, liquid fuels, solvents and other chemicals (Bhat 2000; Butt et al. 2008). The current technological knowledge and available facilities provide means by which the processes regarding the obtaining of xylanases and process optimisation are improved. A wide range of techniques from molecular biology to analytical chemistry and biochemical engineering have been reported as enabling researchers to progress and provide better products continuously.

This chapter comprises the fundamental aspects related to endo-β-1,4-xylanase, namely its early appearance and applications which have encouraged much effort and investment, along with the most recent studies conducted worldwide and their main outcomes leading relevant improvements and achievements in the field.

6.2 Plant Cell Wall Structure

Plant cell wall contains a variable amount of different chemical compounds making up their structures. The cell wall itself is composed of three layers. The compounds present in a plant cell wall are cellulose and hemicellulose, pectin and lignocellulose. After cellulose, hemicellulose is the most abundant natural polysaccharide in plants and accounts to one-third of plant biomass. However, it varies according to plant species due to environmental conditions (Caffall and Mohnen 2009; Schaedel et al. 2010; Yin et al. 2010). Hemicellulose contributes not only to biological processes in nature but also in our daily life, through the employment of its components in several industrial sectors from feedstock to paper making and bread baking (Yin et al. 2010).

Schulze (1891) first introduced the term ‘hemicellulose’ for the fractions isolated or extracted from plant materials with diluted alkali. It is an amorphous heteropolymer formed by aldopentoses (xylose and arabinose) and aldohexoses (glucose, galactose, mannose), forming a short polymer chain presenting lower molar mass, when compared to cellulose (Jacobsen 2000). It is present in hardwood (angiosperm) as well as in softwood (gymnosperm). Hardwood hemicelluloses are xylans, whereas softwood consists of glucomannans. The portion present in

hardwood is the most important due to its higher amount. Xylan backbone is highly branched by side chains consisting of xylose, arabinose and galucuranic acids (Saha 2003). According to the type of sugar residue hemicelluloses are framed into four categories: xylans, secondary cell walls of hardwood and herbaceous plants; xyloglucans, primary cell walls of higher plants (bound to cellulose); mannans, secondary cell walls of conifers and *Leguminosae* (Schaedel et al. 2010).

The degradation of hemicelluloses is mostly carried out by microorganisms that can be found either free in nature or as part of the digestive tract in higher animals. Such degradation is accomplished with the aid of enzymatic complexes and leads to the production of several important industrial compounds.

6.3 Endo-β-1,4 Beta Xylanases

Xylan is the major component in hemicellulose in hardwood, but is less abundant in softwood and accounts for one-third of all renewable organic carbon available on earth (Prade 1996; Kulkarni et al. 1999). Yet, it is a polysaccharide formed by monomers of 1,4-β-D-xylose with side chain components, such as arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl and *p*-coumaroyl residues, depending on the plant source (Biely 1985; Knob and Carmona 2008).

In the biodegradation of xylan the molecule is broken down into its units, namely xylose, a pentose monosaccharide that serves as a primary source of carbons to bacteria and fungi, and was the first sugar isolated from wood in 1886 (Koch 1886; Sohpal et al. 2010). Xylose itself is composed by arabinofuranosyl, glucuronyl and acetyl groups. Due to its heterogeneity and complex nature, the complete breakdown of xylan requires the synergistic action of a large variety of hydrolytic enzymes (Biely 1985; Coughlan and Hazlewood 1993). The sugar is hydrolysed into its monomers by action of different enzymes, among them endo-beta-1,4-xylanase.

Xylanases (EC 3.2.1.8) are classified into glycoside hydrolase (GH) families and specific information can be found in the Carbohydrate-Active Enzyme (CAZy) database (Coughlan and Hazlewood 1993; Biely et al. 1997; Pastor et al. 2007). The protein presents more conserved folds in some families than their amino acid sequences and these families are grouped into clans (Coughlan and Hazlewood 1993; Biely et al. 1997). Endo-xylanases are found in families 5, 7, 8, 10, 11, 26 and 43. The enzymes from families 10 (formerly known as F) and 11 (formerly known as G) have been widely described in the literature. Family 10 endo-xylanases posses higher molecular weight, compared to the ones of family 11, structurally composed of a cellulose-binding domain and a catalytic domain connected by a linker peptide (Biely et al. 1997). Family 11 endo-xylanases contain lower molecular weight enzymes. Based on their isoelectric points, family 11 xylanases are further sub-grouped into alkaline and acidic enzymes. It is considered that, due to their relatively small sizes, these endo-xylanases can pass through the pores of hemicelluloses network performing efficient hydrolysis (Törrönen and Rouvinen 1997).

These xylan degrading enzymes are expressed by a wide variety of fungi (Wase et al. 1985; Bakir et al. 2001; Li et al. 2006) and bacteria (Mamo et al. 2006), but also actinomycetes (Ball and McCarthy 1988), and yeast (Hrmova et al. 1984). Even if there is a great variety of xylanases and their folds, mechanisms of action, substrate specificities, hydrolytic activities and physicochemical characteristics being diverse, mostly attentions have been driven to two of the xylanases containing carbohydrolase families from families 10 and 11 and are based on their amino acid sequence homologies (Henrissat 1991). These enzymes catalyse the 1,4-beta-D-xylosidic linkages in xylan (Collins et al. 2005). As described in the literature (Biely et al. 1983), the enzymes not only hydrolyse the substrate but also catalyse various biomolecular reactions, such as transglycosylation and degradation of oligosaccharides. The authors explained the dependence of enzymatic activity on the substrate concentration and the preference of xylanases from different sources, as those examined from *A. niger* and *C. albidus*, for cleaving glycosidic bonds.

The thermophilic fungus *Thermomyces lanuginosus*, first isolated in 1899 by Tsiklinskaya from a potato, is referred as the most suitable microorganism for the cellulose-free xylanases production both in shake flask and bioreactor cultivations; however, due to intraspecies variability the enzymatic expression may be affected. According to extensive studies on the molecular, physiological and ecological properties of *T. lanuginosus* strains have been carried out (Singh et al. 2003). Furthermore, the cultivation conditions of each strain require optimisation since the strains are influenced by nutrient and growth conditions used in their cultivation. In addition, *Aspergillus niger*, *Trichoderma reesei* and *Streptomyces* have been frequently explored for the production of xylanases (Pradeep et al. 2013). With the advancements of recombinant DNA technology, the gene responsible for the enzyme started being cloned and expressed in other microorganisms, enabling researchers to end up with purer products, presenting higher enzymatic activity and in increased amounts. The enzyme has been cloned in bacteria, mostly *Escherichia coli*, and filamentous fungi such as *Trichoderma reesei* and *Fusarium oxysporum* have also been employed for this purpose (He et al. 2009; Moukouli et al. 2011). In most cases, when it comes to the heterologous expression of xylanase, the host models used are widely applied yeast *Pichia pastoris* and in some cases *E. coli* (He et al. 2009; Cai et al. 2011; Moukouli et al. 2011).

6.4 Molecular Features in Process and Process Optimisation

As described by Harris and Ramalingam (2010) endo-xylanases cleave the glycosidic bonds in the xylan backbone. The enzymes have been differentiated according to the end products they release from the hydrolysis of xylan (for instance xylose, xylobiose and xylotriose and arabinose). Therefore, xylanases may be classified as non-debranching (arabinose non-liberating) or debranching (arabinose liberating) enzymes.

Due to the absent of standardisation of the various existing sources of the enzyme and process parameters Bailey et al. (1992) compared the measurement of xylanase activity among twenty different laboratories. According to the authors, the major source of variation between apparent xylanase activities was probably the substrate chosen, although small differences in protocols were also significant. After standardisation of substrate 260 and method, the interlaboratory standard variation of the results decreased from 108 to 17 % from the mean.

Based on its technical and economic importance and vast range of applications, endo-xylanases have been extensively investigated regarding its expression and process optimisation, particularly over the last few years. Due to the current conditions of operations it is desired that the employed enzyme presents thermostability and an adequate pH range in the production process. The molecular features are of upmost concern since detailed knowledge is required to obtain more efficient processes and appropriate amounts of product with high quality. In order to overcome enzyme limitations under diverse conditions and enhance stability, among other important issues related to enzyme yield and industrial scale production, many research groups have focused on molecular engineering techniques in search for enzymes with superior performance, and substantial results have been reported. Moreover, hybrid xylanases have been created and tend to be increasingly applied in the coming years to enable such progress. Because the processes in which endo-xylanases are applied are undertake in harsh conditions, such as the pH in the case of pulp bleaching, there are specific requirements to improve the process conditions (Biely et al. 1983; Nakamura et al. 1993; Stephens et al. 2007). Therefore, it is crucial to understand the properties of the enzyme, its structure and tune its features as possible in order to achieve optimal efficiency (van Dyk et al. 2010).

An endo-xylanase isolated from the soil showed high sequence homology with the xylanases from *Bacillus pumilus* and *Clostridium acetobutylicum* in the N-terminal region, with stability in alkaline pH (9.0) and optimum temperature for the activity around 50 °C (Nakamura et al. 1993). The influence of medium compounds in the induction and repression of xylanase has been described (Gomes et al. 1994) demonstrating that arabinose, glucose, lactose and xylose were ineffective in the induction of xylanase from *Thermoascus aurantiacus*. The study showed yet that while wood xylan, birch wood glucuronoxylan and methyl/3-D-xylopyranoside (MXP) induced xylanase, glucose acted as a repressor in that case. Purkarthofer and Steiner (1995) also studied the effect of carbohydrates on the production of inducible β-xylanase of *Thermomyces lanuginosus*, concluding that D-pentoses as well as xylan and xylobiose served as inducers, but D-lyxose, D-arabinose, D-ribose and L-arabinose were significantly not as pronounced as xylose, xylobiose and xylan. The crystallographic structure of endo-xylanase I from family G, which typically exhibit acidic pH, resulting in a proposed model to serve as an explanation of the mechanisms that govern the low pH optimum of the enzyme (Krengel and Dijkstra 1996). By solving the structure of an endo-xylanase from *Aspergillus niger* employing molecular replacement Krengel and Dijkstra

(1996) revealed aspects of the enzyme conformation and purposed that two glutamate residues would be involved in the catalysis.

De Groot et al. (1998) cloned the gene encoding xylanase from *Agaricus bisporus* and determined its regulation by heterologous screening techniques. Georis et al. (1999) presented a recombinant xylanase from *Streptomyces* sp. The authors studied the cleavage site between the signal peptide and the protein and compared the similarities and strict identities of the enzyme with other family 11 xylanases. By means of crystallography the group of Professor Mizuno in Japan (Fujimoto et al. 2000) depicted the structure of the xylan-binding domain endo-beta-xylanase family 10 from *Streptomyces olivaceoviridis*. Turunen et al. (2001) reported the synergistic effect of a xylanase II from *Trichoderma reesei* brought about by stabilising mutations to access the information about its thermal stability, catalytic function and pH-dependent properties. Zhengqiang et al. (2001) isolated two xylanases genes from the bacterium *Thermotoga maritima*. The genes were subsequently cloned and expressed in *E. coli* and the authors found that the optimum condition for the enzyme activity was at 90 °C and pH of 6.14. A disulphide bridge into the N-terminal region of *Trichoderma reesei* endo-1,4-beta-xylanase was engineered by Fenel et al. (2004), increasing the enzyme thermostability by about 15 °C. Subsequently Fenel et al. (2006) achieved high stability using a mutant xylanase from *Trichoderma reesei*, which increased brightness in sulphate pulp bleaching. Brutus et al. (2004) showed the cloning, purification and expression of endo-xylanase from *Penicillium funiculosum* in *E. coli* and *Pichia pastoris* as hosts. Wakiyama et al. (2008), Jun et al. (2009) and Do et al. (2013) also published studies related to the expression of xylanase in the same host. The group of Professor Murray contributed to the elucidation of the structure of a endo-xylanase (family 10) from *Thermoascus aurantiacus*, showing that the glycone subsite of the enzyme makes extensive direct and indirect interactions with the arabinose side chain and concluding that xylan side chains are not just accommodated but can actually constitute significant substrate specificity determinants in the enzyme under study (Vardakou et al. 2005).

Another study explored the function of three enzymes, namely xylosidase–arabinosidase from *Thermoanaerobacter ethanolicus* and xylanase from *Thermomyces lanuginosus*, with the aim to achieve more efficiency in the decomposition of arabinoxylan agricultural residue for the bioconversion into fuels or industrial chemicals (Xue et al. 2009). The multifunctional enzyme was expressed in *E. coli* and exhibited enhanced enzymatic activity in the degradation of arabinoxylan into its monomer constituents when compared to the corresponding free enzymes. Vieira et al. (2009) reported the effect of temperature and binding of substrate on the dynamics of the family 11 xylanase from *Bacillus circulans* and its molecular dynamics simulations in the presence of xylobiose. The study indicates that the thermophilicity of the 11/G xylanases might be increased by rational design based on two variables: mutations that lead to changes in the flexibility of the thumb loop and mutations of residues in the substrate-binding cleft that aim to optimise the xylanase–substrate intermolecular interaction. With that the authors purposed that the residues may be considered as preferential targets for site-directed mutagenesis

experiments that modulate the enzyme substrate affinity, thus increasing the catalytic efficiency of the enzyme. The work Michaux et al. (2010), based on crystallographic techniques, with an acidophilic xylanase from *Scytalidium acidophilum*, a fungi isolated from an uranium mine in acidic waters (pH 2.0), revealed features about the stability and acidophilic adaptation. The conclusions were that various sequence and structure modifications may be responsible for the acidophilic characteristic of the enzyme. They would be the presence of aspartic acid hydrogen bonded to the acid/base catalyst; the nature of specifically conserved residues in the active site; the negative potential at the surface and the decreased number of salt bridges and hydrogen bonds in comparison with highly alkaline enzymes.

A xylanase cloned and expressed in *E. coli* by Hwang et al. (2010) had its activity not affected by most salts, such as NaCl, LiCl, KCl, NH(4)Cl, CaCl(2), MgCl(2), MnCl(2) and CsCl(2) at 1 mM; however, the enzymatic activity was affected by CuSO(4), ZnSO(4) and FeCl(3). As described, xylobiose was the major product obtained. The presence of NaCL at 12.5 proved to be not only beneficial but also the optimum condition to an endo-xylanase from thermophilic bacterium *Thermoanaerobacterium saccharolyticum* isolated from a hydrothermal vent (Hung et al. 2011). Furthermore, the expression of endo-xylanase has been described in homologous (Anasontzis et al. 2011) and heterologous (Damásio et al. 2011) models and applied in the production of ethanol and functional foods, respectively. In 2012 Trevizano et al., by means of direct evolution, improved the thermostability of endo-xylanase. The mutant enzymes generated through error-prone PCR presented a good performance in extreme pH conditions, retaining their ability to hydrolyse birchwood and oat spelt xylans, according to the authors. Kim et al. (2012) studied the intra-molecular hydrophobic interactions of xylanase, applying network analysis to interpret such events, and noticed that the enzyme half-life was increased by 78-fold, highlighting the advantages of interpreting collective hydrophobic interaction patterns. Verma and Satyanarayana (2012) also reported a thermostable gene from *Geobacillus thermoleovorans* expressed in *E. coli* with enzymatic production of 27-fold higher than the wild strain. Thermostabilisation was once more investigated by Li et al. (2013). Using *Dictyoglomus thermophilum* as an enzymatic source of xylanase, the researchers stabilised the enzyme by an engineered N-terminal disulphide bridge. The stabilisation was then tested against high temperatures, being achieved at 100–110 °C and the ionic liquid used in the study appeared to affect the functioning of the enzyme's active site to a greater extent than the stability of an already thermostable protein structure. Recently, Mander et al. (2014) produced a cellulase-free xylanase from *Streptomyces* sp. using an agricultural residue (wheat bran) as a growth substrate. The outcome was that the enzyme was not only able to hydrolyse commercially available pure beechwood xylan to xylose, xylobiose and xylotriose, but also abundantly available lignocellulosic agricultural residues in nature such as wheat bran to xylooligosaccharides. Other studies have also approached the thermal and alkaline stability, as well as enzyme–substrate interaction of hybrid xylanases, as the ones reported by Song et al. (2014), Stephens et al. (2014) and Chen et al. (2014).

Lately, the enzymatic stability was investigate by Li et al. (2015a, b) employing a disulphide mutant at pressure and temperature as high as 500 MPa and 80 °C, respectively. Likewise, Qian et al. (2015) found that an engineered xylanase was stable at 55 °C in a broad active pH range (5.5–10), being purposed as a candidate for industrial applications given its high specific activity, stability and soluble protein yield. Huy et al. (2015) observed the synergistic action of a endo glucanase together with endo-xylanase, with increased reducing sugar release of birchwood xylan (6.4 %), beechwood xylan (13 %) and arabinoxylan (15.8 %) and larger increase of reducing sugar release from pretreated barley straw that addition at the start or by treatment with endo-xylanase alone, showing a great potential application for hemicellulose saccharification.

6.5 Techniques Used in Xylanases Process

A specific range of techniques has been used to enable researchers and producers transforming what nature provides us, making important and always better goods in daily life. From molecular biology to analytical chemistry and biochemistry engineering, these methods and their improvements assure better results and more efficient processes to be conducted.

Regarding the recombinant DNA techniques used in the process of obtaining, characterising and optimising key factors of the enzyme, polyacrylamide gel electrophoresis (SDS–PAGE) is a widely used technique to determine molecular mass of proteins, thus aiding the characterisation of such biomolecules (Pribowo et al. 2012). The technique has been applied through the decades as a key tool to identify xylanases and is part of the essential technique repertoire (Biely et al. 1983; Coughlan and Hazlewood 1993; Takahashi et al. 2013). Polymerase chain reaction (PCR) and gene cloning comprises the paramount employed in the analysis and improvement of the enzyme production and analysis of its properties (Stephens et al. 2014).

Among the analytical chemistry techniques, UV/vis spectrophotometry has been employed to analyse the enzymatic activity of xylanases (Sonia et al. 2005). Chromatographic methods, namely high performance liquid chromatography (HPLC), have been used to purify the enzyme as demonstrated in a recent work (Pribowo et al. 2012). Column chromatography is another method to purify the enzymes from cultivation (Heck et al. 2006), as well as thin layer chromatography (TLC) (Chanwicha et al. 2015), used in the analysis of xylanases. Along with the above-mentioned methods, mass spectrometry finds its use in the xylanases process as described by Jänis et al. (2007) for the determination of steady-state kinetic parameters for a xylanase-catalysed hydrolysis of xylooligosaccharides. Puchart and Biely (2008) carried out the production of endo-beta-1,4-xylanase using *Thermomyces lanuginosus*, and the authors studied the compounds formed employing mass spectrometry, suggesting that the fungus might be of importance regarding the conversion of xylan into xylooligosaccharides.

6.6 Biomass Production Methods

Xylanolytic enzymes from various sources, and most importantly from microorganisms, have been studied along the last decades to understand their physical and biochemical characteristics, due to their applications in several fields (Polizeli et al. 2005).

Xylanases from natural sources are produced only to relatively low yields; therefore, the enzyme production cannot meet the demand of our societies. In view of this, heterologous expression approaches have been implemented to design xylanases with the desired characteristics for the production at the industrial scale. The expression of fungal xylanase genes in *Saccharomyces cerevisiae*, *Pichia pastoris* and other microorganisms has been reported in the literature (Karaoglana et al. 2014). The enzyme properties and yield somehow vary depending on the gene source, host system and secretion signal sequences, besides the process parameters.

Until the advent of recombinant DNA technology, enzymes were produced by fermentation of the microorganisms that express the enzymes. Cultivation of filamentous fungi for large-scale protein production is very complex, often ending up in many interfering enzymes. Purification of target enzymes from a pool of proteins requires several purification steps thereby increasing their costs (Kormelink et al. 1993). Recombinant DNA technology allows large-scale expression of these enzymes in both homologous and heterologous protein expression hosts (Puchart and Biely 2008; Juturu and Wu 2012).

Regarding the methods of obtaining endo-β-1,4-xylanases, solid-state fermentation (SSF) is the most present in the literature (Filho et al. 1993; Pandey 2002; Sonia et al. 2005; Chanwicha et al. 2015). SSF is the most suitable method for xylanase production, since it deals with low water activity environment and allows the use of agro waste substrates like corn and sugarcane bagasse. The method received more attention after during 1950–1970 with the steroid transformation using fungal cultures and the production of proteins for cattle feed enrichment. Later on SSF would gain more importance with the design of bioreactors and the microbial production of food, feed and enzymes such as lipase, phytase, cellulase, ligninase, amylase, glutaminase, pectinase and xylanase, besides the production of organic acids, secondary metabolites, fatty acids and biofuel (Pandey 2002; Bhargav et al. 2008). Compared to submerged fermentation (SmF), it facilitates the recovery of the final product, requires less energy (no need for vigorous agitation) and has high volumetric production, being thus eco-friendly and industrially favourable (Filho et al. 1993; Pandey 2002; Bhargav et al. 2008). The method is applied under microbial conditions in which cultures are closer to their natural habitat, considering that fungi grow in nature on solid substrates such as pieces of wood, seeds, stems and roots, therefore having their activity increased. The process can still be classified on the grounds of substrate nature, which can be pure or mixed (Bhargav et al. 2008).

Key factors have to be satisfied if appropriate production is intended to be achieved. Among these are moisture and water contend. This is due to the fact that water activity of the substrate influences the microbial activity, spore production

and metabolites secreted. Another issue to be addressed is the removal of heating since in SSF a large amount of heat if generated based on the low thermal conductivity of the substrates used. As important are the biomass determination and the mass transfer during the process, where O_2 and CO_2 diffusion, nutrient absorption and metabolites formation, airflow into and out of the SSF system, types of substrate, mixing of substrate, bioreactor design, space between particles, variation in particle size and microorganisms all play an important role (Bhargav et al. 2008).

Among the aspects to be considered when working with solid-state fermentation are the selection of the suitable microorganism and substrate, optimisation of the process parameters and isolation and purification of the product. Studies have been conducted in this direction as reported by Sonia et al. (2005), who investigated the endo-xylanase production by *Thermomyces lanuginosus* in SSF using sorghum straw as substrate, obtaining high levels of xylanases. In another study the method was employed by Irfan et al. (2014), in order to obtain endo-xylanase from *Trichoderma viride.* The authors evaluated the enzymatic production using different substrates such as wheat bran, rice polish, rice husk, soybean meal, sunflower meal, sugarcane bagasse or corn cobs and found that sugar cane was the best substrate in that occasion.

For practical reasons, immobilisation of microorganism or enzymes on solid materials offer several advantages, including repeated usage of the catalyst, ease of product separation and improvement of enzyme stability (Beg et al. 2001). Endo-xylanase has also been successfully immobilised in other microorganisms as *Trichoderma reesei* (Haapala et al. 1996) and *Bacillus pumilus* (Kapoor et al. 2008).

6.7 Technological Developments of Endo-Beta-1,4-xylanase

Apart from its use in the pulp and paper industry, xylanases are also used as food additive, in wheat flour for improving dough handling and quality of baked products, for the extraction of coffee, plant oils and starch, and in the improvement of nutritional properties of animal feed, and in combination with pectinase and cellulase for clarification of fruit juices (Biely 1985; Wong et al. 1988; Maat et al. 1992; Beg et al. 2001).

6.8 Biobleaching

Xylanases and other side-cleaving enzymes have been used in pulp bleaching primarily to reduce lignin and increase the brightness of the pulp. This process is necessary due to the presence of residual lignin and its derivatives in the pulping

process, which causes the resultant pulp to gain a characteristic brown colour. The use of xylanase in bleaching pulp requires the use of enzymes with special characteristics (Beg et al. 2001).

A key requirement is to be cellulose-free, to avoid damaging the pulp fibres, as cellulose is the primary product in the paper industry (Srinivasan and Rele 1999; Subramaniyan and Prema 2000). In pulp bleaching, the enzyme must tolerate high temperature and pH after alkaline cooking to be economical (Turunen et al. 2001). The process of lignin removal from chemical pulps to produce bright or completely white finished pulp is called 'bleaching'. It is necessary for aesthetic reasons and for improvement of paper properties, because the left-over residual lignin after sulphite pulping imparts an undesirable brown colour to the paper. The bleaching of kraft pulp uses large amounts of chlorine-based chemicals and sodium hydrosulfite. These bleaching chemicals cause several effluent-based problems in the pulp and paper industries. Byproducts from using these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent and bioaccumulate, and cause numerous harmful disturbances in biological systems (Onysko 1993). In response to public interests, paper industries are currently changing practices to minimise the use of chlorine-based chemicals. The available options are oxygen delignification, extended cooking and substitution of chlorine dioxide for chlorine, hydrogen peroxide and ozone. But most of these methods involve high capital investment for process change. Thus, an alternative and cost-effective method, i.e., use of enzymes, has provided a very simple and economic way to reduce the use of chlorine and other bleaching chemicals. Biobleaching involves using microorganisms and enzymes for bleaching pulp. It relies on the ability of some microorganisms to depolymerize lignin directly and on the use of microorganism or enzymes that attack hemicellulose and hence favour subsequent depolymerization (Jimenez et al. 1997).

Several criteria are essential for choosing a microorganism to produce xylanases. To give the desired bleaching effect, the resulting enzyme preparation must be completely free of any cellulase activity (Srinivasan and Rele 1999; Subramaniyan and Prema 2000; Beg et al. 2001), since any cellulase activity will have serious economic implications in terms of cellulose loss, degraded pulp quality and increased effluent treatment cost.

Use of hemicellulolytic enzymes was the first large-scale application of enzymes in the pulp and paper industry (Viikari et al. 1986). Limited hydrolysis of hemicellulose in pulps by hemicellulases (mainly xylanases) increased the extractability of lignin from the kraft pulps and reduced the chlorine required in subsequent bleaching. The xylanase from *T. reesei* has been reported to act uniformly on all accessible surfaces of kraft pulp and to be effective during biobleaching (Saake et al. 1995; Suurnakki et al. 1996; Bhat 2000).

The enzyme is already on the market and has been successfully applied in the bleaching process as demonstrated by Shatalov and Pereira (2007), nevertheless many efforts have been done with the aim to improve its performance.

An xylanase with highly specific activity towards xylan was reported by Li et al. (2005), with a narrow optimum pH range (7.0–7.5) and decrease in the chlorine

consumption of just over 28 %. Battan et al. (2007) employed *Bacillus pumilus* to produce xylanase, achieving an increase of 13-fold after process optimisation. The enzyme was used in the biobleaching of eucalyptus kraft pulp. It was observed an improvement in the brightness as well as in the whiteness, besides a decrease in the yellowness of the material. Other parameters such as viscosity tensile strength, breaking length, burst factor and burstness also presented better properties. Moreover, the reduction in the consumption of chloride used was of 20 %. Another study, from the group of professor Hazlewood, in California, screened 200 xylanases and assessed their performance in biobleaching of softwood and hardwood from different regions in the US, Canada and South America (Esteghlalian et al. 2008). The enzyme with the best properties was a xylanase from family 11, although alkali-tolerant enzymes from the family 10 were also identified and tested. The later enzyme, however, presented less effective bleachability, leading to a minor reduction of chemicals at the same enzyme activity level when compared with the selected candidate of family 11.

Maalej-Achouri et al. (2012) reported a reduction of 40 % of chloride consumption using xylanase from *Talaromyces thermophilus* for the bleaching of kraft pulp. The synergism of xylanase and a commercial laccase resulted in a higher enzymatic activity when the sample was treated for 3 h at 50 °C. The enzyme showed optimal activity under slight basic pH (7–8). Zheng et al. (2012) obtained 50 % reduction of chloride consumption and 3.65 % increase in the brightness on cotton stalk pulp using a recombinant xylanase. The researchers also used the enzyme in the saccharification of paper sludge in combination with mixed cellulolytic enzymes and found that the efficiency of recycled material was increased by 10 %. Besides that, the recombinant enzyme presented thermal stability in the range of 30–80 °C and in the pH from 4 to 9 being, as proposed by the group, suitable for industrial applications in pulp biobleaching and bioethanol production. Other studies have demonstrated the usefulness and importance of using endo-xylanases in the biobleaching process with enhanced brightness, whiteness and significant reduction of chemicals in the process, contributing thereby to better environmental practices (Saleem et al. 2009; Kumar et al. 2009; Li et al. 2010; Ko et al. 2011; Lin et al. 2013).

6.9 Baking

Hemicellulases, especially endo-xylanases have also been used to improve the quality of dough, bread, biscuits, cakes and other bakery products (Poutanen 1997; Butt et al. 2008). Although the endo-xylanases are known to exhibit many beneficiary effects during dough handling and baking, improving the texture and shelf life of bread, their actual mechanism of action is not well understood. It has been

hypothesised that the ability of endo-xylanases to hydrolyse arabinoxylan present in dough facilitates the redistribution of water in both dough and bread, and is responsible for the observed favourable effects on dough handling, bread volume, texture and stability.

The enzyme was first introduced in the baking industry in the 1970s. They affect rheological properties of dough, as well as the organoleptics properties of the bread (Qi Si and Drost-Lustenberger 2002; Collins et al. 2006). Besides that, the addition of endo-xylanases during dough processing is expected to increase the concentration of arabino-xylo-oligosaccharides in bread, which have beneficiary effects on human health. Recently, arabinases, a-L-arabinofuranosidases, arabinoxylan a-L-arabinofuranohydrolases and esterases have been reported to play important roles in improving the texture, quality and sensory attributes of bakery products (Poutanen 1997). However, a suitable combination of these enzymes is vital for achieving maximum benefit during dough processing and baking.

The hole of endo-xylanase purified from *Trichoderma reesei* in rye baking was investigated by Autio et al. (1996), using two rye varieties, Danko (Polish cultivated in Sweden) and Muskate (Canadian). The authors found a lesser viscosity in the bread mass after adding endo-xylanase. In relation to the fermentation assay, the dough in both varieties tested increased with the use of xylanase with slight difference between the samples analysed; however, the bread volume was not increased. This fact might be related to the degradation of arabinoxylans and beta glucans, leading to the release of the gas during the process, which plays an important role in the bread volume. The enzyme also influenced the softening of the dough. Besides that, it was observed that the amount of xylan present in the rye also influences properties as the molecular weight when xylanase was used, since it affects the amount of xylooligosaccharides generated. Collins et al. (2006) studied the properties of endo-xylanase family 8 in the baking of Argentinean long breads and Belgian hard rolls. The increase in the bread loaf volume was higher when using xylanase 8 than with the compared xylanase 10. Furthermore, the cut width was also increased with xylanase 8.

6.10 Hydrolysis of Xylan into Xylooligosaccharides

The hydrolysis of xylan by endo-xylanases has already been reported during the last decades in order to elucidate the structure of such xylooligosaccharides and the enzymatic activity (Gorbacheva and Rodionova 1977; Meagher et al. 1988; Kormelink et al. 1993; Anand and Vithayathil 1996; Vardakou et al. 2005; Rantanen et al. 2007; Yang et al. 2011; Li et al. 2012, 2014).

Recently, the manufacture of xylooligosaccharides (XOS) from lignocellulosic materials as novel sweeteners and functional foods attracts growing interest. Oligosaccharides are generally defined as saccharides containing between 2 and 10 sugar moieties (Wang et al. 2009; Damen et al. 2012). As one of the non-digestible oligosaccharides (NDOs), XOS exhibit many important and interesting functional

properties, including non-toxicity, non-metabolism by human digestive system, promotion of bowel function, calcium absorption, lipid metabolism, and growth of beneficial intestinal bacteria, and reduction of colon cancer by forming short-chain fatty acids. Because xylan is stable in alkaline media, it can be obtained by dissolving in caustic liquors and then precipitating with organic solvents. Then enzymatic hydrolysis was extensively employed to degrade xylan to produce XOS, due to less undesirable byproducts and specialised equipments. During this bioconversion process, the branch structure of natural xylan requires the coordination of several hydrolases to breakdown. 1,4-b-D-xylanase xylanohydrolase hydrolyses 1,4-b-D-xylosidic bonds within the b-(1,4)-linked D-xylosyl backbone of xylan randomly and liberate b-anomeric XOS (Belkacemi and Hamoudi 2003).

Due to the fact that chemical methods are less specific and may result in removal of side groups and considering the importance of xylooligosaccharides in xylan, arabinoxylans have been investigated and its production has been optimised, in this way, resulting in better process conditions. Enzymes, specific glycosidic linkages can be split, resulting in unmodified oligosaccharides (Kormelink et al. 1993; Vardakou et al. 2005). The arabinoxylan backbone can be randomly hydrolysed using endo-xylanases (EC 3.2.1.8), producing a mixture of various oligosaccharides. Arabinose side groups are liberated by beta-L-arabinofuranosidases (EC 3.2.1.55) and exo-xylosidases (EC 3.2.1.37) release xylose units from the non-reducing end of xylo-oligosaccharides (Biely 1985). The fragments released by xylanase represent usually a complex mixture of arabinoxylooligosaccharides, as reported by Puchart and Biely (2008).

Guerfali described the synergistic action of an immobilised endo-beta-1,4-xylanase from *Talaromyces thermophilus* (Guerfali et al. 2011). The potential of the combined use of endo-xylanase and arabinofuranosidase was highlighted as a potential catalyst source and final application in food processing. Likewise, Várnai et al. (2011) studied the combined action of endo-xylanase and endo-b-mannanase, leading to a higher liberation of glucose than that theoretically expected in glucomannan in softwood.

The obtainment of xylooligosaccharides using a plethora of microorganisms as endo-xylanases source as well as its engineering in order to optimise the process and improve efficiency has been continuously explored, especially in the last few years in order to provide the needs the modern societies demand (Gonçalves et al. 2012; Verma and Satyanarayana 2012; Zhao et al. 2012; Chen et al. 2013; Kim et al. 2014; Bibi et al. 2015; McCleary et al. 2015; Yang et al. 2015).

6.11 Feedstock

The animal feed industry is an important sector of agro-business with an annual production of 600 million tons of feed, worth 50 billion US dollars. Of the total feed produced, the major share is taken by poultry, pigs and ruminants (up to 90 %), while the pet foods and fish farming account for 10 % (Bhat 2000).

Xylans are major constituents in the non-nutritional constituent of feed in monogastric animals since the animals do not naturally produce the enzymes required for the breakdown of these components. Another reason for the use of the enzyme is that, with increasing concerns associated with antibiotic resistance, there is pressure to reduce the use of antibiotics in feed. Alternatively probiotics can be added to feed as a replacement for antibiotics. Since probiotics are effective in enhancing the immune system, increasing body weight gain, reducing diarrhea and improving feed conversion efficiency. Cereals (namely barley, wheat, rye and oats) are major feed components for monogastric animals. The cell walls of cereals are primarily composed of carbohydrate complexes referred to as nonstarch polysaccharides, such as β-glucans in barley and wheat and arabinoxylans in rye and oats. Due to low digestibility of β-glucans and arabinoxylans and their propensity to form high-molecular-weight viscous aggregates in the gastrointestinal tract, these polymers exhibit in antinutritive effect. Therefore, addition of specific enzymes such as xylanase or β-glucanase into wheat- or barley-based diets for nonruminant animals decreases viscosity and consequently reduces the antinutritional effect of nonstarch polysaccharides, leading to better production performance (Liu et al. 2005). Xylanases have been extensively used to improve the performance in animal feed (Beachemin et al. 1999; Deng et al. 2006; Cai et al. 2011). In feed application, the use of the enzyme requires a set of parameters. It has to be stable in high temperature during feed preparation, but the catalytic activity is needed at the body temperature of domestic animals. The addition of xylanase in animal feed to degrade xylans converts the more complex polysaccharides into fermentable energy sources. This helps to maintain a lower pH, which helps exclude potential enteropathogens. The enzyme has a major economic importance in developing countries, where the potential of improved dairy of ruminants is of significant importance (Phakachoed et al. 2012). Improvements in animal performance due to the use of enzyme additives can be attributed mainly to improvements in ruminal fibre digestion resulting in increased digestible energy intake (Phakachoed et al. 2012).

Grounded on role of the enzyme in the animal feed, several researchers have concentrated their efforts in the investigation properties and optimisation process of xylanases in this regard. Liu et al. (2005) cloned and expressed three rumen microbial fibrolytic enzyme genes in a strain of *Lactobacillus reuteri* and investigated the probiotic characteristics of these genetically modified lactobacilli. The genes of *Neocallimastix patriciarum* xylanase, *Fibrobacter succinogenes* β-glucanase and the *Piromyces rhizinflata* cellulase were cloned in a strain of *L. reuteri* isolated from the gastrointestinal tract of broilers. The enzymes were expressed and secreted under the control of the *Lactococcus lactis lacA* promoter. According to the authors the *L. reuteri* transformed strains not only acquired the capacity to break down soluble carboxymethyl cellulose, β-glucan, or xylan but also showed high adhesion efficiency to mucin and mucus and resistance to bile salt and acid. Other studies followed this pattern and investigated in more detail the performance of xylanase in the field (Panwar et al. 2013; Pedersena et al. 2015).

6.12 Xylitol

Xylitol is the second most abundant polyol derived from xylose (Kirilin et al. 2012). Its application hits a broad spectrum in the actual scenario and more are promising to come true (Aranda-Barradas et al. 2010; Kaialy et al. 2014). The sugar alcohol is metabolised in the body by insulin-independent pathways and presents good sweetness as sucrose, however, being less caloric. For these reasons the molecule has been applied in the food industry (present in chewing gums, sweets, soft drinks and ice creams, for instance) as an artificial sweetener in the treatment of diabetes and erythrocytic glucose-6-phosphate dehydrogenase deficiency (Ping et al. 2013).

It has also been used as a starting material in organic chemistry reactions, as a potential anticancer compound, in the biomedical field, as a dental caries inhibitor and oral biofilm formation (Huang et al. 2011; Li et al. 2015a, b; Cardoso et al. 2014; Ma et al. 2014). The molecule has a strong market position, in the range of 90–340 million dollars a year, with a production of 20,000–40,000 tons per year, and therefore has been extensively studied regarding its production parameters (Granström et al. 2007; Aranda-Barradas et al. 2010; Li et al. 2015a, b).

Kirilin et al. (2012) described the aqueous phase reforming (APR) process to produce hydrogen. The work led to interesting results about the stability of hydrogen formed and the conversion of xylitol. Apart from that, the researchers also obtained higher yield and selectivities of xylitol than sorbitol towards hydrogen. Methods to improve the performance in the food industry have also been attempted as the microencapsulation of xylitol in gum arabic by complex coacervation method (Santos et al. 2015). Saleh et al. (2014) explored the obtainment of xylitol through olive stones. The researchers employed response surface methodology (RSM) to optimise the hydrolysis conditions, analysing treatment temperature and process time as key factors. Another study (Zhang et al. 2015) conducted at high temperature, using engineered *Kluyveromyces marxianus*, resulted in high yield, demonstrating the potential of thermo-tolerant organisms in the biomass conversion of xylitol, using glycerol as the best substrate in this case. The simultaneous of ethanol and xylitol production by pinch analysis was performed by Franceschin et al. (2011).

The group of professor Miyatake in Japan recently reported synthesis of xylitol microcapsules to encapsulate phase-change materials (PCM), a material capable of storing and releasing large amount of energy, thereby improving its durability while adding the advantages of smaller particles in relation to the bulk material (Makuta et al. 2015).

6.13 Juice and De-inking Processes

Together with pectinases, cellulases and other hemicellulases, xylanases is part of complex macerate enzymes, used in the extraction and clarification of juices. These enzymes improve the texture and decrease the viscosity of the juices, contributing to a more palatable end product. These enzymes participate in two steps of juice production: after crushing, to macerate the fruit pulp either to partial or complete liquefaction, which not only increases the juice yield and reduces the processing time, but also improves the extraction of valuable fruit components, and after the juice extraction, where mainly pectinases are used for its clarification, thereby lowering the viscosity of fruit juice prior to concentration and increasing the filtration rate and stability of the final product (Bhat 2000).

The application of enzymes in de-inking has been intensively studied in both laboratory and pilot scales, but the technique has not yet been commercialised (Buchert et al. 1998). The two principal approaches in using enzymes for de-inking include the hydrolysis of soy-based ink carriers by lipase and the release of ink from fibre surfaces by cellulases, xylanases and pectinases. The main advantage of enzymatic de-inking is the avoidance of the use of alkali. De-inking, using enzymes at acidic pH, also prevents the alkaline yellowing, simplifies the de-inking process, changes the ink particle size distribution and reduces the environmental pollution. In addition, the enzymatic de-inking improves the fibre brightness, strength properties, pulp freeness and cleanliness as well as reduces fine particles in the pulp. Xylanase treatment has been reported to increase the strength properties, while cellulase treatment improved the brightness and freeness of the pulp (Prasad 1993).

6.14 Final Remarks

Endo-beta-1,4-xylanases comprise a series of enzymes obtained mostly in fungi and applied in a wide range of processes. The roles of the enzyme in the chemical, food and paper industries position this biomolecule as an important and interesting biomaterial. The complexity of its obtaining and production optimisation has gathered researchers from various fields during the last decades to collaborate and contribute to advance towards better manufacturing processes.

Efforts have been applied in order to push through advancements that may result in a finer understanding of the functionality and behaviour of the xylanases and its relation with other molecules. Back in 1988, Meagher et al. determined the subsite map of these hydrolases secreted by *A. niger* in order to get insights in the bond cleavages in oligosaccharides and much progress have been noticed since then.

Molecular biology has been used as an important technique to perform such tasks as enhancing the thermo stability of the enzyme, thereby enabling the makers of goods to obtain the best possible products with maximum efficiency and expand the range of applications to the ever growing needs and opportunities (Zhang et al. 2010;

Deesukon et al. 2011; Karaoglana et al. 2014). The advancements in molecular biology have provided tools to clone and express xylanases in heterologous hosts and produce hybrid enzymes, thereby enhancing the quality and increasing the yield of the product by tuning specific features, key factors in its production.

In short, the function of endo-xylanase in the production of paper, chemicals and in the food industry has proved to be one of the central points in such fields and will indeed continue to expand, fostering technological advancements and benefiting us the best products through the most efficient processes.

Acknowledgments The author thanks Prof. V.K. Gupta for the invitation to write this chapter. The previous work in the field was supported by the Coordination for the Improvement of Higher Level Personnel (CAPES) and São Paulo Research Foundation (FAPESP), Brazil.

References

Anand L, Vithayathil PJ (1996) Xylan-degrading enzymes from the thermophilic fungus *Humicola Zanuginosa* (Griffon and Maublanc) Bunce: action pattern of xylanase and p-glucosidase on xylans, xylooligomers and arabinoxylooligomers. J Ferment Bioeng 81:511–517

Anasontzis GE, Zerva A, Stathopoulou PM, Haralampidis K, Diallinas G, Karagouni AD, Hatzinikolaou DG (2011) Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated bioprocessing (CBP) of lignocellulosics. J Biotechnol 152:16–23

Aranda-Barradas JS, Garibay-Orijel C, Badillo-Corona JA, Salgado-Manjarrez E (2010) A stoichiometric analysis of biological xylitol production. Biochem Eng J 50:1–9

Autio K, Harkonen H, Parkkonen T, Frigard T, Poutanen K, Siika-aho M, Aman P (1996) Effects of purified endo-beta-xylanase and endo-beta-glucanase on the structural and baking characteristics of rye doughs. LWT Food Sci Technol 29:18–27

Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270

Bakir U, Yavascaoglu S, Guvenc F, Ersayin A (2001) An endo-beta-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization. Enzyme Microb Technol 29:328–334

Ball AS, McCarthy AJ (1988) Saccharification of straw by actinomycete enzymes. J Gen Microb 134:2139–2147

Battan B, Sharma J, Dhiman SS, Kuhad RC (2007) Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. Enzyme Microb Technol 41:733–739

Beachemin KA, Rode LM, Karren D (1999) Use of enzymes in feed lot finishing diets. Can J Anim Sci 79:243–246

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microb Biotechnol 56:326–338

Belkacemi K, Hamoudi S (2003) Enzymatic hydrolysis of dissolved corn stalk hemicelluloses: reaction kinetics and modeling. J Chem Technol Biotechnol 78:802–808

Bhargav S, Panda BP, Ali M, Javed S (2008) Solid-state fermentation: an overview. Chem Biochem Eng Q 22:49–70

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Bibi Z, Shahid F, Qader SAU, Aman A (2015) Agar–agar entrapment increases the stability of endo-beta-1,4-xylanase for repeated biodegradation of xylan. Int J Biol Macromol 75:121–127

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286–290

Biely P, Vrsanska M, Gorbacheva IV (1983) The active site of an acidic endo-1,4-beta-xylanase of *Aspergillus niger*. Biochim Biophys Acta 743:155–161

Biely P, VrSanskh M, Tenkanen M, Kluepfel D (1997) Endo-beta-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151–166

Brutus A, Villard C, Durand A, Tahir T, Furniss C, Puigserver A, Juge N, Giardina T (2004) The inhibition specificity of recombinant *Penicillium funiculosum* xylanase B towards wheat proteinaceous inhibitors. Biochim Biophys Acta 1701:121–128

Buchert J, Oksanen T, Pere J, Siika-aho M, Suurnakki A, Viikari L (1998) Applications of *Trichoderma reesei* enzymes in the pulp and paper industry. In: Harman GF, Kubicek CP (eds) Trichoderma & gliocladium–enzymes, biological control and commercial applications, Taylor and Francis Ltd., London, UK, vol 2, pp 343–63

Butt MS, Tahir-Nadeem M, Ahmad Z, Sultan MT (2008) Xylanases in baking industry. Food Technol Biotechnol 46:22–31

Caffall KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344:1879–1900

Cai H, Shi P, Bai Y, Huang H, Yuan T, Yang P, Luo H, Meng K, Yao B (2011) A novel thermoacidophilic family 10 xylanase from *Penicillium pinophilum* C1. Process Biochem 46:2341–2346

Cardoso CAB, de Castilho ARF, Salomao PMA, Costa EN, Magalhaes AC, Buzalaf MAR (2014) Effect of xylitol varnishes on remineralization of artificial enamel caries lesions in vitro. J Dent 42:1495–1501

Chanwicha N, Katekaew S, Aimi T, Boonlue S (2015) Purification and characterization of alkaline xylanase from *Thermoascus aurantiacus* var. levisporus KKU-PN-I2-1 cultivated by solid-state fermentation. Mycosci 56:309–318

Chen S, Kaufman MG, Miazgowicz KL, Bagdasarian M, Walker ED (2013) Molecular characterization of a cold-active recombinant xylanase from *Flavobacterium johnsoniae* and its applicability in xylan hydrolysis. Bioresour Technol 128:145–155

Chen C-C, Luo H, Han X, Lv P, Ko T-P, Peng W, Huang C-H, Wang K (2014) Structural perspectives of an engineered beta-1,4-xylanase with enhanced thermostability. J Biotechnol 189:175–182

Collins T, Gerday C, Feller C (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microb Rev 29:3–23

Collins T, Hoyoux A, Dutron A, Georis J, Genot B, Dauvrin T, Arnaut F, Gerday C, Feller G (2006) Use of glycoside hydrolase family 8 xylanases in baking. J Cereal Sci 43:79–84

Coughlan MP, Hazlewood GP (1993) β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnol Appl Biochem 17:259–289

Damásio ARL, Silva TM, Almeida FBR, Squina FM, Ribeiro DA, Leme AFP, Segato F (2011) Heterologous expression of an *Aspergillus niveus* xylanase GH11 in *Aspergillus nidulans* and its characterization and application. Process Biochem 46:1236–1242

Damen B, Pollet A, Dornez E, Broekaert WF, van Haesendonck I, Trogh FI, Arnaut F, Delcour JA, Courtin CM (2012) Xylanase-mediated in situ production of arabinoxylan oligosaccharides with prebiotic potential in whole meal breads and breads enriched with arabinoxylan rich materials. Food Chem 131:111–118

de Groot PWJ, Basten DEJW, Sonnenberg ASM, Van Griensven L, Visser JLDJ, Schaap PJ (1998) An endo-1,4-beta-xylanase-encoding gene from *Agaricus bisporus* is regulated by compost-specific factors. J Mol Biol 277:273–284

Deesukon W, Nishimura Y, Harada N, Sakamoto T, Sukhumsirichart W (2011) Purification, characterization and gene cloning of two forms of a thermostable endo-xylanase from *Streptomyces* sp. SWU10. Process Biochem 46:2255–2262

Deng P, Li D, Cao Y, Lu W, Wang C (2006) Cloning of a gene encoding an acidophilic endo-beta-1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*. Enzyme Microb Technol 39:1096–1102

Do TT, Quyen DT, Nguyen TN, Nguyen VT (2013) Molecular characterization of a glycosyl hydrolase family 10 xylanase from *Aspergillus niger*. Protein Expr Purif 92:196–202

Esteghlalian AR, Kazaoka MM, Lowery BA, Varvak A, Hancock B, Woodward T, Turner JO, Blum DL, Weiner D, Hazlewood GP (2008) Prebleaching of softwood and hardwood pulps by a high performance xylanase belonging to a novel clade of glycosyl hydrolase family 11. Enzyme Microb Technol 42:395–403

Fenel F, Leisola M, Jänis J, Turunen O (2004) A de novo designed N-terminal disulphide bridge stabilizes the *Trichoderma reesei* endo-1,4-beta-xylanase II. J Biotechnol 108:137–143

Fenel F, Zitting A-J, Kantelinen A (2006) Increased alkali stability in *Trichoderma reesei* endo-1,4-beta-xylanase II by site directed mutagenesis. J Biotechnol 121:102–107

Filho EXF, Puls J, Coughlan MP (1993) Physicochemical and catalytic properties of a low-molecular-weight endo-l,4-beta-D-xylanase from *Myrothecium verrucaria*. Enzyme Microb Technol 15:535–540

Franceschin G, Sudiro M, Ingram T, Smirnova I, Brunner G, Bertucco A (2011) Conversion of rye straw into fuel and xylitol: a technical and economical assessment based on experimental data. Chem Eng Res Des 89:631–640

Fujimoto Z, Kuno A, Kaneko S, Kobayashi SYH, Kusakabe I, Mizuno H (2000) Crystal structure of *Streptomyces olivaceoviridis* E-86 beta-xylanase containing xylan-binding domain. J Mol Biol 300:575–585

Georis J, Giannotta F, Lamotte-Brasseur J, Devreese B, van Beeumen J, Granier B, Frere J-M (1999) Sequence, overproduction and purification of the family 11 *endo*b-1,4-xylanase encoded by the *xyl1* gene of *Streptomyces* sp. S38. Gene 237:123–133

Gomes DJ, Gomes J, Steiner W (1994) Factors influencing the induction of endo-xylanase by *Thermoascus aurantiacus*. J Biotechnol 33:87–94

Gonçalves TA, Damásio ARL, Segato F, Alvarez TM, Bragatto J, Brenelli LB, Citadini APS, Murakami MT, Ruller R, Leme AFP, Prade RA, Squina FM (2012) Functional characterization and synergic action of fungal xylanase and arabinofuranosidase for production of xylooligosaccharides. Bioresour Technol 119:293–299

Gorbacheva IV, Rodionova NA (1977) Studies on xylan-degrading enzymes II. Action pattern of endo-1,4-beta-xylanase from *aspergillus niger* str. 14 on xylan and xylooligosaccharides. Biochim Biophys Acta 484:94–102

Granström TB, Izumori K, Leisola M (2007) A rare sugar xylitol. Part II: biotechnological production and future applications of xylitol. Appl Microb Biotechnol 74:273–276

Guerfali M, Gargouri A, Belghith H (2011) Catalytic properties of *Talaromyces thermophilus*-l-arabinofuranosidase and its synergistic action with immobilized endo-beta-1,4-xylanase. J Mol Catal B Enzym 68:192–199

Haapala R, Parkkinen E, Suominen P, Linko S (1996) Production of endo-1,4-beta-glucanase and xylanase with nylon-web immobilized and free *Trichoderma reesei*. Enzyme Microb Technol 18:495–501

Harris AD, Ramalingam C (2010) Xylanases and its application in food industry: a review. J Exp Sci 1:01–11

He J, Yu B, Zhang K, Ding X, Chen D (2009) Expression of endo-1,4-beta-xylanase from *Trichoderma reesei* in *Pichia pastoris* and functional characterization of the produced enzyme. BMC Biotechnol 9:56

Heck JX, Soares LHB, Hertz PF, Ayub MAZ (2006) Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation. Biochem Eng J 32:179–184

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Hrmova M, Biely P, Vrsanska M, Petrakova E (1984) Induction of cellulose- and xylan-degrading enzyme complex in the yeast *Trichosporon cutaneum*. Arch Microb 138:371–376

Huang CF, Jiang YF, Guo GL, Hwang WS (2011) Development of a yeast strain for xylitol production without hydrolysate detoxification as part of the integration of co-product generation within the lignocellulosic ethanol process. Bioresour Technol 102:3322–3329

Hung K-S, Liu S-M, Tzou W-S, Lin F-P, Pan C-L, Fang T-Y, Sund K-H, Tang S-J (2011) Characterization of a novel GH10 thermostable, halophilic xylanase from the marine bacterium *Thermoanaerobacterium saccharolyticum* NTOU1. Process Biochem 46:1257–1263

Huy ND, LeNguyen C, Seo J-W, Kim D-H, Park S-M (2015) Putative endoglucanase PcGH5 from *Phanerochaete chrysosporium* is a b-xylosidase that cleaves xylans in synergistic action with endo-xylanase. J Biosci Bioeng 119:416–420

Hwang T, Lim HK, Song HY, Cho SJ, Chang J-S, Park N-J (2010) Cloning and characterization of a xylanase, KRICT PX1 from the strain *Paenibacillus* sp. HPL-001. Biotechnol Adv 28:594–601

Irfan M, Nadeem M, Syed Q (2014) One-factor-at-a-time (OFAT) optimization of xylanase production from *Trichoderma viride*-IR05 in solid-state fermentation. J Radiat Res Appl Sci 7:317–326

Jacobsen SE, Wyman CE (2000) Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Appl Biochem Biotechnol 84–86:81–96

Jänis J, Pulkkinen P, Rouvinen J, Vainiotalo P (2007) Determination of steady-state kinetic parameters for a xylanase-catalyzed hydrolysis of neutral underivatized xylooligosaccharides by mass spectrometry. Anal Biochem 365:165–173

Jimenez L, Martinez C, Perez I, Lopez F (1997) Biobleaching procedures for pulp and agricultural residues using Phanerochaete chroysosporium and enzymes. Process Biochem 4:297–304

Jun H, Bing Y, Keying Z, Daiwen C (2009) Functional characterization of a recombinant thermostable xylanase from *Pichia pastoris*: a hybrid enzyme being suitable for xylooligosaccharides production. Biochem Eng J 48:87–92

Juturu V, Wu JC (2012) Microbial xylanases: engineering, production and industrial applications. Biotechnol Adv 30:1219–1227

Kaialy W, Maniruzzaman M, Shojaee S, Nokhodchi A (2014) Antisolvent precipitation of novel xylitol-additive crystals to engineer tablets with improved pharmaceutical performance. Int J Pharm 477:282–293

Kapoor M, Nair LM, Kuhad RC (2008) Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. Biochem Eng J 38:88–97

Karaoglana M, Yildiza H, Inan M (2014) Screening of signal sequences for extracellular production of *Aspergillus niger* xylanase in *Pichia pastoris*. Biochem Eng J 92:16–21

Kim T, Jeong JC, Yoo YJ (2012) Hydrophobic interaction network analysis for thermostabilization of a mesophilic xylanase. J Biotechnol 161:49–59

Kim HM, Lee KH, Kim KH, Lee D-S, Nguyen QA, Bae H-J (2014) Efficient function and characterization of GH10 xylanase (Xyl10 g) from *Gloeophyllum trabeum* in lignocellulose degradation. J Biotechnol 172:38–45

Kirilin AV, Tokarev AV, Kustov LM, Salmi T, Mikkola J-P, Yu DM (2012) Aqueous phase reforming of xylitol and sorbitol: comparison and influence of substrate structure. Appl Catal A Gen 435–436:172–180

Knob A, Carmona EC (2008) Xylanase production by *Penicillium sclerotiorum* and its characterization. World Appl Sci J 4:277–283

Ko C-H, Tsai C-H, Tu J, Yang B-Y, Hsieh D-L, Jane W-N, Shih T-L (2011) Identification of Paenibacillus sp. 2S-6 and application of its xylanase on biobleaching. Int Biodeterior Biodegrad 65:334–339

Koch E (1886) Pharm. Z. Russland, 26, 657. Ber. them. Ges., Ref., 20, 145. 1887

Kormelink FJM, Hoffmann RA, Gruppen H, Voragen AGJ, Kamerling JP, Vliegenthart FG (1993) Characterisation by H NMR spectroscopy of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by digestion with endo-(1 + 4)-P-D-xylanase III from *Aspergillus awamori*. Carbohydr Res 249:369–382

Krengel U, Dijkstra BW (1996) Three-dimensional structure of endo-1,4-beta-xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. J Mol Biol 263:70–78

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microb Rev 23:411–456

Kumar KS, Manimaran A, Permaul K, Singh S (2009) Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. J Biosci Bioeng 107:494–498

Li XT, Jiang ZQ, Li LT, Yang SQ, Feng WY, Fan JY, Kusakabe I (2005) Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. Bioresour Technol 96:1370–1379

Li L, Tian H, Cheng Y, Jiang Z, Yang S (2006) Purification and characterization of a thermostable cellulase-free xylanase from the newly isolated *Paecilomyces themophila*. Enzyme Microb Technol 38:780–787

Li X, She Y, Sun B, Song H, Zhu Y, Lv Y, Song H (2010) Purification and characterization of a cellulase-free, thermostable xylanase from *Streptomyces rameus* L2001 and its biobleaching effect on wheat straw pulp. Biochem Eng J 52:71–78

Li X, Li E, Zhu Y, Teng C, Sun B, Song H, Yang R (2012) A typical endo-xylanase from *Streptomyces rameus* L2001 and its unique characteristics in xylooligosaccharide production. Carbohydr Res 359:30–36

Li H, Kankaanpää A, Xiong H, Hummel M, Sixtac H, Ojamo H, Turunen O (2013) Thermostabilization of extremophilic *Dictyoglomus thermophilum* GH11 xylanase by an N-terminal disulfide bridge and the effect ofionic liquid [emim]OAc on the enzymatic performance. Enzyme Microb Technol 53:414–419

Li J, Zhou P, Liu H, Xiong C, Lin J, Xiao W, Gong Y, Liu Z (2014) Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. Bioresour Technol 155:258–265

Li H, Voutilainen S, Ojamo H, Turunen O (2015a) Stability and activity of *Dictyoglomus thermophilum* GH11 xylanaseand its disulphide mutant at high pressure and temperature. Enzyme Microb Technol 70:66–71

Li Z, Guo X, Feng X, Li C (2015b) An environment friendly and efficient process for xylitol bioconversion from enzymatic corncob hydrolysate by adapted *Candida tropicalis*. Chem Eng J 263:249–256

Lin X-Q, Han S-Y, Zhang N, Hu H, Zheng S-P, Ye Y-R, Lin Y (2013) Bleach boosting effect of xylanase A from *Bacillus halodurans* C-125 in ECF bleaching of wheat straw pulp. Enzyme Microb Technol 52:91–98

Liu J-R, Yu B, Liu F-H, Cheng K-J, Zhao, X (2005) Expression of rumen microbial fibrolytic enzyme genes in probiotic Lactobacillus reuteri. Appl Environ Microbiol 71:6769–6775

Ma P, Li T, Wu W, Shi D, Duan F, Bai H, Dong W, Chen M (2014) Novel poly(xylitol sebacate)/hydroxyapatite bio-nanocomposites via one-step synthesis. Polym Degrad Stab 110:50–55

Maalej-Achouri I, Guerfali M, Romdhane IB-B, Gargouri A, Belghith H (2012) The effect of *Talaromyces thermophilus* cellulase-free xylanase and commercial laccase on lignocellulosic components during the bleaching of kraft pulp. Int Biodeterior Biodegrad 75:43–48

Maat J, Roza M, Verbakel J, Stam H, da Silra MJS, Egmond MR, Hagemans MLD, van Garcom RFM, Hessing JGM, van Derhondel C, van Rotterdam C (1992) Xylanases and their application in baking. In: Visser J, Beldman G, van Someren MAK, Voragen AGJ (eds) Xylan and xylanases. Elsevier, Amsterdam, pp 349–360

Makuta T, Kadoya K, Izumi H, Miyatake M (2015) Synthesis of cyanoacrylate-covered xylitol microcapsules for thermal storage. Chem Eng J 273:192–196

Mamo G, Hatti-Kaul R, Mattiasson B (2006) A thermostable alkaline active endo-beta-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterization. Enzyme Microb Technol 39:1492–1498

Mander P, Choi YH, Pradeep GC, Choi YS, Hong JH, Cho SS, Yoo JC (2014) Biochemical characterization of xylanase produced from *Streptomyces* sp. CS624 using an agro residue substrate. Process Biochem 49:451–456

McCleary BV, McKie VA, Draga A, Rooney E, Mangan D, Larkin J (2015) Hydrolysis of wheat flour arabinoxylan, acid-debranched wheat flour arabinoxylan and arabino-xylo-oligosaccharides by b-xylanase, a-L-arabinofuranosidase and b-xylosidase. Carbohydr Res 407:79–96

Meagher MM, Tao BY, Chow JM, Reilly PJ (1988) Kinetics and subsite mapping of A D-xylobiose- and D-XmsE producing *Aspergiflus niger* endo-(1+4)-/W-xylanase. Carbodydr Res 173:273–283

Michaux C, Pouyez J, Mayard A, Vandurm P, Housen I, Wouters J (2010) Structural insights into the acidophilic pH adaptation of a novel endo-1,4-beta-xylanase from *Scytalidium acidophilum*. Biochimie 92:1407–1415

Moukouli M, Topakas E, Christakopoulos P (2011) Cloning and optimized expression of a GH-11 xylanase from *Fusarium oxysporum* in *Pichia pastoris*. New Biotechnol 28:369–374

Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993) Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. Strain 41 M-1. Appl Environ Microb 59:2311–2316

Onysko KA (1993) Biological bleaching of chemical pulps: a review. Biotechnol Adv 11:179–198

Pandey A (2002) Solid-state fermentation. Biochem Eng J 13:81–84

Panwar D, Srivastava PK, Kapoor M (2013) Production, extraction and characterization of alkaline xylanase from *Bacillus* sp. PKD-9 with potential for poultry feed. Biocatal Agric Biotechnol 3:118–125

Pastor FIJ, Gallardo Ó,Sanz-Aparicio J, Díaz P (2007) Xylanases: molecular properties and applications. In: Polaina J, MacCabe AP (eds) Industrial enzymes. Springer, Dordrecht, The Netherlands, pp 65–82

Pedersena MB, Dalsgaarda S, Arenta S, Lorentsena R, Knudsen KEB, Yua S, Lærke HN (2015) Xylanase and protease increase solubilization of non-starch polysaccharides and nutrient release of corn- and wheat distillers dried grains with solubles. Biochem Eng J 98:99–106

Phakachoed N, Lounglawan P, Suksombat W (2012) Effects of xylanase supplementation on ruminal digestibility in fistula tednon-lactating dairy cows fed rice straw. Livest Sci 149:104–108

Ping Y, Ling H-Z, Song G, Ge J-P (2013) Xylitol production from non-detoxified corncob hemicellulose acid hydrolysate by *Candida tropicalis*. Biochem Eng J 75:86–91

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microb Biotechnol 67:577–591

Poutanen K (1997) Enzymes: an important tool in the improvement of the quality of cereal foods. Trends Food Sci Technol 8:300–306

Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13:101–131

Pradeep GC, Choi YH, Choi YS, Seong CN, Cho SS, Lee HJ, Yoo JC (2013) A novel thermostable cellulase free xylanase stable in broad range of pH from *Streptomyces* sp. CS428. Process Biochem 48:1188–1196

Prasad DY (1993) Enzymatic deinking of laser and xerographic office wastes. Appita J 46:289–292

Pribowo A, Arantes J, Saddler VN (2012) The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolyzing steam pretreated corn stover. Enzyme Microb Technol 50:195–203

Puchart V, Biely P (2008) Simultaneous production of *endo*-beta-1,4-xylanase and branched xylooligosaccharides by *Thermomyces lanuginosus*. J Biotechnol 137:34–43

Purkarthofer H, Steiner W (1995) Induction of endo-P-xylanase in the fungus *Thermomyces lanuginosus*. Enzyme Microb Technol 17:114–118

Qi Si J, Drost-Lustenberger C (2002) Enzymes for bread, pasta and noodle products. In: Whitehurst RJ, Law BA (eds) Enzymes in food technology. Sheffield Academic Press, Sheffield, pp 19–56

Qian C, Liu N, Yan X, Wang Q, Zhou Z, Wang Q (2015) Engineering a high-performance, metagenomic-derived novelxylanase with improved soluble protein yield and thermostability. Enzyme Microb Technol 70:35–41

Rantanen H, Virkki L, Tuomainen P, Kabel M, Schols H, Tenkanen M (2007) Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus endo*-1,4-beta-D-xylanase. Carbohydr Polym 68:350–359

Saake B, Clark T, Puls J (1995) Investigations on the reaction mechanism of xylanases and mannanases on sprucewood chemical pulps. Holzforschung 49:60–68

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291

Saleem M, Tabassum MR, Yasmin R, Imran M (2009) Potential of xylanase from thermophilic *Bacillus* sp. XTR-10 in biobleaching of wood kraft pulp. Int Biodeterior Biodegrad 63:1119–1124

Saleh M, Cuevas M, García JF, Sánchez S (2014) Valorization of olive stones for xylitol and ethanol production from dilute acid pretreatment via enzymatic hydrolysis and fermentation by *Pachysolen tannophilus*. Biochem Eng J 90:286–293

Santos MG, Bozza FT, Thomazini M, Favaro-Trindade CS (2015) Microencapsulation of xylitol by double emulsion followed by complex coacervation. Food Chem 171:32–39

Schaedel C, Bloechl A, Richter A, Hoch G (2010) Quantification and monosaccharide composition of hemicelluloses from different plant functional types. Plant Physiol Biochem 48:1–8

Schulze E (1891) Information regarding chemical composition of plant cell membrane. Ber Dtsch Chem Ges 24:2277–2287

Shatalov AA, Pereira H (2007) Xylanase pre-treatment of giant reed organosolv pulps: direct bleaching effect and bleach boosting. Ind Crops Prod 25:248–256

Singh S, Madlala AM, Prior BA (2003) *Thermomyces lanuginosus*: properties of strains and their hemicellulases. FEMS Microb Rev 27:3–16

Sohpal VK, Dey A, Singh A (2010) Investigate of process parameters on xylanase enzyme activity in *Melanocarpus albomyces* batch culture. In: WCE 2010, London, UK

Song L, Dumon C, Siguier B, André I, Eneyskaya E, Kulminskaya A, Bozonnet S, O'Donohue MJ (2014) Impact of an N-terminal extension on the stability and activity of theGH11 xylanase from *Thermobacillus xylanilyticus*. J Biotechnol 174:64–72

Sonia KG, Chadha BS, Saini HS (2005) Sorghum straw for xylanase hyper-production by *Thermomyces lanuginosus* (D2W3) under solid-state fermentation. Bioresour Technol 96:1561–1569

Srinivasan MD, Rele MV (1999) Microbial xylanases for paper industry. Curr Sci 77:137–142

Stephens DE, Rumbold K, Permaul K, Prior BA, Singh S (2007) Directed evolution of the thermostable xylanase from *Thermomyces lanuginosus*. J Biotechnol 127:348–354

Stephens DE, Khan FI, Singh P, Bisetty K, Singh S, Permaul K (2014) Creation of thermostable and alkaline stable xylanase variants by DNA shuffling. J Biotechnol 187:139–146

Subramaniyan S, Prema P (2000) Cellulase-free xylanases from Bacillus and other microorganisms. FEMS Microbiol Lett 183:1–7

Suurnakki A, Heijnesson A, Buchert J, Westermark U, Viikari L (1996) Effect of pulp surfaces on enzyme-aided bleaching on kraft pulps. J Pulp Pap Sci 22:591–596

Takahashi Y, Kawabata H, Murakami S (2013) Analysis of functional xylanases in xylan degradation by *Aspergillus niger* E-1 and characterization of the GH family 10 xylanase XynVII. SpringerPlus 2:447

Törrönen A, Rouvinen J (1997) Structural and functional properties of low molecular weight endo-1,4-β-xylanases. J Biotechnol 57:137–149

Trevizano LM, Ventorim RZ, de Rezende ST, Junior FPS, Guimaraes VM (2012) Thermostability improvement of *Orpinomyces* sp. xylanase by directed evolution. J Mol Catal B Enzym 81:12–18

Turunen O, Etuaho K, Fenel F, Vehmaanpera J, Wu X, Rouvinen J, Leisola M (2001) A combination of weakly stabilizing mutations with a disulfide bridge in the alpha-helix region of *Trichoderma reesei* endo-1,4-beta-xylanase II increases the thermal stability through synergism. J Biotechnol 88:37–46

van Dyk JS, Sakka M, Sakka K, Pletschke BI (2010) Characterisation of the multi-enzyme complex xylanase activity from *Bacillus licheniformis* SVD1. Enzyme Microb Technol 47:174–177

Vardakou M, Flint J, Christakopoulos P, Lewis RJ, Gilbert HJ, Murray JW (2005) A Family 10 *Thermoascus aurantiacus* xylanase utilizes arabinose decorations of xylan as significant substrate specificity determinants. J Mol Biol 352:1060–1067

Várnai A, Huikko L, Pere J, Siika-aho M, Viikari L (2011) Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour Technol 102:9096–9104

Verma D, Satyanarayana T (2012) Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Bioresour Technol 107:333–338

Vieira DS, Degrève L, Ward RJ (2009) Characterization of temperature dependent and substrate-binding cleft movements in *Bacillus circulans* family 11 xylanase: a molecular dynamics investigation. Biochim Biophys Acta 1790:1301–1306

Viikari L, Ranva M, Kantelinen A, Sandquist J, Linko M (1986) Bleaching with enzymes. In: Third international conference in biotechnology in pulp and paper industry, Stockholm, pp 67–69

Wakiyama M, Tanaka H, Yoshihara K, Hayashi S, Ohta K (2008) Purification and properties of family-10 endo-1,4-β-xylanase from *Penicillium citrinum* and structural organization of encoding gene. J Biosci Bioeng 105:367–374

Wang J, Sun BG, Cao YP, Tian Y, Wang CT (2009) Enzymatic preparation of wheat bran xylooligosaccharides and their stability during pasteurization and autoclave sterilization at low pH. Carbohydr Polym 77:816–821

Wase DAJ, Raymahasay S, Wang CW (1985) Production of/ -D-glucosidase, endo- 1,4-/ -D-glucanase and D-xylanase from straw by *Aspergillus fumigatus* IMI 255091. Enzyme Microb Technol 7:225–229

Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305–317

Xue Y, Peng J, Wang R, Song X (2009) Construction of the trifunctional enzyme associating the *Thermoanaerobacter ethanolicus* xylosidase-arabinosidase with the *Thermomyces lanuginosus* xylanase for degradation of arabinoxylan. Enzyme Microb Technol 45:22–27

Yang H, Wang K, Song X, Xu F (2011) Production of xylooligosaccharides by xylanase from *Pichia stipitis* based on xylan preparation from triploid *Populas tomentosa*. Bioresour Technol 102:7171–7176

Yang Q, Gao Y, Huang Y, Xu Q, Luo X-M, Liu J-L, Feng J-X (2015) Identification of three important amino acid residues of xylanaseAfxynA from *Aspergillus fumigatus* for enzyme activity and formation of xylobiose as the major product. Process Biochem 50:571–581

Yin Y, Chen H, Hahn MG, Mohnen D, Xu Y (2010) Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 81. Plant Physiol 153:1729–1746

Zhang S, Zhang K, Chen X, Chu X, Sun F, Dong Z (2010) Five mutations in N-terminus confer thermostability on mesophilic xylanase. Biochem Biophys Res Commun 395:200–206

Zhang J, Zhang B, Wanga D, Gao X, Hong J (2015) Improving xylitol production at elevated temperature with engineered *Kluyveromyces marxianus* through over-expressing transporters. Bioresour Technol 175:642–645

Zhao L-C, Wang Y, Lin J-F, Guo L-Q (2012) Adsorption and kinetic behavior of recombinant multifunctional xylanase in hydrolysis of pineapple stem and bagasse and their hemicellulose for Xylo-oligosaccharide production. Bioresour Technol 110:343–348

Zheng H, Liu Y, Liu X, Han Y, Wang J, Lu F (2012) Overexpression of a *Paenibacillus campinasensis* xylanase in *Bacillus megaterium* and its applications to biobleaching of cotton stalk pulp and saccharification of recycled paper sludge. Bioresour Technol 125:182–187

Zhengqiang J, Kobayash A, Ahsan MM, Lite L, Kitaoka M, Hayashi K (2001) Characterization of a thermostable family 10 endo-xylanase (XynB) from *Thermotoga maritima* that cleaves p-nitrophenyl-P-D-xyloside. J Biosci D Bioeng 92:423–428

Chapter 7
Microbial Xylanases: Sources, Types, and Their Applications

Hesham Ali El Enshasy, Subeesh Kunhi Kandiyil, Roslinda Malek and Nor Zalina Othman

Abstract Biomass conversion to an utilizable energy sources such as monomer sugars using enzymatic hydrolysis has been emerged as the current technology which promises the future energy. In nature, bioconversion process of biomass is mediated by a group of biofunctional hydrolytic enzymes. These enzymes generally work in cooperative synergetic action to facilitate enhanced effective degradation of biomass. Xylanase is one of the crucial hydrolytic enzymes involved in hydrolysis of xylan, the hemicellulose which constitutes 15–30 % of the plant biomass. This chapter discusses in detail about the enzymatic hydrolysis of xylan by the xylolytic enzyme endo-1,4-β-xylanase, its occurrence in nature and mode of action, structure and classifications, current methods for its production, purification, and characterization. In addition, the major and recent industrial applications of this enzyme were highlighted as well.

7.1 Introduction

Nowadays, the governments promote extensive researches for the development of an alternative transportation fuel from a renewable energy sources, realizing the upcoming major energy crisis due to the depletion of petroleum-derived fuels. As an initiative, the department of energy in US has started producing biofuels with a set target of 60 billion gallon per year by 2030. Europe also has the similar targets by that time to replace 25 % of petroleum-based liquid transportation fuel by biofuels (Himmel et al. 2007). However, it is a challenging target since the biofuel production using substrates such as sugar cane and corn has limited capacity to supply such a huge volumes. Apart from this, in many countries people protest against the use of food crops for biofuel production and many debates that it leads to a major food crisis. As a remedy, researchers found out that the lignocellulose

H.A. El Enshasy (✉) · S.K. Kandiyil · R. Malek · N.Z. Othman
Institute of Bioproduct Development (IBD), Faculty of Chemical Engineering (FKK), University Teknology Malaysia (UTM), 81310 Johor Bahru, Malaysia
e-mail: henshasy@ibd.utm.my

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*, Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_7

biomass which consists of 75 % polysaccharide sugars can be used as a significant feedstock for biofuel production (Lynd et al. 1991). If the attraction is more, lignocellulose biomasses are renewable energy source and exist abundant in nature and it can be derived predominantly from agricultural wastes (Gomez et al. 2008). Lignocelluloses are heteropolysaccharides that give structural rigidity to all kinds of plant. They account for approximately more than 50 % of the total biomass in the nature, synthesized (estimated 10–50 $\times$ 10^{12} tons year^{-1}) by plants through photosynthesis (Claassen et al. 1999). Lignocelluloses are composed of three major polymeric constituents such as cellulose (an unbranched linier polymers of long β-1-4 glucan between 2000 and 27,000 units), hemicellulose (a heterologous polysaccharides which are complex in nature with sugar monomeric subunits such as D-xylose, D-mannose, D-galactose, and L-arabinose) and lignin (complex phenolic structure act as linkage between cellulose and hemicellulose.

The name hemicellulose was first introduced by Schulze (1891). These heteropolysaccharides are abundant in plant cell wall and have complex structures when compared to cellulose due to the enormous number of sugar monomeric side chains (500–3000). It creates a cross-linked network between the cellulose microfibrils and lignin and plays a major role in structural integrity of plant cell walls. It also regulates the rate of expansion of primary cell wall and acts as a shield over cellulose to protect it from enzymatic hydrolysis. Hemicellulose is the second most abundant renewable biomass that is available after cellulose within the lignocellulose matrix representing about 20–35 % of total biomass (Saha 2003). Table 7.1 summarizes the approximate composition of various biomass materials. Classification of hemicellulose was made based on the types of sugar monomer units present on them. As mentioned above, hemicelluloses are composed of xylan, mannan, galactan, and arabinan polymers. Among these xylan is the most frequently occurring polymer composed mainly of β-D-xylopyranosyl residues which linked by β-1,4-glycosidic bonds (Beg et al. 2001).

In hardwoods plants, hemicellulose primarily consists of xylans and glucomannans whereas in softwood plants, it is composed of arabinoglucuronoxylans

Table 7.1 Approximate composition (as a percentage) of various biomass materials

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Bermuda grass	47.8	13.3	19.4	Li et al. (2010)
Reed canary straw	42.6	29.7	7.6	Bridgeman et al. (2008)
Corn cobs	35–39	38–42	4.5–6.6	Okeke and Obi (1994)
Rice straw	41	21.5	9.9	Lee (1997)
Bagasse	38.1	26.9	18.4	Lee (1997)
Coffee pulp	24.0	8.9	19.4	Dijkerman et al. (1997)
Coconut fiber	17.7	2.2	34	Dijkerman et al. (1997)
Rice hulls	24–29	12–14	11–13	Okeke and Obi (1994)
Corn stove	39	19.1	15.1	Lee (1997)
Wheat straw	36.6	24.8	14.5	Lee (1997)
Sawdust	45.0	15.1	25.3	Dijkerman et al. (1997)

Table 7.2 The hemicellulolytic enzymes and their substrates (Shallom and Shoham 2003)

Enzymes	Substrates
Endo-β-1,4-xylanase	β-1,4-Xylan
Exo-β-1,4-xylosidase	β-1,4-Xylooligomers
α-L-Arabinofuranosidase	α-Arabinofuranosyl, (1–3) xylooligomers, α-1,5-arabinan
Endo-α-1,5-arabinanase	α-1,5-Arabinan
α-Glucuronidase	4-*O*-methyl-a-glucuronic acid, (1–2) xylooligomers
Endo-β-1,4-mannanase	β-1,4-Mannan
Exo-β-1,4-mannosidase	β-1,4-Mannooligomers, mannobiose
β-Galactosidase	α-1,6-Galactopyranose, mannooligomers
β-Glucosidase	β-1,4-Glucopyranose, mannopyranose
Endo-galactanase	β-1,4-Galactan

(xylans), xyloglucans, glucomannans, and arabinogalactans. Xylans are the major form of hemicellulose made of D-xylopyranosyl units which are linked by β-1,4-glycosidic bonds. β-mannan-based polymers are the second major form of cellulose which are made of β-1,4-linked mannose and/or glucose residues. Galactoglucomannans which consist of α-1,6-linked galactose side chains are also grouped under these polymers. They are found abundant in hardwood hemicellulose, which comprised varying ratios of D-mannopyranose units α-(1–6)-substituted galactopyranose side chains with *O*-acetyl side chains and β-1-4-linked-D-glucopyranose units. Arabinan and arabinogalactans are also considered as hemicelluloses which are generally composed of α-1,5-linked L-arabinofuranosyl units. Table 7.2 summarizes the major enzymes involved in the breakdown of cross-linked hemicelluloses. Those groups of enzymes which are involved in hydrolysis of xylan are generally known as xylanases. The current book focuses on one of the crucial xylolytic enzymes known as endo-1-4-β-xylanase.

7.2 Enzymatic Hydrolysis of Xylan

In most terrestrial plant species, secondary cell wall thickening takes place by the deposition of xylan and they are found in-between cellulosic fiber sheets and lignin with complex binding relationship through hydrogen bonding. Xylan thus forms a think wall over cellulose and enables the protection from degradation by different cellulose degrading enzymes. Xylan constitutes 15–30 % of the plant biomass in hardwood and 7–12 % of the plant biomass in softwood. Since they are the major hemicellulose group in most of the plant species, they have been considered as one among the renewable source of energy in the form of biomass. Structure of xylan is complex in nature as they are composed of β-1,4 linked-D-xylopyranose units with α-L-arabinofuranose and 4-*O*-methyl-α-D-glucuronopyranosyl acid side chains. Figure 7.1 shows the major enzymes involved and their site of action during the enzymatic hydrolysis is xylan (Polizeli et al. 2005).

Fig. 7.1 Enzymatic hydrolysis of xylan (Polizeli et al. 2005)

Hardwood xylan (e.g., birchwood xylan) consists of more than 70 β-1,4 linked-D-xylopyranose units linked by β-1,4 glycosidic bonds and 4-*O*-methyl-α-D-glucuronopyranosyl acid side chains which are found attached at second carbon of every tenth xylopyranose units. Acetylation rate is higher in these groups and it is quite frequent at second and third carbon atom on xylopyranose ring. In softwood xylan, acetylation rate is zero but instead of an acetyl group they possess α-L-arabinofuranose linked by α-1-3 glycosidic bond at the third carbon atom on xylopyranose ring. Based on the side chains of the xylan backbone, they are classified into different groups such as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. These four groups are heterogenic in terms of their degree and nature of branching. In many plants, xylans are found partially acetylated, which protect them from complete degradation by xylolytic enzymes. This could be the reason behind the fact that the complete degradation of xylan is accomplished only by synergetic interaction of acetyl xylan esterase and endo-xylanase enzymes.

Due to the heterogeneity and complex chemical structure of polymer, the complete breakdown of xylan requires the synergetic action of several hydrolytic enzymes which are specific in their mode of actions. Generally, xylans are not attacked randomly, but the bonds selected for hydrolysis depend on several biochemical factors that determine the nature of the substrate molecules such as the chain length, the degree of branching, and the presence of substituents.

The major xylolytic enzymes involved in xylan hydrolysis are as follows:

- Endo-1,4-β-xylanase—cleaves long xylan backbone into short xylooligosaccharides.
- β-xylosidases—also attacks the β-1,4-glycosidic linkages to liberate xylose from short oligosaccharides.
- α-L-arabinofuranosidases—remove L-arabinofuranose side chains.
- α-D-glucuronidases—hydrolyze the methyl glucuronate residues.
- Acetyl xylan esterases—hydrolyze acetate groups on xylan backbone.
- Ferulic acid esterases—hydrolyze the aromatic acids groups linked on arabinofuranoside residues.

Endo-1,4-β-xylanase: (EC 3.2.1.8) cleaves the glycosidic bonds between the D-xylopyranose units on the xylan backbone and as a result, short xylooligosaccharides are formed. β-D-xylosidases: (EC 3.2.1.37) liberate xylose from short xylooligosaccharides which are formed during xylan hydrolysis by endo-1,4-β-xylanase. These enzymes are classified based on their relative affinity for the xylooligosaccharides into distinct entities such as xylobiases and exo-1,4-β-xylanases. Even though they are two different enzymes, they have been recognized as β-D-xylosidases in general, and releases β-D-xylopyranosyl residues from small xylooligosaccharides and xylobiose. Their affinity for the xylooligosaccharides is inversely proportional to its complexity. Xylobiose was found to be the best substrate for these enzyme groups. These groups of enzymes play important role in xylan hydrolysis when there is endo-1,4-β-xylanase inhibition due to the accumulation of short oligomers of β-D-xylopyranosyl units, by hydrolyzing these products and remove the cause of inhibition thereby enhancing the xylan hydrolysis (Andrade et al. 2004). The molecular weights of these enzyme groups are relatively high, between 60 and 260 kDa and their pH optima is between 4.0 and 5.0. Generally, these enzymes are thermophilic in nature and their optimum temperature varies from 40° to 80°. However, in most cases they exhibit highest activity around 60 °C (Rizzatti et al. 2001). α-L-arabinases: These enzymes remove L-arabinose side chains which are substituted at second and third carbon on β-D-xylopyranosyl ring of arabino xylan. They have been classified as exo-α-L-arabinofuranosidase (EC 3.2.1.55) and endo-1,5-α-L-arabinase (EC 3.2.1.99), based on their distinct mode of action. The first group, which is most common, degrades branched arabinans and *p*-nitrophenyl-α-L-arabinofuranosidase, whereas the second group hydrolyzes only the linear arabinans (de Vries et al. 2000). The α-D-glucuronidases (EC 3.2.1.131) can hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues found in glucuronoxylan. They are classified under glycoside hydrolase family 67 and rarely found in nature. During biodegradation of glucuronoxylan, these

enzymes are able to release glucuronic acid. However, the enzyme activity is only limited on short xylooligomers due to the partial hindrance by acetyl group present in xylan backbone (Puls et al. 1991). Acetylxylan esterase: (EC 3.1.1.6) it acts by removing the *O*-acetyl groups from second and third carbon on β-D-xylopyranosyl ring of acetyl xylan. This enzyme plays important role in xylan hydrolysis by removing *O*-acetyl side chains, since *O*-acetyl groups in acetyl xylan interfere the activity of endo-1,4-β-xylanase and β-D-xylosidases on xylan backbone. Xylan extraction from hardwoods is usually mediated by alkali treatment, resulting in acetyls hydrolysis, and probably this could be the reason of the late discovery of this enzyme (Shao and Wiegel 1995; Blum et al. 1999). Ferulic acid esterase: Ferulic acid esterases (EC 3.1.1.-) act by hydrolyzing the bond between arabinose side chain and ferulic acid group attached (Crepin et al. 2004). The cooperative functioning among the above-mentioned xylolytic enzymes enhance the complete biodegradation of xylan. It was also observed that acetylxylan esterase activity on xylan results in deacetylation of xylan backbone and these deacetylated xylans are more easily hydrolyzed by endo-xylanase. Similarly, there was a significant increase in hydrolysis efficiency when arabinoxylan was pretreated by α-arabinofuranosidase. This was due to the removal of arabinan side chains which act as hindrance to endo-xylanase activity (de Vries et al. 1999).

7.3 Occurrence of Endo-1,4-β-xylanase

Endo-1,4-β-xylanase are also known as endo-(1→4)-β-xylan 4-xylanohydrolase, endo-1,4-xylanase, xylanase, β-1,4-xylanase, endo-1,4-xylanase, endo-β-1,4-xylanase, 1,4-β-xylan xylanohydrolase, β-xylanase, β-1,4-xylan xylanohydrolase, endo-1,4-β-xylanase, β-D-xylanase, 4-β-D-xylan xylanohydrolase, endo-(1→4)-beta-xylan 4-xylanohydrolase, beta-1,4-xylanase, endo-beta-1,4-xylanase, endo-1, 4-beta-D-xylanase, 1,4-beta-xylan xylanohydrolase, beta-xylanase, beta-1,4-xylan xylanohydrolase, endo-1,4-beta-xylanase, beta-D-xylanase, and 4-beta-D-xylan xylanohydrolase. In nature, the endo-1,4-β-xylanase enzymes are widely distributed among eukaryotes and prokaryotes. The occurence of these enzymes was also reported in higher eukaryotes such as plants, protozoa, small insects, and several marine species. A Japanese pear fruit was reported producing endo-xylanase during its over-ripening period. Endo-xylanase of molecular weight 55 kDa was also isolated from wheat flour (Cleemput et al. 1997). Apart from these sources, members of higher animals such as insects and fresh water mollusc were also reported as xylanase producers (Yamura et al. 1997). However, bacteria, fungi, and actinomycetes were found to be the largest source of xylanase enzyme.

There have been several reports on microbial endo-1,4-β-xylanase since 1960. Nevertheless, most of these xylanase enzymes remained unnoticed. During 1980s first studies on xylanase-mediated bio-bleaching in pulp industry was reported and only since then the scientific world realized the great impact of xylanase enzymes in industrial applications. As mentioned above, a number of microorganisms including

bacteria and fungi have the capacity for hydrolyzing xylans by synthesizing a variety of xylolytic enzymes. Early reports reveal that many of these microbial species are plant pathogens as they play an important role in degradation and invasion of plant tissues. It also shows that xylanases can elicit defense mechanisms in plants by a collective functioning with other cellulolytic enzyme groups.

7.3.1 Fungal Endo-1,4-β-xylanase

Many of the fungal species that are pathogenic to plants produce plant cell wall polysaccharide degrading enzymes. Endo-1,4-β-xylanase is one among the major group of such enzymes and they result in partial degradation of cell wall structures to the region of penetration (Subramaniyan 2000). A hypothetical model of fungal invasion on plant tissue is shown in Fig. 7.2.

A number of fungal species have been used to produce xylanase since they are the major producer of xylanases in nature. Table 7.3 shows the major fungal species used to produce xylanases. *Phanerochaete chrysosporium*, a potent plant pathogen, reported to produce xylanase activity of 15–20 U mL^{-1} along with considerable amount of cellulose activity (Copa-Patino et al. 1993). Other study also showed that the thermophilic fungus *Thermomyces lanuginosus* has the capacity of high xylanase production up to 3576 U mL^{-1} (Singh et al. 2000). Many fungal species have been reported as xylanase producers; however, species with high xylanase activity and negligible cellulase activity are found to be very rare in nature. It was also observed that the pH optima of many of these fungal xylanases are between pH 5 and 6 even though they are stable at pH between 3 and 8. On the other hand, bacterial xylanases showed slightly higher pH optima that makes them suitable for many of the industrial applications. Thermal tolerance of the majority of fugal xylanase reported so far has been found below 50 °C and in most cases this

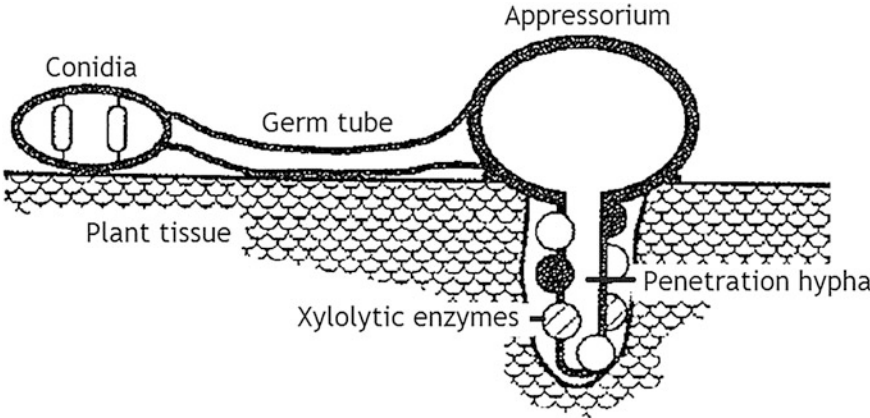


Fig. 7.2 Fungal invasion on plant tissue by xylolytic degradation (Prade 1996)

Table 7.3 Major fungal xylanase and its characteristics

Source	Molecular weight (kDa)	Optimum conditions		References
		Temperature (°C)	pH	
Aspergillus awamori	39, 23, 26	45–55	4.0–5.5	Kormelink et al. (1993)
A. fumigatus	19, 8.5	55	5.5	Silva et al. (1999)
A. kawachii	35, 26, 29	60, 55, 50	5.5, 4.5	Ito et al. (1992)
A. nidulans		55	8	Taneja et al. (2002)
A. oryzae	35	60	5	Kitamoto et al. (1999)
A. nidulans	22, 34	62, 56	5.5, 6.0	Fernández-Espinar et al. (1994)
A. sojae	32.7, 35.5	60, 50	5.0, 5.5	Kimura et al. (1998)
A. sydowii	33	50	4	Ghosh and Nanda (1994)

particular property of fungal xylanase makes them less favorable for application in harsh industrial conditions. Reduced xylanase yield in fermenter studies is another major problem associated with fungi xylanase. This was due to poor oxygen transfer, shear force during fermentation, and the enzyme production process in fungi is highly regulated by growth morphology (El Enshasy et al. 1999, 2006; El Enshasy 2007). Generally, the fungal species has filamentous growth pattern, the fungal growth in fermenters is restricted due to the shear stress, and eventually this results in poor xylanase production (Dean et al. 1991).

7.3.2 *Bacterial Endo-1,4-β-xylanase*

Since 1980s, several bacterial strains which are capable of producing alkaline and highly thermophilic xylanase enzymes have been reported. Among these wide spectrum of bacteria, *Bacillus*s species are the most predominant producers of endo-1,4-β-xylanase enzyme with negligible amount of cellulose activity under optimized growth conditions. Other author showed a high yield of xylanase up to 506 IU mL^{-1} by *Bacillus* SSP-34 (Subramaniyan and Prema 2002). In general, bacterial endo-1,4-β-xylanase is suitable for industrial application since they have a wide pH optima and improved thermostability when compared to fungal xylanases. Ratto et al. (1992) reported *Bacillus circulans* strain with xylanase activity of 400 IU mL^{-1}. They observed the optimal enzyme activity was at pH 7 and retained 40 % of activity at high pH up to 9.2. Many other researchers reported cellulose-free xylanase enzyme from *Bacillus stearothermophilus* strain T6 (Khasin et al. 1993; Lundgren et al. 1994). A relatively high xylanase activity was reported in *Bacillus* sp. strain NCL when it was grown in zeolite-induced medium (Balakrishnan et al. 2000). Thermophile bacteria such as *Rhodothermus marinus* produced approximately 1.8–4.03 IU mL^{-1} of thermostable xylanase along with

Table 7.4 Major bacterial xylanase and its characteristics

Source	Molecular weight (kDa)	Optimum conditions	
		Temperature (°C)	pH
Aeromonas caviae ME1	20	50	7
Bacillus amyloliquefaciens	18.5–19.6	80	9
B. circulans WL-12	85	–	–
Bacillus sp. *strain* SPS-0	99	75	–
Bacillus sp. W1 (JCM2888)	21.5, 49.5	65, 70	4.5–10, 4.5–7
Bacillus sp. *strain* 41-1(36)	36	50	9
Bacillus sp. *strain* TAR-1	40	75	6
Bacillus stearothermophilus T-6	43	75	6.5
Streptomyces T-7	20.6	60	4.5–5.5
Streptomyces sp. No 3137	50, 25, 25	60–65	6-May
Thermotoga maritima	–	85	6.5
T. thermarum	–	80–100	7

Modified from Subramanya and Prema 2000

detectable amount of cellulose activity. Other strain such as *Bacillus circulans* showed high yield of xylanase (400 IU mL^{-1}) which has pH optima of 7 but with a high stability at alkaline pH (Beg et al. 2000). The concept of TCF (total chlorine free) bleaching of pulp was developed during this period and it was achieved with xylanase from *Bacillus stearothermophilus* strain T6 which has optimum activity at pH 6.5 (Kohli et al. 2001). A detailed description of major bacterial xylanase and their characteristics are given in Table 7.4

7.4 Biosynthesis and Regulation of Microbial Endo-1,4-β-xylanase

Biosynthesis of xylanase enzyme by microorganisms and the phenomenon of xylanase induction are less investigated at molecular level due to the difficulty in setting up a cell-free system under experimental conditions. However, Srivastava and Srivastava (1993) introduced a hypothetical model of xylanase biosynthesis and regulation to describe similar conditions. Xylans are comparatively complex in structure with high molecular weight and thus it is usually hard to be utilized directly by microbes. Xylans are converted into smaller molecules such as xylose, xylobiose, xylotriose, or xylooligosaccharides by the constitutively produced small amount of endo-1,4-β-xylanase enzymes. These simpler molecules can be easily utilized by microbial cell as the carbon source (Zhao et al. 1997). These low molecular weight molecules can act as inducer for further xylanase expression and this has been one of the possible explanations for direct induction of xylanase gene.

Xylanases are usually secreted in the media which contains pure xylan or xylan residues in cultures of different species such as *Aspergillus awamori*, *Trametestrogii*,

and *Streptomyces* sp. QG-11-3 (Beg et al. 2001). However, several exceptional cases have also been reported such as in *Cellulomonas flavigena*; xylan acts as a poor inducer of xylanase gene (Avalos et al. 1996). In some rare cases, for example, in yeast strain *Trichosporon cutaneum*, xylanase biosynthesis is induced by certain positional isomers. In many bacterial species, the xylanase induction is also possible with various sugars such as D-xylose, D-maltose, D-glucose, and D-arabinose. On the other hand, in many fungal species, the natural lignocelluloses such as corn cobs, rice straw, sugarcane bagasse, and wheat bran were found to be capable of triggering the xylanase induction (Gupta et al. 2000). Other study conducted by Kumar and Satyanarayana (2011) reported on the wheat bran-mediated xylanase induction in *Bacillus halodurans* TSEV1 strain. However, the hypothetical mechanism of direct induction is questionable when the transportation across the cell wall can be blocked by larger molecules. Based on the study of Gomes et al. (1994), there has been a universally supported concept based on intracellular β-xylosidases. They explains that the larger xylooligomers formed during xylan hydrolysis are directly transported into the cell matrix and it was further degraded into xylose residues by β-xylosidase which are present in the cytoplasm.

Hydrolysis of xylooligomers by hydrolytic transporter proteins during their transportation through cell membrane is another possible explanation for above phenomenon. There are also rare cases in which the high xylose concentration in the media inhibits the xylanase gene expression (Strauss et al. 1995). Exclusion of inducer transport across the cell membrane is another possible reason for poor gene expression. In similar cases such as in *E. coli* it has been observed that the presence of glucose in the media prevents the lactose transportation through the cell membrane which is inducer for *lac* operon (Borralho et al. 2002). It was also observed that the xylanase biosynthesis is mediated by complex metabolic pathway in which the inducer level and the level of repressor molecule to that particular inducer vary with the organism and their growth medium. For example, the xylanase production by *Streptomycetes* sp. was increased when cellulose substrates are used in the growth media, whereas in *Cellulomonas favigena*, sugarcane bagasse was found to be the best inducer of xylanase enzyme (Alejandro et al. 2007). Addition of xylose in fermentation media increased xylanase production to a significant amount in *Bacillus* sp. (Gupta and Kar 2009). However, other studies showed that the presence of readily utilizable sugars such as glucose, xylose, and galactose can suppress xylanase biosynthesis in other strains such as in *Streptomyces* sp. (Bajaj and Singh 2010) and in *T. reesei* (Mach-Aigner et al. 2010).

7.5 Classification and Structures of Xylanase

Xylanases are considered as one of the major hydrolyzing enzyme groups as they mediate the xylan degradation to simpler utilizable units. Activity of these enzymes depends on the substrate specificity as well as substrate complexity. Wong et al. (1988) classified xylanases into two major groups based on the end product of

hydrolysis reaction. They were debranching enzymes which liberate arabinose and non-debranching enzymes which do not liberate arabinose residues from α,1,3,L-arabinofuranosyl. A few years later, another classification system was introduced based on the physiological properties of xylanase enzymes such as isoelectric point (PI) and molecular weight (MW). Therefore, xylanases were classified into two major groups: end xylanase enzymes with molecular weight lower than 30 kDa and basic pH and those with molecular weight higher than 30 kDa and acidic pH. However, few years later, this classification system was found incorrect since it matches for only a few xylanase enzymes.

Henrissat and Bairoch (1993) proposed a broad classification system based on the similarities in amino acid sequence and catalytic module. The glycoside hydrolases are further classified into different families. This classification system has got wide acceptance since it gives information regarding the structural properties, the catalytic mechanisms of the enzyme, and their evolutionary relationship within the group. Xylanases were grouped under glycoside hydrolases (GH) 5, 8, 10, and 11 families. However, the enzymes with multi-domain that exhibit detectable amount of xylanase activity are grouped into GH7, 16, 43, and 62 families (Cantarel et al. 2009). Table 7.5 comprises major xylolytic enzymes, their classification into CE, GH families, and their current crystallographic structure. Some of these GH families are further classified into super families that represent a more distant common evolutionary ancestor. Major GH family xylanase and their general structures are shown in Fig. 7.3 (Table 7.6).

Table 7.5 Xylolytic enzymes, their classification into CE, GH families, and the their current crystallographic structure status

Enzyme	Enzyme family		Structure status
	EC	GH	
Endo-β-1,4-xylanase	3.2.1.8	5, 8, 10, 11 and 43	GH5-Cryst. (1BQC) GH8-Cryst. (1IS9) GH10-1FH7, 1CLX, 1XYZ GH11-1H4G, 1BCX, 1IGO GH43-(1GYD)
Exo-β-1,4-xylosidase/β-xylosidases	3.2.1.37	3, 39, 43, 52 and 54	GH3-Cryst. (1EX1) GH39-Cryst. GH43-(1GYD)
α-L-Arabinofuranosidase	3.2.1.55	3, 43 and 51	GH3-(1EX1) GH43-(1GYD) GH51-Cryst
Endo-α-1,5-arabinanase	3.2.1.99	43	1GYD
α-Glucuronidase	3.2.1.139	67	1K9D, 1GQI
Acetyl xylan esterase	3.1.1.72	1	(1JJF)
		5	1QOZ, 1G66
Ferulic acid esterases	3.1.1.73	1	1JJF, 1GKK

Source Shallom and Shoham 2003

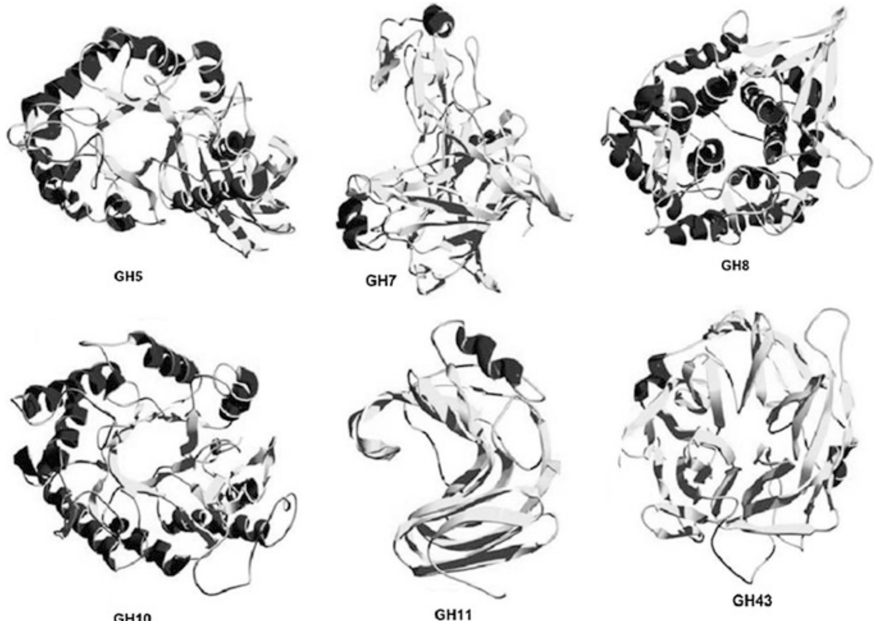


Fig. 7.3 Structures of xylanase major GH family xylanase (Collins et al. 2005a, b)

Table 7.6 CBMs of known structure linked to xylanases (Berrin and Juge 2008)

CBMs family	Source of xylanase	GH family	PDB code
CBM-2	*Cellulomonas fimi*	10A, 11A	1EXG, 2XBD, 1HEH
CBM-4	*Rhodothermus marinus*	10A	1K45
CBM-6	*Clostridium thermocellum*	11A	1UXX, 1NAE, 1UY4
CBM-9	*Thermotoga maritima*	10A	1I8A
CBM-10	*Cellvibrio japonicus*	10A	1QLD
CBM-13	*Streptomyces olivaceovidris*	10A	1XYF
CBM-15	*Cellvibrio japonicus*	10C	1GNY
CBM-22	*Clostridium thermocellum*	10B	1DYO
CBM-36	*Paenibacillus polymyxa*	43A	1UX7

7.5.1 GH10 Xylanases

Most of the glycoside hydrolases which are classified under GH10 are endo-β-1,4-xylanases. Apart from these enzyme, G10 group also consists of a small number of endo-β-1,3-xylanases (EC 3.2.1.32), which cleave β-1,3-glycosidic linkages randomly in β-1,3-xylans backbone. A typical GH10 xylanases has a wide spectrum of substrate specificity as they are able to hydrolyze various forms of xylans in nature. These enzymes are capable to attack not only the linear forms of xylan but also the highly branched heteroxylans and xylosomes. Kolenova et al. (2006)

analyzed the hydrolysis product from glucuronoxylan and confirmed that GH10 xylanases can attack xylan at its non-reducing end if there are two or more unbranched xylose residues which are present on backbone. It was also found that GH10 xylanases can even hydrolyse the highly branched arabinoxylan into small fragments. Plant xylanases which have been identified so far are grouped under GH10 family. In recent years many studies were carried out to characterize these plant xylanases and it was observed that these enzymes have limited substrate specificity (Chithra and Muralikrishna 2008). The study of Van Campenhout et al. (2007) was focused on the characterization of GH10 xylanase isolated from barley and they reported that the enzyme can release small xylose units from various substrates. GH10 xylanases, in addition to their xylolytic activity, are also active against glucose-derived substrates like aryl-cello-oligosaccharides (Charnock et al. 1997; Andrews et al. 2000).

Three-dimensional structure of xylan was first described by Atkins (1992). Subsequently, more studies were carried out using NMR spectrometry and X-ray crystallography techniques to obtain more carbohydrate structure details. It was found that under crystallized condition, the xylan backbone exhibits a threefold left-handed confirmation and the geometry showed that there is no effect on β-1,4 glycosidic bonds by the side chains of those attached. Electron diffraction pattern study confirmed the structure and the hexagonal morphology. The hydrogen at the fifth carbon atom on xylose ring has binding properties either intra- or interchain. The study also suggested that the structural confirmation of D-xylose ring indicated the di-equatorial binding property of both 1–4 and 1–3 glycosidic linkages.

The X-ray crystallographic image of endo-β-1,4-xylanases enzyme isolated from *Thermoascus aurantiacus* has been used to explain the general structure of GH family 10 xylanase (Fig. 7.4). It was found that the 32.5 kDa long polypeptide chain with a β/α-fold TIM barrel structure consists of eight major β strands which are arranged side by side and parallel, forming a cylinder in the center followed by eight major α helixes. In addition, around six short helixes are also found attached with the polypeptide chain. The catalytic domains of family 10 xylanases are found in cylindrical shape and the overall side view resembles a 'Salad bowl' (Fig. 7.5). In this configuration, the catalytic sites are seen relatively closer to the carboxyl terminus end of xylan backbone. Since the molecular weight of these xylanase is higher, they always exhibit low degree of oligosaccharide polymerization. Both the disulfide bond and salt bridges present on the xylan structure improve its thermostability. In the overall structure, the top phase of the molecule which is β-barrel side has larger than the bottom face, the α-β turns. This was due to the elaborate architecture of β–α loops (Natesh et al. 1999).

7.5.2 GH11 Xylanases

GH11 xylanase unlike the other xylanase families, they only consist of endo-β-1,4-xylanases that are exclusive enzymes cleaves β-1,4-xylosidic bonds between xylose monomers. These enzymes are often sub-classified based on their

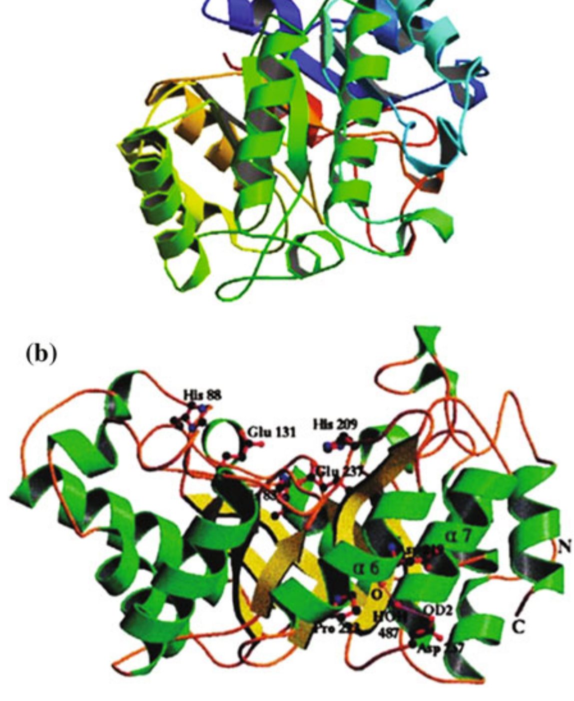


Fig. 7.4 **a** A front view showing the (α/β) 8 TIM barrel fold, **b** salad bowl view exposing the substrate binding cleft of endo-1,4-β-xylanase from *T. aurantiacus* (Natesh et al. 1999)

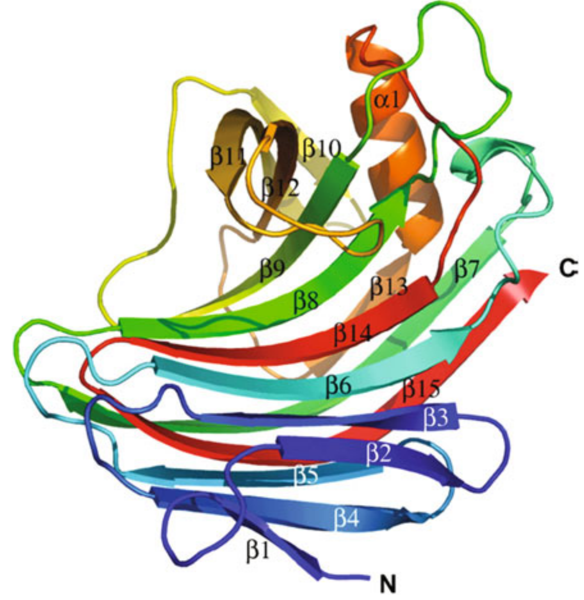


Fig. 7.5 Three-dimensional structure of NpXyn11A. The protein schematic is color ramped from the N terminus (*blue*) to the C terminus (*red*) (Vardakou et al. 2005)

PI values into acidic and alkaline xylanases (Joshi et al. 1997). Many studies demonstrated the correlation between the pH optima and the amino acid residues which are present adjacent to the catalytic site of these enzymes. In many cases, it was observed that the acidic xylanase (pH < 5) consists of asparagine, whereas in alkaline xylanase has arginine (Krengel and Dijkstra 1996; Fushinobu et al. 1998). This was also confirmed by other study which reported that arginine introduction to bacterial xylanase shifts the pH optimum to acidic range (Pokhrel et al. 2013). Heteroxylan, xylobiose, and xylotriose are the major subunits formed during GH11 xylanase-mediated xylan hydrolysis. Further hydrolytic activities on these subunits were found negligible; however, hydrolysis products such as xylotetrose, xylopentose, and xylohexose are further hydrolyzed by GH11 xylanases (Cervera-Tison et al. 2009). These enzymes cleave the xylose backbone at unsubstituted regions which are quite away from the branched xylose present at non-reducing end. It was also found that GH11 xylanases require minimum of three unsubstituted consecutive xylose residues for the primary binding and initiation of hydrolysis (Biely et al. 1997; Katapodis and Christakopoulos 2008).

Ko et al. (1992) reported the first structural description of a GH11 xylanase that was isolated from *Bacillus pumilus*. But his analysis was not very precise and he failed to deposit the 3D structure in protein database. To date, more than 100 GH11 xylanase 3D structures from different fungal and microbial species have been solved and made available in CAZy database. Xylanases isolated from a thermophilic fungi *Thermomyces lanuginosus* are widely used to explain the general structure of GH11 xylanases. The structure shows a globular protein composed of two β-sheets (β-a, β-b) with a molecular weight of 25 kDa. The outer β-sheet (β-a) is composed of five antiparallel β strands with polar and uncharged amino acids such as threonine and serine. The inner β-b sheet is made up of nine antiparallel β strands of which the outer sides are developed to catalytic sites and the inner sides are found attached with β-a forming a hydrophilic core (Torronen et al. 1994). In general, most of the GH11 xylanases are made of only a single catalytic domain and found in β-jelly roll structure. It also resembles the shape of partially closed right hand in which two β-sheets represent the 'fingers,' the loops between β-strands of β-b such as B7 and B8 represent the 'thumb,' and the twisted inner sides of β-b and α-helix represent the 'palm' of the hand (Gruber et al. 1998).

While comparing these two major GH families, the GH11 family consists only of 'true xylanases' displaying substrate specificity toward D-xylose containing xylan substrates. Their catalytic versatility is lower when compared to GH10 xylanases and the products from its action such as xylobiose and xylotriose usually required further hydrolysis by other xylanase enzymes. The GH10 xylanases can mediate hydrolysis until the release of xylose as end product. Another interesting feature of GH10 xylanases is their ability to tolerate glucose-derived to a certain extend in addition to their xylanolytic activity that exhibits in addition to their xylanolytic activity (Collins et al. 2005a). It is generally accepted that GH10 can cleave decorated regions on AX backbone and its activity is less hampered by α-L-arabinofuranosyl and acetyl or 4-*O*-methyl-D-glucuronate side chains present on xylan

backbone, whereas GH11 xylanases are very specific in their action as they cleave only at the unsubstituted regions (Biely et al. 1997). This property is also reflected in the shape of their active binding sites, where GH10 xylanase has a shallow groove active site which has less affinity toward the unsubstituted regions and GH11 xylanases possess a cleft-shaped active site which has higher affinity toward unsubstituted consecutive xylose.

7.5.3 GH8 and GH5 Xylanases

In addition to the above-mentioned GH families, GH8 family xylanase is another most studied xylanase which consists of both endo- and exo-xylanases enzymes. Exo-xylanases are those enzymes which reduce xylooligosaccharides (XOS) and release xylose subunits. A very few number of endo-xylanase have been identified so far that belong to GH8 family such as xylanases isolated from *Pseudoalteromonas haloplanktis* (Collins et al. 2005a); GH8 exo-oligoxylanases was also isolated from *Bifidobacterium adolescentis* (Lagaert et al. 2007) and from *Bacillus halodurans* (Honda and Kitaoka 2004). In general, the diversity in substrate specificity of these enzymes is large in nature and rXyn8 isolated from *P. haloplanktis* is the best example which shows highest activity on mixed linkage (β-1,3- and β-1,4-bonds) homoxylan (Pollet et al. 2010). Studies on three-dimensional structure of GH8 xylanase isolated from P. *haloplanktis* xylanase show that it is a single-domain molecule with six (α/α)-fold barrel which is found similar to GH48 enzymes (De Vos et al. 2006). Glycoside hydrolases 5 (GH5) is another largest enzyme family which consists of more than 1800 entries in the CAZy database. Under this family, several enzymes with apparent activity on xylan have been reported. Endo-xylanases from *Aeromonas punctuate* (Suzuki et al. 1997) and *Meloidogyne incognita* (Mitreva-Dautova et al. 2006) are the major reported GH5 xylanases so far. However, certain enzymes in this group such as xylanase from *H. jecorina* also exhibit exo-activity. Larson et al. (2003) was the first one who reported the structure of GH5 xylanase (XynA) isolated from *E. chrysanthemi.* In recent years, many other X-ray analysis of GH5 xylanase has been reported. St John et al. (2009) reported X-ray crystallographic analysis of XynC from *B. subtilis*, which shows a multidomain protein (consist of a catalytic domain along with a C-terminal CBM) with (α/β) 8-fold barrel.

7.6 Catalytic Mechanisms of Endo-1,4-β-xylanase

Hydrolysis of glycosidic bonds is carried out mainly by two different catalytic residues that are present in the active site of the enzyme. Hydrolysis takes place at the anomeric center of the substrate molecules either by inversion or retention of the configuration and this has been depended on factors such as the structure and

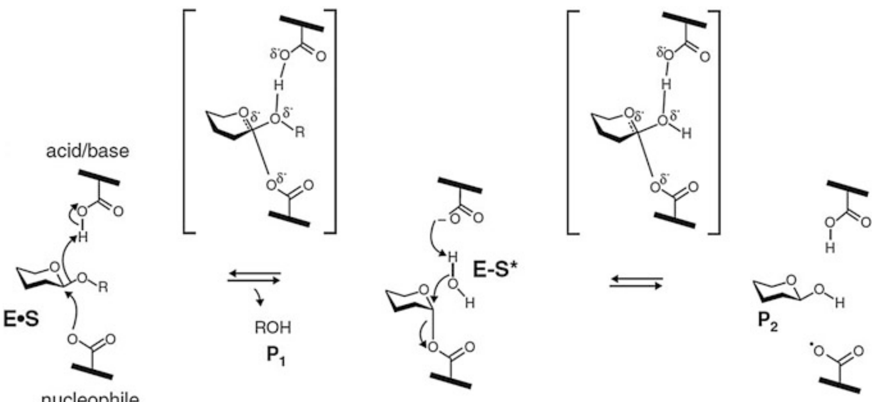


Fig. 7.6 A hypothetical model of catalytic mechanism of GH xylanase. The double-displacement reaction mechanism for retaining glycosidases (Koshland mechanism). Modified from Paës et al. (2012)

position of catalytic residues on the enzymes (Collins et al. 2005b). Many of the endo-1,4-β-xylanase exhibit a double-displacement mechanism in their operation on specific substrate molecules (Fig. 7.6). In most cases, the first acid/base catalytic residue initiate the hydrolysis by protonating the xylopyranosyl linkages between the xylan monomers and this process is termed as glycosylation. The second catalytic residue which acts as a nucleophile attacks the same linkage and results in the formation of enzyme–glycosyl intermediate while passing through an oxocarbenium-ion-like transition state. In consecutive steps which are also termed as deglycosylation, the first catalytic residue exhibits basic properties by activating the incoming water molecule and abstracting a proton from it. The activated water molecule readily attacks the anomeric carbon of the enzyme–glycosyl complex formed in the previous step and release the product with an α-configuration at the anomeric carbon (Sidhu et al. 1999; Chiku et al. 2008).

7.7 Factors Affecting Endo-1,4-β-xylanase Functionality

7.7.1 GH Family Origin

Among the above-mentioned families, endo-xylanases GH10 and GH11 are the most studied. Endo-xylanase enzymes with relatively higher molecular weight are grouped into family F/10 and they are found with cellulose-binding domain and a catalytic domain that are usually connected by linker peptide. Enzyme belonging to this family also found to have a (β/α)-fold TIM barrel structure (Biely et al. 1997), whereas the family G/11 xylanase is generally of low molecular weight and is further

classified into two major groups based on their PI value. Similarly, β-xylosidases, another major xylolytic enzyme is grouped under GH classification system into various families such as 3, 39, 43, 52, and 54. Studies showed that β-xylosidases from the families 3, 39, 52, and 54 use a retaining mechanism to hydrolyze xylooligomers, while those from family 43 mediate the hydrolysis by inverting the anomeric configuration. Advanced bioinformatics tools such as Basic Local Alignment Search (BLAST) and pair-wise alignments of the protein sequences have been used to identify the xylanase enzymes and compared with closely related enzymes within the families. The sequences of family 10 xylanase are generally used to identify mutually exclusive enzymes using BLAST search. Further confirmations are also made using X-ray crystallographic studies. A continuous update to all these GH family classifications is provided by the carbohydrate-active enzyme database (CAZy) since there is a direct relationship between the protein folding and the sequence. This system is universally accepted as it can provide structural features of enzymes that help to predict their sole substrate specificity; it helps to identify and reveal the evolutionary relationships between the enzyme groups, and also act as a convenient tool to derive mechanistic information.

7.7.2 Carbohydrate-Binding Modules (CBMs)

The X-ray crystallographic studies on the xylanase structure reveal that most of them have a modular structure composed of a catalytic site and one or more carbohydrate-binding modules (CBMs) that are interconnected by flexible linkages (Kulkarni et al. 1999; Subramaniyan and Prema 2002; Collins et al. 2005b). A CBM is defined as a specially arranged amino acid sequence with high affinity to carbohydrate molecules within a carbohydrate-active hydrolytic enzyme. These unique sites are designed to bind with specific polysaccharides on plant tissue and mediate the structural damage by enzymatic hydrolysis (Bolam et al. 2001; Boraston et al. 2002). Similar to the catalytic modules of glycoside hydrolases, CBMs are classified into 64 families according to their amino acid sequence similarity and this classification is available in CAZy database (Tomme et al. 1994). A study of Boraston and his group reported a detailed overview about the structure and functions of CBMs (Boraston et al. 2000). However, a very few members of GH11 xylanases carry CMB. Among these only a few of them have been experimentally demonstrated, but others are hypothetically explained based on their sequential similarities with well-studied ones. It have been also reported that certain CBMs from Xyl-11 are specific for cellulose and this characteristic feature helps the xylanase to localize the substrates indirectly even when they are in close association with cellulose. Major CBMs of known structure which are linked to xylanases are presented in Table 7.7. Xylanases isolated from fungi such as *Penicillium funiculosum* XynB (Brutus et al. 2004) and *Neocallimastix patriciarum* XynS20 (Liu et al. 2008) are found to have CMB1 modules. It was also reported that the

Table 7.7 Sensitivity of GH11 xylanases to proteinaceous inhibitors (XIP-1, TAXI-1 and TLXI) (Gebruers et al. 2008)

Xylanase source	XIP-1	TAXI-1	TLXI
Fungi			
Penicillium purpurogenum	−	+	−
Fusarium graminearum	+	−	−
P. funiculosum	+	+	+
P. funiculosum	+	+	+
Trichoderma longibrachiatum	+	+	−
T. viride	+	+	+
Bacteria			
Bacillus agaradhaerens	−	−	−
B. subtilis	−	+	−
Fibrobacter succinogenes	−	−	−

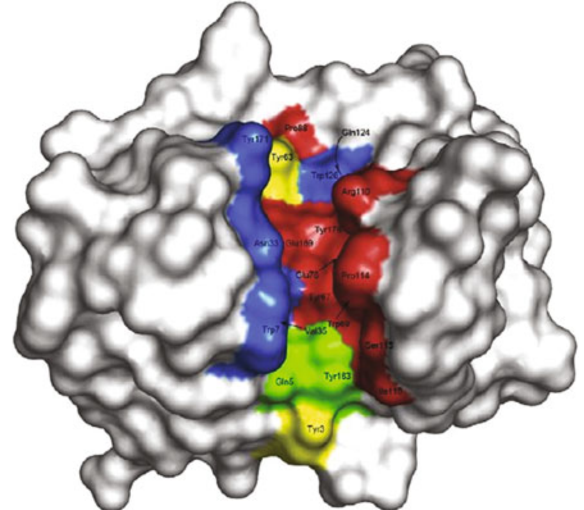


Fig. 7.7 Spatial conservation of residues paving the catalytic cleft of Xyl-11 (Paës et al. 2012)

occurrence of a C-terminal CBM1 in Xyl-11 isolated from *Podospora anserina* found to have significantly improved ability to reduce the sugar during the hydrolysis of wheat straw (Couturier et al. 2010). Figure 7.7 shows the three-dimensional structure of NpXyn11A which belongs to GH11. Some GH11 xylanases, apart from the CBMs, also consist of dockerin domains that are key elements which help in the formation of cellulose-binding multi-enzyme complex known as cellulosomes. These enzyme complexes are originally observed in *Clostridium thermocellum* (Lamed et al. 1983). In addition to cellulolytic enzymes, cellulosomes are also found with hemicellulase such as xylosidases, xylanases, and arabinofuranosidases. Other research showed also the similar results in XynC isolated from *Fibrobacter succinogenes* S85 (Marrone et al. 2000).

7.7.3 Xylanase Enzyme Inhibitors

Almost all the plant species have developed a self-defense mechanism as response to the pathogenic microbial attack by hydrolases enzymes. Xylanase inhibition by the proteinaceous inhibitors is one among those defense response widely seen in both soft- and hardwood plants. A numerous studies have been carried out to understand this defense mechanism since 1990s and it was found that *Triticum aestivum* xylanase inhibitor (TAXI) and xylanase inhibitor protein (XIP) are the two major groups of proteins responsible for Xyl-11 in specific (Debyser et al. 1999; Rouau et al. 2006). Apart from the above two groups, the third group of inhibitor known as thaumatin-like xylanase inhibitor (TLXI) also been isolated from wheat. It was also found that none of these xylanase inhibitors are effective against plant origin xylanase enzymes (Dornez et al. 2010a, b). Occurrence of these inhibitors seems to be a significant hindrance in xylanase-mediated industrial process such as bread making and brewing as they inhibit effective hydrolysis of substrates (Sorensen and Sibbesen 2006). Therefore, it has been an important topic of research to understand the mode of action of these inhibitors. Sensitivity of GH11 xylanases to proteinaceous inhibitors (XIP-1, TAXI-1, and TLXI) are as given in Table 7.7 (Fig. 7.8).

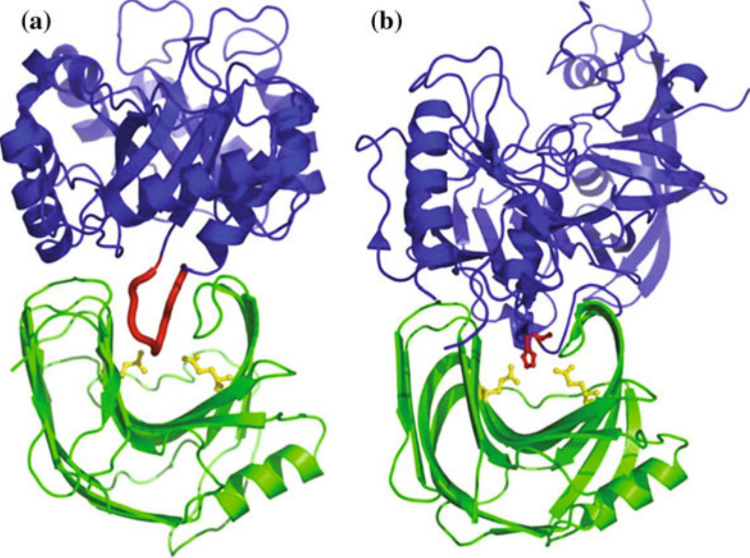


Fig. 7.8 **a** Structure of the inhibition complex between *P. funiculosum* Xyl-11 (in *green*) and XIP-I inhibitor (in *blue*). XIP-I inhibiting loop is in *red*, catalytic residues are in *yellow*. **b** Structure of the inhibition complex between *B. subtilis* Xyl 11 (in *green*) and TAXI-IA inhibitor (in *blue*). His374 residue is in *red*, catalytic residues are in *yellow* (Paës et al. 2012)

7.7.3.1 *Triticum Aestivum* Endo-xylanase Inhibitor (TAXI)

These are proteins with pI values of ≥ 8.0, which occur in two different molecular forms known as A and B (Gebruers et al. 2008). Form A is seen as full-length proteins with molecular mass near to 40 kDa, whereas the form B consists of two polypeptide chains (a longer chain and a shorter chain of size 28–30 and 10–12 kDa, respectively) that bound together by disulfide bonds (Croes et al. 2009). These inhibitors are seen largely in wheat grains during the early stages of grain development and specifically inhibit GH11 xylanase disrespect to their origin (Tables 7.8, 7.9). Further analysis of originally identified TAXI inhibitor proteins revealed that it is a mixture of two different proteins of different pI values. They are named as TAXI-I and TAXI-II and have different inhibition activities on GH11 endo-xylanase isolated from *A. niger* (Gebruers et al. 2004). Other research carried out by Raedschelders et al. (2004) identified and isolated the genes coding for these two inhibitor proteins from ray and barley grains and based on this study Fierens et al. (2004) developed a *Pichia pastoris*-based expression system for the large-scale production. By 2005, two more new TAXI class named TAXI-III and TAXI-IV were also identified (Igawa et al. 2005). TAXI-type inhibitors are found to be very specific in their inhibitory characteristics. The available research data shows that they inhibit only GH11 endo-xylanases of bacterial and fungal origin. However, the subclass TAXI-II was turned to be an exception, as they are unable to inhibit GH11 endo-xylanases such as XynBc1 and ExlA isolated from *B. cinerea* and *A. niger*, respectively (Brutus et al. 2005).

Table 7.8 Occurrence of TAXI-type inhibitors in different cereals (Raedschelders et al. 2004)

Cereal	Inhibitor proteins		
	TAXI-I	TAXI-II	Inhibitor level (ppm)
Wheat	+	+	ca. 38
Durum wheat	+	+	ca. 11
Rye	+	−	ca. 21–37
Barley	+	−	ca. 4–5
Maize, rice, oats, buckwheat	−	−	−

Table 7.9 Occurrence of XIP-type inhibitors in different cereals (Raedschelders et al. 2004)

Cereal	Inhibitor proteins	
	XIP-I	Inhibitor level (ppm)
Wheat	+	ca. 80
Durum wheat	+	ca. 14
Rye	+	ca. 95
Barley	+	ca. 3
Maize	+	ca. 2
Rice, oats, buckwheat	−	−
Sorghum	−	−

7.7.3.2 Endo-xylanase Inhibiting Proteins (XIP)

Basically, these are glycosylated single-chained proteins with molecular weight of 30 kDa and its pI value falls between 8.0 and 9.0. The crystallography study shows that it has similar structural features of GH18 with typical (α/β) 8-fold TIM barrel shape (Payan et al. 2003). However, they are found to be passive on GH18 enzymes such as chitinases due to the high degree of mismatch at its enzyme biding site. In general, these inhibitors consist of two independent enzyme-binding sites that mediate binding with almost all GH10 and GH11 endo-xylanases that are fungal origin. However, GH11 endo-xylanase from *Neocallimastix patriciarum* (Payan et al. 2004) and GH10 endo-xylanase from *Aspergillus aculeatus* (Flatman et al. 2002) are found to be insensitive to XIP. Similar to TAXI-type inhibitors, XIP-type inhibitors are also categorized into different polymorphic families based on the recent proteomics studies. Followed by identification of Xip-I (the most common inhibitor in the XIP) class, Durand et al. (2005) identified Xip-II class in *T. turgidum* ssp. In addition to this, Xip-III type inhibitor encoding gene was discovered in *T. aestivum* and was observed that these genes are transcribed very rarely under stressed leaves. Later on, several other XIP-type genes were also identified known as Xip-R genes, OsXip that are of plant origin (Takahashi-Ando et al. 2007). Biely et al. (2008) confirmed the existence of XIP-type inhibitors in maize leaves and roots during the first week after germination.

7.7.3.3 Thaumatin-like Xylanase Inhibitor (TLXI)

Thaumatin-like endo-xylanase inhibitors (TLXI) consist of a single amino acid chain with molecular weight of 18 kDa. This type of inhibitor was first discovered in wheat by Fierens et al. (2007). These proteins have high pI and usually exhibit glycosylation at varying extents that found to have a very high stability (no denaturation was observed at 100 °C at extreme pH ranges even after 120 min). These inhibitors are grouped into class 5 pathogenesis-related proteins as they show very high sequential similarity with certain antifungal proteins (Trudel et al. 1998). Even though there are no crystallographic studies that have been reported on structure of TLXI inhibitor, the structure has been predicted and shows the existence of five disulfide bridges and three β-sheets (Fierens et al. 2009). The inhibitory mechanism of these proteins was found to be specific against Xyl-11. The xylanase inhibitors were recently reviewed by Dornez et al. (2010a, b).

7.8 Recombinant Endo-1,4-β-xylanase

The advances in molecular biology and genetic engineering opened up several new applications of recombinant DNA technology. The new opportunity for the construction of GM microbial strains with selected enzyme machinery was one of the

greatest breakthroughs. On the basis of this, more efficient endo-1,4-β-xylanase-producing microorganisms were constructed to improve the biodegradation of hemicellulosic residues and thus find many industrial applications. In this section, we will highlight in detail the recent advance in recombinant xylanase production. Moreover, studies on xylanase gene regulation and biosynthesis at molecular level and various tools applied to enhance the novel characteristic of xylanase enzymes will be discussed.

Several xylanases have been isolated and identified from various sources. However, very limited numbers of these enzymes are found to be suitable for industrial applications due to their poor performance in harsh process conditions. Poor thermostability is the biggest issue associated with most of the xylanases. Enzymes with higher thermostability can enhance the efficiency of industrial process with an increased residual time. Therefore, there has been a huge demand for thermostable xylanases in pulp and paper industry as they are applied during pulp bleaching process which usually carried out at temperatures more than 90 °C. Most of the xylanases we have today are not able to withstand this high temperature. However, highly thermophilic xylanases have been reported from thermophilic bacteria such as *G. thermoleovorans* and *Thermotoga* sp. (Sharma et al. 2007). Alkali stability is also another major problem associated with industrial applications. It was observed that most of the highly thermophilic xylanases are less stable under alkaline conditions which limit their potential use in industries. Most of the xylanases reported so far have optimal activity at either acidic or neutral pH (Wang et al. 2010). Exceptional cases are also reported, such as xylanases from *Bacillus halodurans* which exhibit high alkali tolerance (Mamo et al. 2006), but these enzymes are found to have poor thermostability. Cellulose-free xylanase is another desirable characteristics required in pulp bleaching industries to mediate efficient hydrolysis without disturbing the cellulosic content of the pulp. Studies also shows that low molecular weight xylanases are more efficient in pulp bleaching as they can easily diffuse into pulp fibers. In practical and economical point of view, growing these extremophilic microorganisms in large scale is not feasible. Moreover, it is difficult to have a single enzyme with all desired characteristics. Conventional enzyme stabilization techniques such as helix capping and salt bridging (Puchkaev et al. 2003) have been practiced to improve the enzyme characteristics. However, these methods are found to be ineffective when the enzyme is required in large quantities. To overcome these problems and enhance the large-scale production for the industrial application, recombinant xylanases were introduced using gene cloning and expression techniques. rDNA technology has been widely used nowadays to make alteration or modifications in existing wild-type xylanase to improve their specific characteristics such as thermostability and alkaline stability. A number of xylanase coding genes from different microbial species have been isolated and expressed successfully in suitable expression hosts. The expression level of xylanase gene in host cell usually depends on several factors such as cloning strategies used, host cell, biosynthesis mechanism, gene regulation, fermentation media, and many bioprocessing parameters. Bacterial and fungal species are generally used to express xylanases in large scale. The cloning vector selection

is usually carried out based on the expression host and a wide variety vectors have been introduced in last few decades. Even though the expression rate is higher in recombinant strain, the enzyme activity is usually less than in the native producer strain. Several reasons have been identified for this activity loss and the intercellular accumulation of the enzyme due to the lack of post-translational modification is suggested to be the key reason (Schlacher et al. 1996).

7.8.1 Cloning and Expression of Fungal Endo-1,4-β-xylanase Gene

It has been more than three decades since filamentous fungi are used in the production of xylanase enzyme. Fungal cells are the widely used biofactories in large-scale production of xylanases and have been considered as more potent xylanase producers as they are capable of secreting much higher amounts of xylanolytic enzymes in fermentation media than bacteria or yeast (Bergquist et al. 2002; Fang et al. 2008). Large numbers of fungal strains are widely used for xylanases production such as many species from *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and *Disporotrichum.* However, in many of these fungi, the difficulty in isolating pure form of xylanase was the major problem since they generally exhibit a low amount of cellulase activity. Recombinant DNA technology has been widely applied to overcome this drawback and production of xylanase expression systems which are free of cellulase activity which improved its acceptability in industrial applications. Apart from the bacterial expression hosts, filamentous fungi are also attractive hosts for xylanase expression. It is because of their ability to secrete the proteins into the fermentation media in large quantities. *Aspergillus* and *Trichoderma* species are the widely used expression hosts in homolog expression system. Kitamoto et al. (1999) successfully overexpressed XynF1 gene in *A. oryzae* under a strong TEF1 promoter in glucose-based submerged fermentation. XynF1, XynF3, and XynG2 are another important xylanase genes expressed efficiently in *A. oryzae* under stronger promoter called P-No 8142 in similar fermentation conditions. *Aspergillus niger* is another attractive host for recombinant xylanases production due to its ability to produce and secrete protein in high capacity up to 30 g L^{-1} in the fermentation media (Hessing et al. 1994). Xylanase coding gene from *T. reesei* was successfully expressed in *A. niger* cD15 stain under G6P-dehydrogenase promoter and glaA terminator which is isolated from *Aspergillus awamori* (Rose and van Zyl 2002). This recombinant xylanase retained its 75 % activity even after 3 h of incubation at 50 °C. The optimal condition of the enzyme activity was observed at temperature between 50 and 60 °C and pH 5–6. Levasseur et al. (2005) also reported on the potential use of *Aspergillus niger* as host to express xynB under promoter sequence isolated from *Aspergillus nidulans*. They showed that, *A. nidulans* can be used as efficient host for xylanase expression and production. Kimura et al. (1998) expressed xynG1 in *A. nidulans* under its own promoter showed that the xylanase expression was induced in the presence of

Table 7.10 Major fungal xylanase expression system

Source of xylanase gene	Expression host	Vector	MWt (kDa)	References
Heterologous expression host				
Aspergillus niger	*P. pastoris* GS115	pHIL-D2	30	Berrin et al. (2000)
A. usamii E001	*E. coli* BL21	pET-28a (+)	20	Zhou et al. (2008a)
Thermobifida fusca NTU22	*P. pastoris* KM71H	pPICZα A	36	Cheng et al. (2005)
Cochliobolus sativus	*E. coli* SOLR	Lambda ZAP	25	Emami and Hack (2001)
A. niger BCC14405	*P. pastoris* KM71	pPICZαA	21	Ruanglek et al. (2007)
Aureobasidium pullulans	*E. coli*	pYES2	–	Ohta et al. (2001)
Penicillium sp. 40	*E. coli* DH5α	pUC119	20.7	Kimura et al. (2000)
Trichoderma reesei Rut C-30	*E. coli* BL21 (DE3)	pET-28a	24	Jun et al. (2008)
T. reesei PC-3-7	*E. coli* JM109	pT7Blue-T	33.1	Ogasawara et al. (2006)
Homologs expression host				
A. oryzae KBN 616	*Aspergillus oryzae* KBN616	pNAN8142	32	Kimura et al. (2000)
A. niger BRFM281	*Aspergillus niger* D15#26	pAN52.3	23	Levasseur et al. (2005)
Acrophialophora nainiana	*Trichoderma reesei* Rut C-30	pHEN11 exp	19	Salles et al. (2007)
Orpinomyces sp. PC-2	*Hypocrea jecorina*	pT3C	28	Li et al. (2007)

glucose in the media. Filamentous fungi enable extracellular secretion of heterologous protein which facilitates the downstream processes and ease the purification and characterization of the produced enzyme. Moreover, most genes from fungi have introns and filamentous fungi are able to recognize them and express with minimal miscoding. In addition, fungal proteins are glycosylated and the protein expression in another filamentous fungi results in similar glycosylation pattern. For expressing highly hemophilic xylanase, many researches have been selected filamentous fungi because of above-mentioned advantages when compared to other expression hosts like bacteria. Table 7.10 shows major fungal xylanase expression system.

Yeast-based heterologous gene expression is considered as an excellent alternative system when based on their ease scaling up compared to fungi. The presence of eukaryotic post-translational modification mechanism is one of the main reasons behind the wide application of yeast expression systems in industry (Cheng et al. 2005). *S. cerevisiae* is one of the important hosts for xylanase production by cloning of xylanase cDNA obtained by RT-PCR through crossing over. The presence of intron sequence (lake of proper splicing of introns) that hinders the xylanase gene expression is the major disadvantage of these expression systems (Moreau et al.

1992). It has been also observed that a significant increase in xylanase expression in yeast transformed with the xynA gene without intron (26.2 U mL^{-1}) when compared to the expression from the gene with intron (16.7 U mL^{-1}) (Li and Ljungdahl 1996). It is also worthy to note that Chavez et al. (2004) made the first successful attempt to clone and express the xynA gene which is isolated from *P. purpurogenum* in *S. cerevisiae*. Isolated xynA gene that consists of eight introns regions was spliced correctly and was integrated on yeast chromosome. The Xln A gene was then expressed under transcriptional control (XlnR and CreA) where xylose or xylan acts as inducer and glucose acts as repressor (van Peij et al. 1997). The methanotropic yeast *Pichia pastoris* is another widely used xylanase expression host which has many advantages over *S. cerevisiae* such as improved secretion efficiency, ease to attain high cell density culture in inexpensive media, and relative ease in scaled up fermentation process (Cereghino and Cregg 2000). Moreover, the presence of strong and regulatory alcohol oxidase (*AOX I* and *AOX 11*) promoters which are involved in methanol metabolism promotes the overexpression of xylanase gene upon the addition of methanol in the fermentation media (Tsai and Huang 2008). Table 7.11 summarizes some examples of successful cloning of fungal xylanase in yeast.

7.8.2 Cloning and Expression of Bacterial Endo-1,4-β-xylanase Gene

La Grange et al. (2000) successfully co-expressed xylanase genes (xln B from *Bacillus pumilus* along with xyn2 isolated from *T. reesei*) in yeast *S. cerevisiae*. *E coli*-based expression systems are widely used to express the xylanase. Endo-1,4-β-xylanase coding genes isolated from *A. usamii* were cloned in *E. coli* BL-21 with the help of PET-28a plasmid vector and expressed successfully and a maximum xylanase activity of 49.4 U mg^{-1} was reported. The gene expression was induced by IPTG and the protein was expressed with 6-X histidine tags that facilitate the purification of expressed protein (Zhou et al. 2008a). Similarly, for the xylanase gene from *B. circulans* Teri-42 when cloned in *E. coli* DH5-alpha using

Table 7.11 Cloning of different fungal xylanase genes in yeast

Xylanase source	Expression host	Vector	MWt (kDa)	References
Aspergillus niger	*P. pastoris* GS115	pPIC9K	20	Liu et al. (2006)
A. niger	*P. pastoris* GS115	pHIL-D2	30	Berrin et al. (2000)
A. niger BCC14405	*P. pastoris* KM71	pPICZαA	21	Ruanglek et al. (2007)
A. sulphureus	*P. pastoris* X33	pGAPZαA	–	Cao et al. (2007)
Aureobasidium pullulans	*P. pastoris* GS115	pPIC3.5	39	Tanaka et al. (2004)
A. pullulans	*S. cerevisiae* INVSc1	pYES2	–	Ohta et al. (2001)
Thermobifida fusca NTU22	*P. pastoris* KM71H	pPICZα A	36	Cheng et al. (2005)
T. fusca	*P. pastoris* GS115	pPIC9K	31	Sun et al. (2007)

pUC19 plasmid vector, the xylanase expression was found to be reduced. However, when the same gene is expressed in *B. subtilis* using plasmid pBA7, 14-fold increase in expression was observed. Therefore, *Bacillus* became more attractive hosts compared to *E. coli* and thus used widely in heterogeneous protein production. *Bacillus* sp. used in industrial fermentation are non-pathogenic in nature and in bacillus-based expression, the proteins are usually secreted out of the cell. Moreover, they are gram-positive bacteria and therefore, they do not produce endotoxins (lipopolysaccharides). These features could be of great advantage by reducing some purification steps in terms of breaking the cells and extraction from large number of intracellular proteins.

7.8.3 Overexpression in Escherichia coli

For the past several decades, *E. coli* has been recognized as the ideal platform for expression of recombinant proteins. Factors such as rapid growth on inexpensive media, non-pathogenicity, easy to make gene modification without effecting the cell growth, easy methods required for isolation and purification of expressed proteins made *E. coli* the most successful expression host for industrial use. In spite of these advantages, due to the lake of specific post-translational modification such as glycosylation and disulfide bond formation, many heterologous proteins were not successfully expressed in *E. coli*. Xylanase was one of those which require N-glycosylation during post-translation stage, whereas *E. coli* only can perform O-glycosylation. However, many exceptional cases such as successful expression of a glycosylated β-xylosidase gene isolated from thermophilic fungi *P. thermophile* have been reported (Teng et al. 2011). It was also observed that the endo-1,4-β-xylanase expression levels are significantly higher in gram-positive hosts such as *Lactobacillus* species and *B. subtilis* compared to *E. coli* as they can perform N-glycosylation.

In general, the recombinant xylanases expressed in *E. coli* are accumulated in the cytoplasm or in periplasmic place in the absence of a proper secretory channel on cell wall (Schlacher et al. 1996). However, in many cases, xylanase activity has also been detected outside the cell (Karlsson et al. 1997; Ebanks et al. 2000). Xylanase expression rate in *E. coli* mainly depends on the efficiency of transcription of xylanase gene which is cloned under promoter sequence. Even though there are several cloning vectors and host strains have been introduced, eukaryotic gene expression in *E. coli* is unable to carryout due to the absence of a functional promoter sequence. Basaran et al. (2001) isolated β-xylanase gene from *Pichia stipites* and successfully cloned and expressed in *E. coli*. The recombinant enzyme activity was low (4 U mg^{-1}) when compared to the activity original enzyme activity (30 U mg^{-1}) in wild stain. Although a variety of cloning vectors have been used, pET expression vector systems were reported to be the most efficient for protein expression in *E. coli*. Xylanase genes isolated from different sources are cloned under the control of a strong T7 promoter which is isolated from bacteriophage genome. This promoter

Table 7.12 Major cloning vectors used in *E.coli*-based expression system

Source of xylanase gene	Cloning vector	References
Bacteria		
AeromoA75:C93nas sp. *212*	pBR 322	Kudo et al. (1985)
Bacillus circulans	pUC 19	Yang et al. (1988)
B. polymyxa	pUC 13, pBR 322	Yang et al. (1988)
B. ruminicola	pUC 18	Whitehead and Hespell (1989)
Butyrivibrio fibrosolvens	pUC 19	Mannarelli et al. (1990)
Caldocellum saccharolyticum	λ1059, pBR 322	Luthi et al. (1990)
Cellulomonas sp.	pUC 18	Bhalerao et al. (1990)
Chainia sp.	pUC 8, λgt 10	Chauthaiwale and Deshpande (1992)
Clostridium stercorarium F9	pBR 322	Sakka et al. (1991)
C. thermocellum	pUC 8	Hu et al. (1991)
Neocallimastix patriciarum	λZAP II	Lee et al. (1993)
Fungi		
Aspergillus oryzae KBN 616	pNAN-d	Kimura et al. (2000)
A. usamii E001	pET-28a(+)	Zhou et al. (2008b)
Aureobasidium pullulans Y-2311-1	pCRII, λ ZapII	Li and Ljungdahl (1996)
Cochliobolus sativus	λ ZAP	Emami and Hack (2001)
Helminthosporium turcicum H-2	pBluescript SK(+)	Degefu et al. (2004)
Neocallimastix patriciarum	pBTac2	Xue et al. (1992)
Penicillium sp. 40	pUC119	Kimura et al. (2000)
Trichoderma reesei Rut C-30	pET-28a	Jun et al. (2008)
T. reesei PC-3-7	pT7Blue-T	Ogasawara et al. (2006)
T. reesei C30	pGEM5Z(+)	Torronen et al. (1994)

remains silent until T7 RNA polymerase is produced by the host cell genome. Table 7.12 shows the major cloning vectors used in *E. coli*-based expression system.

Ogasawara et al. (2006) amplified xynIII gene from *T. reesei* genomic DNA using a RT-PCR and was cloned and expressed in *E. coli* JM109 using pAG9-3 plasmid vector. When the xylanase gene expression was induced by IPTG, they could produce 26 mU mL^{-1} of xylanase activity in cellular extract, which was very low. However, the XynIII activity increased by about 500-fold (13.2 U mL^{-1}) compared to the activity in soluble supernatant, when the xylanase inclusion bodies were refolded in 8 mM urea solution. Zhou et al. (2008a) reported the maximum xylanase activity up to 49.4 U mg^{-1} when they cloned and expressed xynII cDNA isolated from *A. usamii* in *E. coli* BL21-Codon plus (DE3) RIL strain using pET-28a (+) expression vector. The xylanase protein expressed is attached to 6X-His-tag which ease protein purification by affinity chromatography. Jun et al. (2008) also used the same pET vector to clone and express a β-xylanase gene, xyn2, isolated from *T. reesei* in *E. coli* and reported the enhanced xylanase activity of 650 U mg^{-1}. In some cases, the heterologous cloning and expression results in the

change in original characteristics of xylanase gene. For example, a non-glycosylated XynA gene when expressed in *E. coli* exhibited significant loss in its substrate specificity when compared to the glycosylated xylanase from the wild strain *P. stipites*. The stability of the enzyme also decreased due to the change in optimum temperature rage. However, improved thermostability and pH stability have been reported when Xyn2 from *T. reesei* was expressed in *E. coli*. Table 7.13 shows the properties of major endo-β-1,4-xylanase enzyme expressed in *E. coli.*

Table 7.13 Properties of major endo-β-1,4-xylanase enzyme expressed in *E. coli*

Source of xylanase gene	MWt (kDa)	Optimum pH	Optimum temperature (°C)	References
Geobacillus thermoleovorans	45	8.5	80	Verma and Satyanarayana (2012)
Paenibacillus macerans IIPSP3	205	4.5	60	Dheeran et al. (2012)
Thermoanaerobacterium saccharolyticum NTOU1	50	6.5	63	Hung et al. (2011)
Clostridium sp. TCW1	53, 60	6	75	Lo et al. (2010)
Actinomadura sp. S14	21, 30 and 40	6	80	Sriyapai et al. (2011)
Alicyclobacillus sp. A4	42.5	7	55	Bai et al. (2010)
Anoxybacillus sp. E2	38.8	7.8	65	Wang et al. (2010)
Nesterenkonia xinjiangensis CCTCC AA001025	22	7	55	Kui et al. (2010)
Bacillus sp. JB 99	20	8	70	Shrinivas et al. (2010)
Paenibacillus sp. DG-22	20	6	60	Lee et al. (2007)
Bacillus licheniformis 77-2	40	8	75	Damiano et al. (2003)
Enterobacter sp. MTCC-5112	43	9	100	Khandeparkar and Bhosle (2006)
Geobacillus sp. MT-1	36	7	70	Wu et al. (2006)
Bacillus sp.	44	6.5, 8.5 and 10.5	50	Sapre et al. (2005)
Bacillus thermantarcticus	45	5, 6	80	Lama et al. (2004)
Bacillus thermoleovorans				
K-3d	40, 69	7	70, 80	Sunna et al. (1997)
Bacillus flavothermus LB3A	80–130	7	70	Sunna et al. (1997)
Thermotoga sp. FjSS3-B. 1	31	5.5	80	Simpson et al. (1991)

7.9 Improvement of Endo-1,4-β-xylanase Characteristics

In general, enzymes are classified based on their conserved regions on them. Xylanases are specific in their identity due to the random variations in amino acid sequence and it was found that some very specific stretches of amino acids defines the structural and functional behavior of enzyme. Existing xylanases can be improved in terms of their activities and other novel characteristics such as substrate specificity, thermostability, and pH stability using protein engineering approaches. Critical amino acid exchanges are usually practiced in order to enhance the characteristics of existing xylanase enzymes. Therefore, before amino acid sequence modification a detailed studies on the native xylanase amino acid sequences are required to fully understand the structural and functional behaviors such as the presence of catalytic domains, specificity of substrate-binding sites, occurrence of thermo-stabilizing domains, and the presence of covalent/non-covalent interactions. The significance of a particular acid or a set of amino acids for novel enzyme characteristics can be identified easily using specific amino acid inhibitors such as *N*-bromosuccinimide (tryptophan), phenylmethylsulfonyl fluoride (serine), and iodo acetate (cysteine). It was also found that cysteine plays critical role in the formation of covalent glycosyl enzyme intermediate during most bacterial xylanase activity. Many of the family 10 xylanases possess three different amino acids such as Phe4, Trp6, and Tyr 343. They were identified as crucial in protein folding and enzyme stability even under extreme conditions. Torronen and Rouvinen (1997) studied the significance of asparagines and aspartic acid on the pH optima of family 11 xylanases. The studies conducted on xylanase isolated from *B. circulans* revealed that the presence of asparagine at 52nd position of amino acid sequence increased the thermal fluctuations at unstable regions. However, when it is substituted with tyrosine, the overall thermostability of the enzyme exhibited significant increased (Joo et al. 2011). It was also reported that the catalytic activity of *B. circulans* xylanase increased when the Asn at position 35 is mutated to Asp (Li and Wang 2011). Other study showed also a significant increase in thermostability (10–15 °C) as a result of point mutation (aspartic acid to asparagine) at position 56 (Yin et al. 2010). However, in many other cases, the point mutation was reported to have negative effects such as loss in substrate-binding affinity, alkaline stability, and thermostability. Simpson et al. (1999) reported the complete loss of carbohydrate-binding module affinity toward the xylan when the arginine was mutated to glycine. In case of *B. circulans* xylanase, change in pH optima was reported when R73V and Q167M were mutated (Yang et al. 2008). Table 7.14 comprises significant achievements made in xylanase protein engineering to increase enzyme stability.

Table 7.14 Significant achievements made in xylanase protein engineering (Verma and Satyanarayana 2012)

Xylanase source	Approaches/alterations	Significant inferences	References
Bacillus sp. NG-27	Deletion of Phe4 and Trp6	Complete loss in activity, while Trp6 and Tyr343 affected folding	Bhardwaj et al. (2010)
B. circulans	Amino acid modification (N52Y)	Slight increase in half-life and Tm as compared to wild type	Joo et al. (2011)
B. circulans	Asn35Asp	Improvement in catalytic activity	Li and Wang (2011)
N. patriciarum	D57N	Enhancement in thermostability by 10–15 °C	You et al. (2010)
G. stearothermophilus	Substitution with N-terminal of *T. fusca*	Significant improvement in thermostability	Sun et al. (2007)
T. reesei	Improvement of C-terminal packaging	Half-life of the mutant xylanase was increased by 63 min	Turunen et al. (2001)
T. lanuginosus DSM5726	Introduction of disulfide bond between Cys100 and Cys154	Improvement in thermostability	Gruber et al. (1998)
B. stearothermophilus No. 236	Introduction of between Ser and Cys) 100 and (Asn to Cys) 150	Enhancement in thermostability by 5 °C	Jeong et al. (2007)
A. niger BCC144505	Substitution of arginine in place of Ser/Thr	Enhancement in the thermostability	Sriprang et al. (2006)
Thermobifida fusca	Error prone PCR to obtain a mutant having T21A, G25P, V87P, I91T, and G217L	Improved stability at alkaline pH, 4.5-fold increase in Km and 12-fold enhancement in Kcat/Km	Wang and Xia (2008)
Thermomyces lanuginosus	Error prone PCR A54T	Alkali-stable mutant	Stephens et al. (2009)

7.10 Industrial Production of Endo-1,4-β-xylanase

Although many microbial species such as bacteria, fungi, and actinomycetes have been reported as xylanase producers, a few number of strains are acceptable by the industrial society. To meet the current and forecasting market demands, xylanase production need to be increased by many folds through efficient production

Table 7.15 Major commercial xylanases produced by SmF techniques (Polizeli et al. 2005)

Commercial name	Distributors	Microorganism	Application
Allzym PT	Alltech	*Aspergillus niger*	Animal feed improvement
Bio-Feed Plus	Novo Nordisk	*Humicola insolens*	Animal feed
EcopulpX-200	Primalco	*Trichoderma reesei*	Cellulose pulp bleaching
Ecosane	Biotec	*Trichoderma reesei*	Animal feed
Grindazym GP, GV	Danisco ingredients	*A. niger*	Bird and pig feed
Irgazyme 40	Nalco-Genencor, Ciba, Geigy	*T. longibrachiatum*	Paper industry and animal feed
Solvay pentonase	Solvay Enzymes	*T. reesei*	Starch and bread making
Xylanase	Seikagaku	*Trichoderma* sp.	Carbohydrate structural studies
Xylanase GS35	Iogen	*T. reesei*	Pulp bleaching

strategies. Therefore, optimizations of production and purification processes are the key to obtain maximum xylanase yield either by solid-state fermentation (SSF) or submerged fermentation (SmF) (Narang and Satyanarayana 2001). Nowadays, the most of commercial xylanases production processes are carried out by SmF as shown in Table 7.15. Cultivation media plays important role in the production of xylanases in industrial scale. A suitable and economically viable media formulation which supports the maximum xylanase production is usually required for large-scale production. Therefore, abundantly available natural agriculture residues are widely used for xylanase production. Agricultural wastes such as corn cob, con stover, and wheat straw were successfully used as suitable substrates for fungal xylanases production (Damiano et al. 2003; Gupta et al. 2000).

7.10.1 Physical and Nutritional Parameters Affecting Endo-1,4-β-xylanase Production

In SSF, biomass and xylanase productions depend on several factors such as the type of microorganism, inoculums size, temperature, oxygen diffusion, the period of cultivation, the substrate used and its pretreatment, size, water activity, relative humidity, pH, and many other factors. On the other hand, for SmF, the temperature, pH, agitation, and dissolved O_2 are the most critical physical parameters affecting xylanase production in SmF. Most of the xylanase-producing microorganisms reported so far are either mesophilic or thermophilic (Coughlan and Hazlewood

1993). It was observed that these microbes show highest xylanase activity and optimum growth at temperature between 40 and 70 °C. However, those extremophilic such as *Thermotoga* sp., *Dictyoglomus*, and *Geobacillus* exhibit even higher optimum temperature range of up to 80 °C (Ko et al. 2011; Sharma et al. 2007; Qureshy et al. 2002). Xylanases of fungal origin usually are found less thermophilic in nature. Even though some exceptional cases have been reported, the optimum temperature for most fungal xylanases is below 55 °C. This has been one of the major drawbacks of fungal xylanase in industrial application. On the other hand, bacterial xylanases exhibit higher thermostability. The pH of the fermentation media is another major factor effecting the xylanase production as well as the enzyme stability. Fungal xylanases usually are highly alkaliphilic as they can be active at wide ranges of pH, usually between 5.0 and 10.0, whereas the bacterial xylanases have quite narrow pH spectrum (Dhillon et al. 2000). Aeration and agitation rates during fermentation are the other crucial factors in case of xylanase production process. Studies show that xylanase production by *Bacillus* sp. is enhanced at an agitation rate of 200–250 rpm and 30 % and above dissolved O_2 saturation (Anuradha et al. 2007; Sanghi et al. 2009). In cretin fungal species which exhibit mycelia growth pattern, higher agitation rate results in poor growth as well as less xylanase production due to the high sheer force. Studies made by Archana and Satyanarayana (1998) and Sharma et al. (2007) deal with the optimization of physical parameters effecting xylanase production.

In addition, carbon and nitrogen sources and their concentration in the fermentation media are found to be not only effecting the xylanase production but also the localization of enzymes. The fungal xylanase producers are able to utilize wide verity lignocellulosic-derived substrates such as corn cob, corn straw, wheat bran, rice straw, and sugarcane bagasse. For production in SSF system, these substrates are used as they are cheaper and easily available raw materials (Chauhan et al. 2007). However, in bacterial cells in SmF, simpler and readily utilizable nutrients are used. Organic nitrogen sources such as tryptone, peptone, yeast extract, and soybean meal are widely used as they enhance the xylanase production as well as biomass. Kapoor et al. (2008) observed a significant increase in xylanase production which was observed when the *Bacillus* sp. SSP-34 was provided with yeast extract in combination with peptone. In similar study, xylanase production by *Bacillus sam*-3 was increased up to 25-folds higher when the inorganic nitrogen source was replaced by soybean meal. Use of corn steep powder also significantly enhanced xylanase production by *T. reesei* (Lappalainen et al. 2000). Apart from these major nutrients, the incorporation of micronutrients such as trace elements, vitamins, and amino acids improved the xylanase production to great extend in cases of thermo-anaerobe-mediated xylanase fermentation. In cases of recombinant strains, the xylanase expression greatly depends on the gene inducers present in the fermentation media. IPTG is the most widely used chemical inducer for '*lac*'-based expression system (Mamo et al. 2007). However, in large-scale production, IPTG is not applicable due to its high cost.

In cases of recombinant xylanase, the expression of xylanase genes usually depends on several aspects such as biosynthesis mechanism, gene regulation, vector

construction, expression host used, expression media, and other growth parameters during the fermentation. For commercial applications, xylanases have to be ideally produced quickly and in large quantities. The conventional production strategies have been followed are found to be less effective to meet the growing need in the present xylanase market. Therefore, many researches are going on for developing recombinant strains for xylanase production. However, a very limited number of these recombinant strains are studied further for the scaling up of xylanase production in pilot scale. Hence, more investigations on this area are required and it is very significant to have more efficient and economically viable process to reduce the cost of xylanase production.

7.10.2 Optimization of Endo-1,4-β-xylanase Production

Media screening and medium composition optimization are usually the preliminary steps involved in bioprocess optimization which are always carried out in shake flask level to select the most suitable medium formulation. Both physical and chemical parameters effecting the cell growth as well as xylanases production are monitored and optimized at this stage with a number of experiments. Basar et al. (2010) observed that the recombinant xylanase production in *E. coli* is a growth-associated process. They observed a rapid growth of *E. coli*, reaching the stationary phase after 16 h and the maximal xylanase production up to 324.72 U mL^{-1} was obtained in defined medium. They also reported the effects of different concentrations of glucose in growth media on cell growth and xylanase production. Farliahati et al. (2009) reported the xylanase expression in recombinant *E. coli* DH5α in different media compositions (defined, semi-defined, and complex). Optimization study of pH and agitation ranges also carried out in shake flask cultivation mode. From these studies, they observed the highest xylanase production (up to 2122 U mL^{-1}) in defined media at initial pH 7 and agitation speed of 200 rpm. A suitable cultivation strategy for the production of two truncated thermostable recombinant xylanases (XynlAN and XynlANC), isolated from *Rhodothermus marinas*, was developed by Karlsson et al. (1998). They found that the fed-batch cultivations of *E. coli* strain BL21 (DE3) with a controlled exponential glucose feed led to high specific production of the recombinant proteins and addition of complex nutrients such as Tryptone Soya Broth (TSB) to the media exhibits positive impact on cell growth rate and cell productivity. Single addition of either lactose or isopropyl-thio-go-galactoside (IPTG) was used for induction. In lactose-induced cells, the production of recombinant xylanase was delayed for approximately 30 min compared to those induced with IPTG, but the specific product levels were comparable at 3 h after induction. Huang et al. (2006) cloned a xylanase gene from *B. subtilis* into *E. coli* and found that the xylanase distributions in extracellular, intracellular, and periplasmic fractions were in ratio 22.4, 28.0, and 49.6 %, respectively, with an optimal enzyme production at pH 6.0 and 50 °C. The

superiority of defined medium for xylanase production using recombinant bacteria was also reported by Farliahati et al. (2009).

Catabolic repression of the xylanases biosynthesis is found to be a common phenomenon usually in the presence of glucose in culture medium. When glucose is abundant in the grown medium, the repression of catabolic enzyme synthesis takes place due to the gene regulation. When the microbe can easily absorb the readily available glucose in the medium, they will not utilize for other complex nutrients by spending energy to metabolize complex nutrients. Xylanase production reported to be reduced due to the catabolic repression that makes the xylanase coding gene in inactive mode. In another study by Basar et al. (2010) they enhanced xylanase production using recombinant *E. coli* by optimizing the media composition and other growth factors. Two basal mediums (defined media and complex media) have been used and found out that the defined media gives the highest growth as well as the xylanase production under experimental conditions. At 20 % enhanced dissolved oxygen tension (DOT) in lab scale bioreactor, they increased xylanase production up to 1784 mL^{-1}.

7.10.3 Application of Response Surface Methodology (RSM) in Optimization of Xylanase Production

The conventional media optimization method based on step-by-step process varying one variable at a time (OFAT) while keeping the other variables constant is a time-consuming process and less reliable in many cases. To overcome this problem, statistical approach using experimental designs was applied. Response surface methodology (RSM) is the most reliable statistical application that has been used for optimization of fermentation media. Optimizations of physiological and nutritional growth factors are found to be very important procedures to reduce the production cost of xylanases in industrial scale. Many researches have been carried out based on RSM which used to understand and evaluate the significant interaction between the physiological and nutritional factors effecting the cell growth and product formation. A prior knowledge about these growth factors and their interactions is necessary for the designing of a more realistic model. Hence, many of the RSM studies are found to be based on the results obtained from OFAT method. Selection of these growth parameters has been done by checking their significance level using a 2FFD Pareto chart before proposing an experimental design. Most significant factors were then used as variables in central composite design (CCD) (Farliahati et al. 2010). The effect of various growth parameters in production of xylanase enzyme was studied by Mullai et al. (2010). In his study, statistical experimental designs (CCD) were constructed with the help of statistical application MINITAB 14. Plackett–Burman (PB) design for seven different variables, NH_4NO_3, KH_2PO_4, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}7H_2O$, and NaCl at two levels +1 and −1 (higher and lower level, respectively) and the

statistical significance were studied by determining the *F*-value. The optimum levels of selected variables from the Plackett–Burman design were investigated using central composite design (CCD). The response was measured in terms of xylanase activity. Counter plots and response surface 3D graphical representations generated could show the interactions of different variables under experimental conditions. Garai and Kumar (2012) optimized the xylanase production by *A. candidus* in SSF. They studied the effect of various nitrogen sources in the production media and they found that xylanase production was enhanced when the ammonium nitrate was used as sole nitrogen source. In the same study, applying RSM-based BoxBehnken design, the researcher also optimized the physical parameters such as time of incubation, temperature, and moisture content which effects xylanase activity. The crude xylanase recovered at the end of solid-state fermentation was used for the scarification of ammonia-treated lignocellulose materials to study the substrate-binding assay. Among the various substrates used, corn comb was found releasing the maximum reduced sugar (438.47 mg g^{-1}) when it was treated with crude xylanase enzyme. In RSM-based optimization, xylanase production was optimized by Box–Behnken design. Initially, Planket–Berman design was employed to identify the key ingredients and process conditions which exhibit the most significant effect on the xylanase expression. In the later stage, using Box–Behnken design, the optimal level of the identified key ingredients in production media as well as the optimum range of process parameters such as cultivation time, pH, temperature, and agitation are identified. Using this approach 6.83-fold increase in xylanase production was obtained under optimized conditions when compared to that of initial un-optimized conditions.

Solid-state fermentation (SSF) is also widely used for large-scale production of xylanase enzymes. It is basically designed to mimic the natural habitat of microbes that are less likely to grow in submerged fermentation (SMF) conditions. In SSF, the solid bed is used as nutrient source as well as the mechanical support for cell growth. *A. niger*, *T. reesei*, and *T. koningii* are the major fungal species used to produce xylanase under SSF conditions (Dhiman et al. 2008; Garg et al. 1998). However, xylanase production by bacteria is commonly carried out in submerged fermentation conditions where the physical parameters such as temperature, pH, and dissolved O_2 level involved are controlled to the optimum levels with more ease, and therefore an enhanced production can be achieved. Esteban et al. (1982) reported that the large-scale production of xylanase in SmF is the growth-associated process. However, several exceptional cases were also been reported such as xylanase production using *Bacillus* sp. (Dey et al. 1992). Later study by Bocchini et al. (2002) reported the production of highly thermostable cellulase-free xylanase in SMF using *B. circulans.* Similar study was also carried out by Sharma et al. (2007) who used *Geobacillus thermoleovorans* AP7 to produce highly thermo-alkali-stable xylanase. The switch to xylanase-producing recombinant *E. coli* is seen as an economic strategy toward higher productivity and easier downstream purification. Sandhu and Kennedy (1984) reported a large-scale production of xylanase by transforming *E. coli* with recombinant plasmids carrying a 1,4-β,D-xylanase gene from *B. polymyxa.* In another study, xylanase gene isolated

from *A. usmani* was cloned in pET-28a expression vector and when it is expressed in *E. coli* BL21, the maximum xylanase activity was found to be 49.4 U mg^{-1}. The gene expression was induced by IPTG and the xylanase was found expressed with 6-X histidine tag that facilitates an easy purification of expressed protein (Zhou et al. 2008b). A very successful cultivation strategy for the production of two truncated thermostable recombinant xylanases (XynIAN and XynIANC), isolated from *Rhodothermus marinas*, was developed by Karlsson et al. (1999). They found that the fed-batch cultivations of *E. coli* strain BL21 (DE3) with a controlled exponential glucose feed led to high specific production of the recombinant proteins with additional complex medium. Highest xylanase production in defined medium was achieved by Farliahati et al. (2009). In this study, xylanase production reached up to 2122.5 U mL^{-1} at pH 7.4 and when $(NH_4)_2HPO_4$ was used as the main nitrogen source. They also reported that the xylanase production was a growth-associated process as the enzyme secretion was greatly dependent on biomass concentration for strain *E. coli* DH5α.

Biswas et al. (2010) used mutant IITD3A of *Melanocarpus albomyces* for the production of high titer cellulose-free xylanase in 14-L stirred tank bioreactor where an optimized (RSM based) production media and process parameters are used. The authors reported the xylanase activity reached up to 415 U mL^{-1} after 24 h by pH recycling strategy (between 7.8 and 8.2) during the production phase. They also reported that the xylanase production is significantly influenced by aeration and agitation. A 5.2-fold increase (overall volumetric productivity of 22,000 U L^{-1} h^{-1}) in activity was observed when the cells exhibited pellet form at an agitation speed of 600 RPM.

Kumar et al. (2009) optimized the xylanase production in lab scale stirred tank bioreactor using mycelial fungal strain *T. lanuginosus* MC 134. Fermentation media contains corn cob as a sole source of carbon which was inoculated with *T. lanuginosus* MC 134 and observed the production in terms of xylanase activity over a period of 9 days at various process parameters. A highest xylanase activity, 2009 U mL^{-1}, was obtained after 6 days of fermentation. However, a significant decrease (40 %) in productivity was observed in bioreactors when compared to shake flask culture. Similarly, xylanase production by several microbial strains using corn cob was reported and from these studies it was clear that the production largely depends on rheological factors in bioreactors and the type of extraction methods used. Ruanglek et al. (2007) successfully cloned and expressed XylB (564 bp) encoding endo-1,4-β-xylanase obtained from *A. niger* BCC14405 in *Pichia pastoris* under AOX1 promoter. A maximum of 7352 U mg^{-1} of xylanase-specific activity was obtained in 2 L lab scale bioreactor with BMGY/BSM medium under exponential feeding strategy. During the batch phase which was aimed to achieve HCDC, the temperature and pH were maintained at 30 °C and 5.0, respectively. Agitation was maintained in the range of 600–1000 rpm. Fed-batch phase was started after 16–20 h by feeding 67 % (v/v) glycerol plus 1 % (v/v) trace mineral solution in exponential manner. Table 7.16 shows examples for xylanase production in lab and pilot-scale submerged fermentation by different fungal strains.

Table 7.16 A comparison of xylanase production in lab and pilot-scale submerged fermentation using fungal strains

Name of the organism	Substrate used	Mode of operation	Xylanase activity (IU L^{-1})	Productivity (IU L^{-1} h^{-1})	References
Aspergillus niger	Xylan (from corncob)	Batch (20 L)	290,000	3820	Yuan et al. (2005)
Aspergillus oryzae	Spent sulfite liquor	Batch (15 L)	199,000	13,100	Chipeta et al. (2008)
Thermomyces lanuginosus DSM 10635	Oat husk hydrolysate	Batch (2 L)	210,000	4380	Xiong et al. (2004)
Thermomyces lanuginosus MC 134	Coarse corncob	Batch (5 L)	2,010,000	13,950	Kumar et al. (2009)
Trichoderma reesei	Oat husk hydrolysate	Fed-batch (5 L)	1,350,000	14,060	Xiong et al. (2005)
Melanocarpus albomyces	Soluble wheat straw extract	Batch with pH cycling (14 L)	550,000	22,000	Biswas et al. (2010)

7.10.4 pH Optima and Stability of Endo-1,4-β-xylanase Enzymes

In general, endo-1,4-β-xylanases are active at wide pH ranges and their pH optima depend on the producer organism and the physiochemical properties of the substrate molecules. Fungal xylanases are usually acidophilic in nature. However, the optimal pH for bacterial xylanases is usually above 5.5. Endo-1,4-β-xylanase from *A. kawachii* reported as the most acidophilic xylanase which has a very low pH optimum of 2. On the other hand, xylanase from *Bacillus* sp. 41 M1 reported as the most alkalophilic xylanase which has pH optimum 9.0 (Nakamura et al. 1993). In certain cases, different pH optimums were observed in different xylanases secreted by same organism. For example, XynI and XynII produced by *H. jecorina* were reported to have pH optimums 3–4 and 5–5.5, respectively. This showed that the pI values of the enzymes are correlated with the amino acid residues at various positions on xylanase. However, among Xyl-11 family, those are acidophilic in nature possess an Asp residues at position 33 even though there are very rare exceptions such as *A. kawachii* IFO 4308 xylanase which has pH optimum close to 5.0 (Zhu et al. 1994). However, many alkalophilic xylanases such as Xyn11 from *A. versicolor* (Carmona et al. 1998), XynC from *Uccinogenes* sp. (Zhu et al. 1994) also displayed an Asp at the same position. Similarly, studies on *P. griseofulvum* Xyl-11 reported that it displays a Ser residue at position 44 and mutating this

residue resulted significant changes in pH optimum of the enzyme (Andre-Leroux et al. 2008).

There have been many different explanations for the factors determining the optimal pH value of xylanases. However, it believes that certain amino acid residues of xylanase structure play key role in the determination of the optimal pH value of the enzyme. In case of acidic pH optima, the Asp/Glu residues near the catalytic cleft become protonated and it decreases the electrostatic repulsion between the substrate and enzyme, whereas in alkaline xylanases, instead of acidic residues more alkaline residues such as arginine are seen. de Lemos Esteves et al. (2005) experimented various mutations in *Streptomyces* sp. S38, and observed a pH optimum shift from 6.0 to 7.5, which enhanced the xylanase enzyme activity several folds higher than the enzyme from the wild type. The pI values of catalytic domain present on xylanase play a key role in its pH activity profile. Many recent studies reveals that, among GH11 xylanase, the residues those contribute positive charges and hydrogen bonds to the catalytic domain help in increasing or decreasing the pI values depending upon the electrostatic interaction between the substrate molecules (Joshi et al. 1997). Apart from these residues, factors such as occurrence of salt bridges are also reported to have significant effects on pH optima (Hakulinen et al. 1998).

7.10.5 Endo-1,4-β-xylanase of Extremophilic Origin

Considerable progress has been made in the isolation of extremophilic microorganisms and their successful cultivation in the laboratory. Commercial applications of the xylanases demand highly thermostable enzyme which can work effectively under elevated temperature. Many advantages such as reduced contamination risk and faster reaction rates have been proposed for the use of thermophiles in biotechnology processes. In general, parameters such as temperature, pH, and chemical and enzymatic stability are important for the industrial applicability of any enzyme. Recently, a simple model, based on the thermodynamic and kinetic parameters for inactivation of the enzyme, has been presented for predicting (1) thermostabilities in the presence of substrate and (2) residual activities of enzymes in harsh processing environments. The study of extremophiles and their enzymes can extend the present understanding of protein chemistry in addition to expanding the potential applications of biocatalysts. Studies of alkaliphiles have led to the discovery of a variety of enzymes which exhibit some unique properties. Biological detergents contain enzymes such as alkaline proteases and/or alkaline cellulases from alkaliphiles. Commercial production became economical only after the discovery of alkaliphilic *Bacillus*-producing CGT with enhanced pH stability. Alkaline xylanases have gained importance due to their application for the development of eco-friendly technologies used in paper and pulp industries. The enzymes are able to hydrolyze xylan which is soluble in alkaline solutions.

7.10.5.1 Alkaliphilic and Acidophilic Endo-1,4-β-xylanase

The pH tolerance of microbial endo-1,4-β-xylanase enzymes depends on the natural growing environment of the microbial species. Endo-1,4-β-xylanase-producing alkalophilic microbes grow optimally at higher pH above 9.0 and those produce acidophilic xylanases grow between pH 1.0–5.0 (Kimura et al. 2000). These organisms are usually isolated from various natural environments such as soil, decomposing organic matters, paper pulp industry wastes, and many other sources. *Bacillus* sp. C-59-2 was the first reported xylanase-producing alkaliphilic microorganism (Nakamura et al. 1993). Since then, there have been a number of reports on xylanases production using alkaliphilic and acidophilic microbes. However, the studies indicated that the intracellular xylanase produced by many of these microbes may not be adapted to extreme environmental growth conditions of their hosts. A large number of alkaliphilic microbes found to produce xylanase at optimal pH close to neutral although their activity can be retained in alkaline conditions. Many exceptional cases are also reported such as XylB from *Bacillus* sp. which has pH optima of 9.0–10 (Gessesse and Mamo 1998). Many acidophilic xylanases of fungal and bacterial origin have also been reported in last two decades. *Cryptococcus* sp. S-2 and *Penicillium* sp. 40 (Kimura et al. 2000) are the most studied extremely acidophilic (pH optima 2–3) xylanase producers.

7.10.5.2 Psychrophilic Endo-1,4-β-xylanase

Even though the xylanase-producing microorganisms are abundant in nature, only a very less number of endo-1,4-β-xylanase-producing psychrophilies have been identified so far. Gram-negative bacteria such as *Pseudoalteromonas haloplanktis* TAH3a (Collins et al. 2005a) and *Flavobacterium frigidarium* (Humphry et al. 2001); gram-positive *Clostridium* sp. PXYL1 (Akila and Chandra 2002); fungal species such as *Alternaria alternate*, *Penicillium* sp., and *Phoma* sp.; and yeast sp *Cryptococcus adeliae* (Petrescu et al. 2000) are the major psychrophilic organisms reported to be endo-1,4-β-xylanase producers. Almost all these microbial species are isolated from Antarctic regions. The best studied two psychrophilic xylanases, which are from *Pseudoalteromonas haloplanktis* and *Cryptococcus adeliae*, were classified under GH 8 and GH10, respectively. The (α/α) 6 barrel structure of these structures is described by Van Petegem et al. (2003). The limited studies revealed that these enzyme groups have very poor stability due to the reduced number of salt bridges present on it but higher catalytic activity even at very low temperature. Georlette et al. (2004) also observed that psychrophilic xylanases are characterized by up to 10-folds higher activity even at mild cool temperature of 5 °C when compared to mesophilic xylanase activity. Moreover, the psychrophilic xylanase displayed higher catalytic and retained 60 % of maximum activity at very low temperature. However, the psychrophilic xylanases are totally unstable at high temperature (temperature 55 °C) and exhibited 12 times shorter half-life compared to mesophilic xylanases (Collins et al. 2005b).

7.10.5.3 Thermophilic Endo-1,4-β-xylanase

A large number of thermophilic and hyper-thermophilic (optimal growth temperature 50–80 °C and more than 80 °C, respectively) microbes which are able to produce xylanases have been isolated from various sources such as thermal hot springs, deep marine fields, self-heating organic debris, etc. Almost all of these enzymes were grouped under families 10 and 11. Extremophile microbial species such as *Caldicellulosiruptor* sp. (Luthi et al. 1990), *Rhodothermus marinus* (Abou Hachem et al. 2000), and *Bacillus stearothermophilus* (Khasin et al. 1993) are the major producers of thermophilic family 10 xylanases, whereas family 11 thermophilic xylanases are less common and have been isolated from extremophiles such as *Bacillus* strain D3 (Connerton et al. 1999), *Chaetomium thermophilum* growing medicinal mushrooms in Egypt as a new strategy for cancer treatment, *dictyoglomus thermophilum* (McCarthy et al. 2000), *paecilomyces variotii* (Kumar et al. 2000), and *thermomyces lanuginosus* (Schlacher et al. 1996).

Optimum activity range of these thermophilic xylanases was found between 65 and 80 °C, whereas the xylanases from *Thermotoga* sp. exhibit optimal activity at 105 °C and pH 5.5. X-ray crystallographic structure and amino acid sequential analysis of many of these thermophilic xylanase indicated very close similarities with mesophilic xylanase and probable reason for the higher thermostability could be the minor changes in amino acid alignment. Many mutagenic studies also have indicated that minor modifications in mesophilic aminoacid array enhanced the thermostability to many folds (Winterhalter and Liebl 1995).

7.10.5.4 Features Responsible for Endo-1,4-β-xylanase Thermostability

Thermostability comparison made between the enzymes are usually misleading since in most cases, the enzymes compared do not share the same experimental conditions such as incubation time, concentration of protein, nature, and concentration of the substrate. Many researchers believe that the high thermostability of xylanases is due to the presence of a network of certain charged amino acid residues near to the catalytic sites. These residues are usually found coupled with the disulfide bridges between β-strand and the α-helix. However, many recombinant thermostable xylanases are expressed in *E. coli* and are found to have thermostability ranging from 70 to 80 °C. When analyzing their protein structure, comparatively longer N-terminal ends are observed which may account for its higher thermostability (Morris et al. 1996). Many other analyzes also propose that the thermostability is due to the presence of highly flexible α-helix and C-terminal regions where the protein unfolding is generally initiated (Kumar et al. 2000). However, several other factors have also been identified that explain the xylanase thermostability such as follows:

- Presence of disulfide bridges which may strengthen the whole protein framework would give more stability to the enzyme.
- Presence of ion and aromatic pairs and their interactions may favor to more stable framework.
- Strategic position of water molecules on the framework, since the readily available water bindings are required for the catalytic activity even at higher temperature.
- Complex folding nature of enzyme. The higher the folding complexity, the higher the stability.
- Occurrence of oligomerization and the presence of certain aromatic residues result in the hydrophobic patches which allows stronger monomeric interactions enhancing the enzyme stability.

Certain GH11 endo-1,4-β-xylanases display disulfide bridges at position between β-strand and α-helix. However, these enzymes do not exhibit higher thermostability, whereas endo-1,4-β-xylanase produced by *Dictyoglomus thermophilum* exhibits thermostability even though that do not have any disulfide bridges on their structure. Therefore, the presence of disulfide bridges does not necessarily account for the thermophilic characters of the enzymes (Table 7.17).

Table 7.17 Techniques used for purification of endo-1,4-β-xylanase enzymes

Source	Purification	Buffer solution	References
Acrophialophora nainiana	Gel filtration and ion exchange chromatographies	50 mM acetate	Salles et al. (2000)
Bacteroides xylanisolvens XB1A	Metal affinity resin and batch/gravity-flow column	50 mM phosphate	Mirande et al. (2010)
Sulfolobus solfataricus	Ultrafiltration and AKTA Fast Protein Liquid Chromatography	50 mM Tris–HCl	Cannio et al. (2004)
Streptomyces sp. AMT-3	Filtration and overnight dialysis	50 mM sodium citrate	Nascimento et al. (2002)
Glaciecola mesophila KMM 241	Precipitation with 60 % saturation of ammonium sulfate and His-Tag Ni-affinity column	50 mM citrate	Guo et al. (2009)
Bacillus sp. YJ6	Sephacryl S-100 h chromatography	20 mM citrate (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.0), sodium carbonate (pH 8.0–11.0)	Yin et al. (2010)
Paenibacillus barcinonensis	Precipitation with 20 % ammonium sulfate and cation exchange chromatography	50 mM phosphate	Valenzuela et al. (2010)
Aspergillus versicolor	DEAE–Sephadex and HPLC GF-510 gel filtration	McIlvaine (pH 4.0–8.0), Tris–HCl (pH 8.0–9.0), glycine–NaOH (9.0–9.5)	Carmona et al. (2005)

7.10.5.5 Improvement of Endo-1,4-β-xylanase Thermostability

In last two decades, thermostability of xylanase enzymes was continuously improved through the protein engineering approaches. In most of these studies, the researches considered to modify the mesophilic xylanase enzyme structure to design a new structural model similar to the thermophilic xylanase. Arase et al. (1993) used chemical random mutagenesis in XYNA from *B. pumilus* and this strategy was successful to increasing the enzymes' half-life from 4 to 12 min at 57 °C. In a similar study, the specific activity and optimum temperature of Xyl1 from *Streptomyces* sp. S38 was enhanced from 2100 to 2500 IU mg^{-1} with the shift of the optimal temperature from 57 to 66 °C (Georis et al. 2000). Other study showed that the replacement of the N-terminal end of mesophilic xylanase xlnB from *Streptomyces lividans* with those from thermostable xylanases TfxA from *T. fusca* by random gene shuffling and observed a significant increase in thermostability (Shibuya et al. 2000). Introduction of two disulfide bridges, one between B9 strand and the α-helix and another between N and C-terminal ends, also enhanced the thermostability of Tx-xyl (Paës and O'Donohue 2006). The authors also reported that the occurrence of residues that met at N-terminal end brought negative impact on xylanase thermostability and temperature optimum which are reduced by 10-folds and 5 °C, respectively. From these engineering strategies applied in xylanases protein engineering, researchers concluded that:

- The α-helix on xylanase structure seems very sensitive to thermal unfolding—making these are more rigid and may enhance the thermostability of the enzyme. However, it may also diminish the catalytic activity (Muilu et al. 1998).
- N-terminal end plays key role in thermostabilization in GH11 xylanase—linking the N-terminal extremity to A2 strand or C-terminal extremity enhanced the thermostability of xylanase. Introduction of an additional complete β strand by mutagenesis also beneficial for improved thermostability.
- Presence of disulfide bridges does not necessarily enhance the thermophilic behavior of the enzymes. Therefore, addition of these bridges does not always bring positive impact on enzyme thermostability.

7.11 Industrial Applications of Endo-1,4-β-xylanase

Industrial production of xylanases has been grown significantly during last few decades based on their increased application. Xylan can be converted into many useful products such as xylitol and furfurol through enzymatic hydrolysis (Subramaniyan and Prema 2002). The hydrolysis could be also carried out through acid treatment process which is relatively easy, fast, and cheap. However, the application of this method in industries is limited due to the formation of toxic end

substances that may have cross reactions with product of interest (Beg et al. 2001). Therefore, enzymatic hydrolysis method is widely applied even though it is a slow process but more efficient and eco-friendly. During industrial application, the xylolytic enzymes are not commonly applied in pure form but in combination with other hydrolytic enzymes to achieve better results. The industrial applications of xylanases cover different areas and the list of applications increased by time and thus increases the market demand of xylanases accordingly.

7.11.1 Animal Feed Industry

Xylanases are widely used in animal feed manufacturing. They are usually applied in combination of other hydrolytic enzymes such as pectinase, protease, cellulose, lipase, glucanase, alginase, and phytase. These enzymes are used to reduce the viscosity of raw materials used in animal feeds. The complex arabinoxylan that is present on the cell wall of most grains may reduce the effective digestion and absorption of nutrients in poultry. Studies on this area have reported that the xylanase-treated feed has better digestibility and nutrient absorption rate when compared to raw feeds (Bedford and Classen 1992). In animal feed industries, the feed conversion rate (FCR) is defined as the ratio of feed consumed to the body weight gained by the animal. A lower FCR means improved feed digestibility and nutrient absorption. Xylanase enzymes are widely incorporated in poultry feed to reduce the FCR. It have been reported that the addition of endo-xylanase is isolated from *Acidothermus cellulolyticus* in poultry feed and observed a significant decrease in FCR, indicating an improved digestibility of feed (Clarkson et al. 2001). Similar results were observed when endo-xylanase is isolated from *Neocallimastix patriciarum* added to canola seed after oil extraction and applied for cattle feeding. The digestibility and stability of nutritive value of silage for cattle are found to be enhanced when it was treated with xylanase along with probiotic lactobacillus. The xylose formed by the de-polymerization of hemicellulose was fermented by the probiotic lactobacillus and thus enhanced the feed nutritive value. It was also reported that xylanase supplementation in the animal diet improved the animal growth through the better nutrition absorbability and enhancement of immune response as well as the gut micro-flora (Gao et al. 2008). In other study, Vandeplas et al. (2010) reported that when *T. viride* xylanase is incorporated in poultry feed, it increased nutrient digestion by 60 % and resulted in a significant increase in chicken weight. Pretreatment of forage crops with xylanase leads to better digestion in cattle and these enzymes are often found in synergy with other cellulolytic enzymes. The assimilation of ruminant feed was also improved many folds by xylanases addition (Yang and Xie 2010).

7.11.2 Food and Beverages Industry

Food and beverages industries use also xylanases in wide range of applications. Xylanases are capable of hydrolyzing the hemicellulose in wheat that enhances the water absorption of dough at leavening level in bread making process. This could possibly enhance the fermentation rate and as a result the softness of the bread and volume was increased (Rouau 1993). Xylanase cocktails are often used to reduce the stickiness of the dough thus enhance the crumb (Goesaert et al. 2005; Butt et al. 2008). Other study showed also that under specific process condition, incorporation of xylanase in the dough increased the shelf life of bread and also improved the volume up to 24 % (Verjans et al. 2010). However, enzyme selection is critical in these conditions since the cereals used for bread making often exhibit the xylanase inhibition by TAXI, XIP, or TLXI (Gebruers et al. 2004). Pasta processing is another area where xylanases are widely used as solubilizing agent of durum wheat semolina without changing its rheological properties (Ingelbrecht et al. 2001; Brijs et al. 2004). It was also reported that xylanase enzymes can improve the separation of wheat flour into starch and gluten (Frederix et al. 2004). However, in certain cases, action of Xyl-11 does not favor the separation due to high viscosity caused by the un-extractable arabino xylanase hydrolysis. In such conditions, synergetic dosages of Xyl-10 and Xyl-11 are used as it can mediate-improved agglomeration of gluten protein even though the wheat quality is very poor (Van Der Borght et al. 2005). Table 7.18 shows some examples of commonly used xylanase preparations for the baking industry. Xylanases are widely used also in juice, beer, and wine making industries. The enzymatic breakdown of fruits and vegetables hemicellulose can increase the clarity and stability of juice and wine. Xylanases can be used along with several other enzymes such as pectinase and amylase to improve the quality of beverage products in terms of aroma, taste, clarity, and stability (Wong et al. 1988). It was reported that up to 27 % decrease in insoluble fibers was achieved in juice clarification by addition of xylanase enzyme isolated from *Sclerotinia sclerotiorum* (Olfa et al. 2007). In beer production, partial hydrolysis of arabinoxylan found in the barley cell wall gives a middy viscous appearance to the fermented beer. In such

Table 7.18 Examples of commonly used xylanase preparations for the baking industry (Collins et al. 2005a, b)

Supplier	Brand	Enzyme source
Beldem-Puratos	Bel'ase B210	*Bacillus subtilis*
	Bel'ase F25	*Aspergillus niger*
Genencor Intl.	Multifectw	*Trichoderma reesei*
Danisco	Grindamyle H	*Aspergillus niger*
DSM	Bakezymew HS, BXP	*Aspergillus niger*
Novozymes	Pentopan Mono	*Thermomyces lanuginosus*
AB enzymes	Veronw 191	*Aspergillus niger*
	Veronw Special	*Bacillus subtilis*

cases, various xylanase enzyme groups are used to increase the filtration performance of beer wort by hydrolysis of viscous AXs fractions. Xylanase inhibitors present in the malt are the major hinderance to xylanase action. The xylanase activity is retained with the help of other synergetic enzymes used in the process (Debyser et al. 1997).

7.11.3 Paper and Pulp Industries

Endo-1,4-β-xylanase enzymes play significant role in paper and pulp industry since they facilitate an eco-friendly bio-bleaching of wood pulp by reducing the use of chemical bleaching agents such as chlorine and chlorine dioxide. For the past few decades, kraft pulp bleaching industry is recognized as the biggest area of xylanase application. Several studies reported that the removal of lignin content can be achieved by xylanase enzymes far better than by other lignin-degrading enzymes (Lundgren et al. 1994). Use of chemical bleaching agents in conventional pulp bleaching methods cause serious environmental pollution issues and this drove the researcher's attention on xylanase enzymes (Subramaniyam and Prema 2002). Table 7.19 shows major endo-1,4-β-xylanase supplier in pulp bleaching industry. Application of endo-1,4-β-xylanase enzymes in pulp bleaching can reduce using chlorine as well as the total energy requirement for this process. Kulkarni et al. (1999) observed that xylanase pretreatment of kraft pulp lowers the usage of chemicals by 10–20 %. The concept of TCF (total chlorine free) bleaching technologies was also recently introduced to make the process more eco-friendly. However, cellulase-free endo-1,4-β-xylanase is required in this process since the cellulose enzyme may degrade the cellulose which is the main target of the industry

Table 7.19 Major xylanase supplier in pulp bleaching industry (Beg et al. 2001)

Supplier	Product trade name
Alko Rajamaki, Finland	Ecopulp
Sandoz, Charlotte, N.C. and Basle, Switzerland	Cartazyme
Clarient, UK	Cartazyme HS, HT and SR 10
Genercor, Finland; Ciba Giegy, Switzerland	Irgazyme 40–4X/Albazyme 40–4X
Novo Nordisk, Denmark	Pulpzyme HA, HB and HC
Biocon India, Bangalore	Bleachzyme F
Rohn Enzyme 0Y; Primalco, Finland	Ecopulp X-100, X-200, and X-200/4
Solvay Interox, USA	Optipulp L-8000
Thomas Swan, UK	Ecozyme
Iogen, Canada	GS-35, HS70

(Bajpai and Bajpai 2001). Relatively lower molecular weight of xylanase enzymes helps them to penetrate more deep into the lignocelluloses substrates and facilitate better hydrolytic actions (Beaugrand et al. 2005; Beg et al. 2001). Higher thermostability and alkaline stability are other key features that make xylanase more favorable for bio-bleaching industry (Buchert et al. 1995). The mechanisms of pulp bleaching by xylanase have been studied and many researchers believe that xylanase hydrolysis takes place at lignin xylan interaction and thus results in a loosely packed cellulosic framework (Shatalov and Pereira 2007). However, it was recently reported the potential use of *Aspergillus oryzae* MDU-4-based fungal xylanase for effective deinking of newspaper pulp where a significant improvement in optical properties such as brightness up to 57.9 % ISO with decreased (211 ppm) residual ink concentration (Chutani and Sharma 2015).

7.11.4 Biofuel Production

The next-generation fuel production is based on renewable sources such as lignocellulosic biomass. As first step, the complex lignocellulosic biomass needs to be breakdown to a mixture of relatively smaller units made of hexoses and pentoses or their monosaccharides that are further fermented to ethanol or other biofuels (Margeot et al. 2009). The process of converting the lignocellulosic feedstocks into biofuels usually involves the following steps:

- Enzyme hydrolysis: the critical step involved where the hydrolysis of lignocelluloses into various sugar monomers by the action of cocktail of hydrolytic enzymes.
- Fermentation: production of biofuel by the utilization of sugar monomers formed by various microbial species, usually carried out by different bacterial and yeast strains depends on the desired type of biofuel.
- Distillation: separation and purification of biofuel produced.

The controlled hydrolysis of lignocelluloses using enzymatic cocktail to produce the desirable products is the challenge involved in this process. Xylolytic enzymes, such as Endo-1,4-β-xylanase in particular, are very essential in biomass hydrolysis as they initiate progressive cell wall in the assembly and thus mediate the cell wall penetration by other cellulolytic enzymes (Beaugrand et al. 2005).

7.11.5 Other Applications

Apart from the traditional applications in pulp beaching industry, xylanases have been used in several other areas as well. The broad spectrum of xylanase application includes the saccharification of xylan to xylose, plant cell protoplasting, coffee

mucilage liquefaction, vegetable softening, extraction of plant pigments and oils, etc. (Kulkarni et al. 1999). In laundry industries, endo-1,4-β-xylanase was effectively used to remove the plant-based stains. The stain-removing capacity of family 11 endo-xylanase was improved when it chemically linked with cellulose-binding domain of cellulases. In textile industries, xylanase enzymes can be used to process the plant fiber as they can decrease the lignin content and improve the color and quality of fibers (Csiszár et al. 2001; Battan et al. 2012). Until now, xylanase applications in pharmaceutical industries are very limited. However, it is used in some dietary supplements in combination with other enzymes (Polizeli et al. 2005). Commercial products such as chewing gum contain xylitol and it can be easily produced by hydrolysis of xylan using xylanase enzymes.

7.11.6 Future Perspectives

The current list of highly diversified applications of xylanases clearly demonstrates the increased demand of this type of enzymes. In addition, the continuous improvement of the enzyme catalytic properties increases the potential uses of this enzyme by adding many new industrial applications. The improvement of catalytic properties of the xylanases by shifting the optimal operation conditions of the enzyme and by isolation of new potential xylanases from extremophilic microbes are further expressed in suitable mesophilic host to industrialize the process. In addition, with the increased knowledge about protein engineering, it become possible also to increase enzyme stability and kinetic properties using different approaches such as changing of amino acid sequence, creation of sulfide bridge within the molecule, and changing the enzyme 3D configuration. All these will help to increase the potential application of xylanases under extreme operation conditions.

However, some recent studies were skeptic about the future application of xylanases in biofuel sector based on the current dramatic drop of oil price which make biofuel non-competitive alternative to fossil fuel in economic point of view. However, xylan biorefinery is not limited to fuel market but the biohydrolysis products of xylan such as pentose oligomers and monomers are also used as intermediate in the production of many biochemicals. Moreover, the increased public awareness about the negative impacts of chemicals on the environment, ecosystem, and human and animal health increased the pressure on chemical industries to replace the current chemical-based catalytic processes to a biobased and eco-friendly processes especially in paper/pulp, leather, and detergent industries. In addition, there is significant increased demand for xylanases application in food and feed industries for the production of safe and high-quality product. These all together support our current estimation of the continuous increase of xylanases demand in many industrial applications.

References

Abou Hachem M, Karlsson EN, Bartonek-Roxa E, Raghothama S, Simpson PJ, Gilbert HJ, Williamson MP, Holst O (2000) Carbohydrate-binding modules from a thermostable *Rhodothermus marinus* xylanase: cloning, expression and binding studies. Biochem J 345:53–60

Alejandro SH, Jesús VE, del María CMH, María EHL (2007) Purifcation and characterization of two sugarcane bagasse-absorbable thermophilic xylanases from the mesophilic *Cellulomonas Xavigena*. Microbiol Biotechnol 34:331–338

Akila G, Chandra TS (2002) A novel cold-tolerant Clostridium strain PXYL1 isolated from a psychrophilic cattle manure digester that secretes thermolabile xylanase and cellulase. FEMS Microbiol Lett 219:63–67

Andrade SV, Polizeli MLTM, Terenzi HF, Jorge JA (2004) Effect of carbon source on the biochemical properties of β-xylosidases produced by *Aspergillus versicolor*. Process Biochem 39:931–938

Andre-Leroux G, Berrin JG, Georis J, Arnaut V, Juge V (2008) Structure-based mutagenesis of *Penicillium griseofulvum* xylanase using computational design. Proteins 72(4):1298–1307

Andrews SR, Charnock SJ, Lakey JH, Davies GJ, Claeyssens M, Nerinckx V, Underwood M, Sinnott ML, Warren RA, Gilbert HJ (2000) Substrate specificity in glycoside hydrolase family 10. Tyrosine 87 and leucine 314 play a pivotal role in discriminating between glucose and xylose binding in the proximal active site of *Pseudomonas cellulosa* xylanase 10A. J Biol Chem 275(30):23027–23033

Anuradha P, Vijayalakshmi K, Prasanna ND, Sridevi K (2007) Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields. Curr Sci 92(9):1283–1286

Arase A, Yomo T, Urabe I, Hata Y, Katsube Y, Okada H (1993) Stabilization of xylanase by random mutagenesis. FEBS Lett 316(2):123–127

Archana A, Satyanarayana T (1998) Cellulase-free xylanase production by thermophilic *Bacillus licheniformis* A99. Indian J Microbiol 38:135–139

Atkins EDT (1992) Three-dimensional structure, interactions and properties of xylans. Xylanas Xylanases 7:39–50

Avalos OP, Noyola TP, Plaza IM, Torre M (1996) Induction of xylanase and *β*-xylosidase in *Cellulomonas flavigena* growing on different carbon sources. Appl Microbiol Biotchnol 46:405–409

Bai Y, Wang J, Zhang Z, Yang P, Shi P, Luo H, Meng K, Huang H, Yao B (2010) A new xylanase from thermoacidophilic *Alicyclobacillus* sp. A4 with broad-range pH activity and pH stability. J Ind Microbiol Biotechnol 37(2):187–194

Bajpai P, Bajpai PK (2001) Development of a process for the production of dissolving kraft pulp using xylanase enzyme. Appita J 54(4):381–384

Bajaj BK, Singh NP (2010) Production of xylanase from an alkali tolerant *Streptomyces* sp. 7b under solid-state fermentation, its purification, and characterization. Appl Biochem Biotechnol 162(6):1804–1818

Balakrishnan H, Srinivasan MC, Rele MV, Chaudhari K, Chandwadkar AJ (2000) Effect of synthetic zeolites on xylanase production from an alkalophilic *Bacillus* sp. Curr Sci 79:95–97

Basar B, Shamzi MM, Rosfarizan M, Puspaningsih NNT, Ariff AB (2010) Enhanced production of thermophilic xylanase by recombinant *Escherichia coli* DH5α through optimization of medium and dissolved oxygen level. Int J Agric Biotechnol 12:321–328

Basaran P, Hang YD, Basaran N, Worobo RW (2001) Cloning and heterologous expression of xylanase from *Pichia stipitis* in *Escherichia coli*. J Appl Microbiol 90(2):248–255

Battan B, Dhiman SS, Ahlawat S, Mahajan R, Sharma J (2012) Application of thermostable xylanase of *Bacillus pumilus* in textile processing. Indian J Microbiol 52(2):222–229

Beaugrand J, Paës G, Reis D, Takahashi M, Debeire P, O'Donohue MJ, Chabbert B (2005) Probing the cell wall heterogeneity of micro-dissected wheat caryopsis using both active and inactive forms of a GH11 xylanase. Planta 222:246–257

Bedford MR, Classen HL (1992) Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through changes in the carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. J Nutr 122:560–569

Beg QK, Bhushan B, Kappor M, Hoondal G (2000) Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. J Ind Microbiol Biotechnol 24:396–402

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56(3–4):326–338

Bergquist P, Te'o V, Gibbs M, Cziferszky A, de Faria FP, Azevedo M, Nevalainen H (2002) Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. Extremophiles 6(3):177–184

Berrin JG, Juge N (2008) Factors affecting xylanase functionality in the degradation of arabinoxylans. Biotechnol Lett 30(7):1139–1150

Berrin JG, Williamson G, Puigserver A, Chaix JC, McLauchlan WR, Juge N (2000) High-level production of recombinant fungal endo-beta-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 19(1):179–187

Bhalerao J, Patki AH, Bhave V, Khurana I, Deobagkar DN (1990) Molecular-cloning and expression of a xylanase gene from *Cellulomonas* sp. into *Escherichia coli*. Appl Microbiol Biotechnol 34(1):71–76

Bhardwaj A, Leelavathi S, Mazumdar-Leighton S, Ghosh A, Ramakumar S, Reddy VS (2010) The critical role of N- and C-terminal contact in protein stability and folding of a family 10 xylanase under extreme conditions. PLoS ONE 5(6):e11347

Biely P, Vrsanska M, Kluepfel M, Tenkanen D (1997) Endo-beta-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57(1–3):151–166

Biely P, Leathers TD, Cziszarova M, Vrsanska M, Cotta MA (2008) Endo-beta-1,4-xylanase inhibitors in leaves and roots of germinated maize. J Cereal Sci 48(1):27–32

Biswas R, Sahai V, Mishra S, Bisaria VS (2010) Bioprocess strategies for enhanced production of xylanase by *Melanocarpus albomyces* IITD3A on agro-residual extract. J Biosci Bioeng 110:702–708

Blum DL, Li XL, Chen H, Ljungdahl LG (1999) Characterization of an acetyl xylan esterase from the anaerobic fungus *Orpinomyces* sp. strain PC-2. Appl Environ Microbiol 65(9):3990–3995

Bocchini DA, Alves-Prado HF, Baida LC, Roberto IC, Gomes E, Da-Silva R (2002) Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. Process Biochem 38:727–731

Bolam DN, Xie HF, White P, Simpson PJ, Hancock SM, Williamson MP, Gilbert HJ (2001) Evidence for synergy between family 2b carbohydrate binding modules in *Cellulomonas fimi* xylanase 11A. Biochemistry 40(8):2468–2477

Boraston AB, Tomme P, Amandoron EA, Kilburn DG (2000) A novel mechanism of xylan binding by a lectin-like module from *Streptomyces lividans* xylanase 10A. Biochem J 350(Pt 3):933–941

Boraston AB, McLean BW, Chen V, Li A, Warren RA, Kilburn DG (2002) Co-operative binding of triplicate carbohydrate-binding modules from a thermophilic xylanase. Mol Microbiol 43 (1):187–194

Borralho T, Chang Y, Jain P, Lalani M, Parghi K (2002) Lactose induction of the lac operon in *Escherichia coli* B23 and its effect on the o-nitrophenyl β-galactoside assay. J Exp Microbiol Immunol 2:117–123

Bridgeman TG, Jones JM, Shield I, Williams PT (2008) Torrefaction of reed canary grass, wheat straw and willow to enhance solid fuel qualities and combustion properties. Fuel 87 (6):844–856

Brijs K, Ingelbrecht JA, Courtin CM, Schlichting L, Marchylo BA, Delcour JA (2004) Combined effects of endoxylanases and reduced water levels in pasta production. Cereal Chem 81:361–368

Brutus A, Villard C, Durand A, Tahir T, Furniss C, Puigserver A, Juge N, Giardina T (2004) The inhibition specificity of recombinant *Penicillium funiculosum* xylanase B towards wheat proteinaceous inhibitors. Biochim Biophys Acta 1701(1–2):121–128

Brutus A, Reca IB, Herga S, Mattei B, Puigserver A, Chaix JC, Juge V, Bellincampi D, Giardina T (2005) A family 11 xylanase from the pathogen *Botrytis cinerea* is inhibited by plant endoxylanase inhibitors XIP-I and TAXI-I. Biochem Biophys Res Commun 337(1):160–166

Buchert J, Teleman A, Harjunpa V, Tenkanen M, Viikari L, Vuorinen T (1995) Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. Tappi J 78 (11):125–130

Butt MS, Nadeem MT, Ahmad Z, Sultan MT (2008) Xylanases and their applications in baking industry. Food Technol Biotechnol 46(1):22–31

Cannio R, Di Prizito N, Rossi M, Morana A (2004) A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity. Extremophiles 8 (2):117–124

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:D233–D238

Cao Y, Qiao J, Li Y, Lu W (2007) De novo synthesis, constitutive expression of *Aspergillus sulphureus* beta-xylanase gene in *Pichia pastoris* and partial enzymic characterization. Appl Microbiol Biotechnol 76(3):579–585

Carmona EC, Brochetto-braga MR, Pizzirani-kleiner AA, Jorge JA (1998) Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*. FEMS Microbiol Lett 166:311–315

Carmona EC, Fialho MB, Buchgnani EB, Coelho GD, Brocheto-Braga MR, Jorge JA (2005) Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor*. Process Biochem 40(1):359–364

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24(1):45–66

Cervera-Tison MC, Andre-Leroux G, Lafond M, Georis J, Juge N, Berrin J-G (2009) Molecular determinants of substrate and inhibitor specificities of the *Penicillium griseofulvum* family 11 xylanases. Biochim Biophys Acta 1794:438–445

Charnock SJ, Lakey JH, Virden R, Hughes N, Sinnott ML, Hazlewood GP, Pickersgill R, Gilbert HJ (1997) Key residues in subsite F play a critical role in the activity of *Pseudomonas fluorescens* subspecies *cellulosa* xylanase A against xylooligosaccharides but not against highly polymeric substrates such as xylan. J Biol Chem 272(5):2942–2951

Chauhan K, Trivedi U, Patel KC (2007) Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. Bioresour Technol 98:98–103

Chauthaiwale VM, Deshpande VV (1992) Molecular cloning and expression of the xylanase gene from *Chainia* in *Escherichia coli*. FEMS Microbiol Lett 99(2–3):265–270

Chavez R, Schachter K, Navarro C, Peirano A, Bull P, Eyzaguirre J (2004) The acetyl xylan esterase II gene from *Penicillium purpurogenum* is differentially expressed in several carbon sources, and tightly regulated by pH. Biol Res 37(1):107–113

Chutani P and Sharma KK (2015) Biochemical evaluation of xylanases from various filamentous fungi and their application for the deinking of ozone treated newspaper pulp. Carbohydrate Polymers 127:54–63

Cheng YF, Yang CH, Liu WH (2005) Cloning and expression of *Thermobifida* xylanase gene in the methylotrophic yeast *Pichia pastoris*. Enz Microb Technol 37(5):541–546

Chiku K, Uzawa J, Seki H, Amachi S, Fujii T, Shinoyama H (2008) Characterization of a novel polyphenol-specific oligoxyloside transfer reaction by a family 11 xylanase from *Bacillus* sp. KT12. Biosci Biotechnol Biochem 72(9):2285–2293

Chipeta ZA, Du-Preez JC, Christopher L (2008) Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. J Ind Microbiol Biotechnol 35:587–594

Chithra M, Muralikrishna G (2008) Characterization of purified xylanase from finger millet (*Eleusine coracana*-Indaf 15) malt. Eur Food Res Technol 227(2):587–597

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EJW, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52(1999):741–755

Clarkson K, Siika-aho M, Tenkanen M, Bower BS, Penttilä ME, Saloheimo MLA (2001) *Trichoderma reesei* xylanase. Patent WO 0149859

Cleemput G, Hessing M, van Oort M, Deconynck M, Delcour JA (1997) Purification and characterization of a [beta]-D-xylosidase and an endo-xylanase from wheat flour. Plant Physiol 113(2):377–386

Collins T, Gerday C, Feller G (2005a) Xylanases, xylanases families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23

Collins T, De Vos D, Hoyoux A, Savvides SN, Gerday C, Van Beeumen J, Feller G (2005b) Study of the active site residues of a glycoside hydrolase family 8 xylanase. J Mol Biol 354 (2):425–435

Connerton I, Cummings N, Harris GW, Debeire P, Breton C (1999) A single domain thermophilic xylanase can bind insoluble xylan: evidence for surface aromatic clusters. Biochim Biophys Acta 1433:110–121

Copa-Patino JL, Broda YG, Broda P (1993) Production and initial characterization of the xylan-degrading system of *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 40:69–76

Coughlan MP, Hazlewood GP (1993) Beta-1,4-D-xylan-degrading enzyme-systems-biochemistry, molecular-biology and applications. Biotechnol Appl Biochem 4:259–289

Couturier M, Haon M, Coutinho PM, Henrissat B, Lesage-Meessen L, Berrin JG (2010) *Podospora anserina* hemicellulases potentiate the *Trichoderma reesei* secretome for saccharification of lignocellulosic biomass. Appl Environ Microbiol 77:237–246

Crepin VF, Faulds CB, Connerton IF (2004) Functional classification of the microbial feruloyl esterases. Appl Microbiol Biotechnol 63:647–652

Croes E, Gebruers K, Carpentier S, Swennen R, Robben J, Laukens K, Witters E, Delcour JA, Courtin CM (2009) A quantitative portrait of three xylanase inhibiting protein families in different wheat cultivars using 2D-DIGE and multivariate statistical tools. J Proteomics 72 (3):484–500

Csiszár E, Urbánszki K, Szakás G (2001) Biotreatment of desized cotton fabric by commercial cellulase and xylanase enzymes. J Mol Catal B Enzym 11:1065–1072

Damiano VB, Bocchini DA, Gomes E, Silva RD (2003) Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of *Eucalyptus* kraft pulp. World J Microbiol Biotechnol 19:139–144

Dautova M, Roze E, Overmars H, de Graaff L, Schots A, Helder J, Goverse A, Bakker J, Smant G (2006) A symbiont-independent endo-1,4-beta-xylanase from the plant-parasitic nematode Meloidogyne incognita. Mol Plant Microbe Interact 19(5):521–529

De Lemos Esteves F, Gouders T, Lamotte-Brasseur J, Rigali S, Frere JM (2005) Improving the alkalophilic performances of the Xyl1 xylanase from Streptomyces sp. S38: structural comparison and mutational analysis. Protein Sci 14(2):292–302

De Vos D, Collins T, Nerinckx W, Savvides SN, Claeyssens M, Gerday C, Feller G, Van Beeumen J (2006) Oligosaccharide binding in family 8 glycosidases: crystal structures of active-site mutants of the beta-1,4-xylanase pXyl from *Pseudoalteromonas haloplanktis* TAH3a in complex with substrate and product. Biochemistry 45(15):4797–4807

De Vries RP, Visser J, de Graaff LH (1999) CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. Res Microbiol 150 (4):281–285

De Vries RP, Kester HC, Poulsen CH, Benen JA, Visser J (2000) Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydr Res 327 (4):401–410

Dean JFD, Gross KC, Anderson JD (1991) Ethylene biosynthesis-inducing xylanase. 3. Product characterization. Plant Physiol 96(2):571–576

Debyser W, Peumans WJ, Van Damme EJM, Delcour JA (1999) *Triticum aestivum* xylanase inhibitor (TAXI), a new class of enzyme inhibitor affecting breadmaking performance. J Cereal Sci 30(1):39–43

Debyser W, Derdelinckx G, Delcour JA (1997) Arabinoxylan solubilization and inhibition of the barley malt xylanolytic system by wheat during mashing with wheat whole meal adjunct: evidence for a new class of enzyme inhibitors in wheat. Am Soc Brew Chem 55:153–156

Degefu Y, Lohtander K, Paulin L (2004) Expression patterns and phylogenetic analysis of two xylanase genes (htxyl1 and htxyl2) from *Helminthosporium turcicum*, the cause of northern leaf blight of maize. Biochimie 86(2):83–90

Dey D, Hinge J, Shendye A, Rao M (1992) Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Can J Microbiol 38:436–442

Dheeran P, Nandhagopal N, Kumar S, Jaiswal YK, Adhikari DK (2012) A novel thermostable xylanase of *Paenibacillus macerans* IIPSP3 isolated from the termite gut. J Ind Microbiol Biotechnol 39(6):851–860

Dhillon A, Gupta JK, Khanna S (2000) Enhanced production, purification and characterisation of a novel cellulase-poor thermostable, alkalitolerant xylanase from *Bacillus circulans* AB 16. Process Biochem 35(8):849–856

Dhiman SS, Sharma J, Battan B (2008) Industrial applications and future prospects of microbial xylanases: a review. BioResources 3(4):1377–1402

Dijkerman R, Ledeboer J, Op den Camp HJM, Prins RA, van der Drift C (1997) The anaerobic fungus *Neocallimastix* sp. strain L2: growth and production of (Hemi) cellulolytic enzymes on a range of carbohydrate substrates. Curr Microbiol 34(2):91–96

Dornez E, Croes E, Gebruers K, De Coninck B, Cammue BPA, Delcour JA, Courtin CM (2010a) Accumulated evidence substantiates a role for three classes of wheat xylanase inhibitors in plant defense. Crit Rev Plant Sci 29(4):244–264

Dornez E, Croes E, Gebruers K, Carpentier S, Swennen R, Laukens K, Witters E, Urban M, Delcour JA, Courtin CM (2010b) 2-D DIGE reveals changes in wheat xylanase inhibitor protein families due to *Fusarium graminearum* DeltaTri5 infection and grain development. Proteomics 10(12):2303–2319

Durand A, Hughes R, Roussel A, Flatman R, Henrissat B, Juge N (2005) Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. FEBS J 272 (7):1745–1755

Ebanks R, Dupont M, Shareck F, Morosoli R, Kluepfel D, Dupont C (2000) Development of an *Escherichia coli* expression system and thermostability screening assay for libraries of mutant xylanase. J Ind Microbiol Biotechnol 25(6):310–314

El Enshasy H, Hellmuth K, Rinas U (1999) Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger*. Appl Biochem Biotechnol 81:1–11

El Enshasy H, Kleine J, Rinas U (2006) Agitation effects on morphology and protein productive fractions of filamentous and pelleted growth forms of recombinant *Aspergillus niger*. Process Biochem 41:2103–2112

El Enshasy H (2007) Filamentous fungal culture—process characteristics, products, and applications. In: Yang ST (ed) Bioprocessing for value-added products from renewable resources. Elsevier Press, Amsterdam. ISBN-10: 0-444-52114-3

Emami K, Hack E (2001) Characterisation of a xylanase gene from *Cochliobolus sativus* and its expression. Mycol Res 105:352–359

Esteban R, Villanueva JR, Villa TG (1982) β-D-xylanases of *Bacillus circulans* WL-12. Can J Microbiol 28:733–739

Fang HY, Chang SM, Lan CH, Fang TJ (2008) Purification and characterization of a xylanase from *Aspergillus carneus* M34 and its potential use in photoprotectant preparation. Process Biochem 43(1):49–55

Farliahati MR, Mohamad R, Puspaningsih NNT, Ariff AB (2009) Kinetics of xylanase fermentation by recombinant *Escherichia coli* DH5α in shake flask culture. Am J Biochem Biotechnol 5(3):110–118

Farliahati MR, Ramanan RN, Mohamad R, Puspaningsih NNTM, Ariff AB (2010) Enhanced production of xylanase by recombinant *Escherichia coli* DH5 alpha through optimization of medium composition using response surface methodology. Ann Microbiol 60(2):279–285

Fernández-Espinar M, Pinaga F, Degraaff L, Visser J, Ramon D, Valles S (1994) Purification, characterization and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. Appl Microbiol Biotechnol 42(4):555–562

Fierens E, Gebruers K, Voet AR, De Maeyer M, Courtin CM, Delcour JA (2009) Biochemical and structural characterization of TLXI, the *Triticum aestivum* L. thaumatin-like xylanase inhibitor. J Enzyme Inhib Med Chem 24(3):646–654

Fierens E, Rombouts S, Gebruers K, Goesaert H, Brijs K, Beaugrand J, Volckaert G, Van Campenhout S, Proost P, Courtin CM, Delcour JA (2007) TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. Biochem J 403 (3):583–591

Fierens K, Geudens N, Brijs K, Courtin CM, Gebruers K, Robben J, Van Campenhout S, Volckaert G, Delcour JA (2004) High-level expression, purification, and characterization of recombinant wheat xylanase inhibitor TAXI-I secreted by the yeast *Pichia pastoris*. Protein Expr Purif 37(1):39–46

Flatman R, McLauchlan WR, Juge N, Furniss C, Berrin JG, Hughes RK, Manzanares P, Ladbury JE, O'Brien R, Williamson G (2002) Interactions defining the specificity between fungal xylanases and the xylanase-inhibiting protein XIP-I from wheat. Biochem J 365(Pt 3):773–781

Frederix SA, Courtin CM, Delcour JA (2004) Substrate selectivity and inhibitor sensitivity affect xylanase functionality in wheat flour gluten–starch separation. J Cer Sci 40:41–49

Fushinobu S, Ito K, Konno M, Wakagi T, Matsuzawa H (1998) Crystallographic and mutational analyses of an extremely acidophilic and acid-stable xylanase: biased distribution of acidic residues and importance of Asp37 for catalysis at low pH. Protein Eng 11(12):1121–1128

Gao J, Zhang HJ, Yu SH, Wu SG, Yoon I, Quigley J, Gao YP, Qi GH (2008) Effects of yeast culture in broiler diets on performance and immunomodulatory functions. Poult Sci 87:1377–1384

Garai D, Kumar V (2012) Response surface optimization for xylanase with high volumetric productivity by indigenous alkali tolerant *Aspergillus candidus* under submerged cultivation. Biotechnology 3:127–136

Garg AP, Roberts JC, McCarthy AJ (1998) Bleach boosting effect of cellulase-free xylanase of *Streptomyces thermoviolaceus* and its comparison with two commercial enzyme preparations on Birchwood kraft pulp. Enzyme Microb Technol 22:594–598

Gebruers K, Brijs K, Courtin CM, Fierens K, Goesaert H, Raedschelders G, Robben J, Sorensen JF, Van Campenhout S, Delcour JA (2004) Properties of TAXI-type endoxylanase inhibitors. Biochim Biophys Acta 1696:213–221

Gebruers K, Beaugrand J, Croes E, Dornez E, Courtin CM, Delcour JA (2008) Quantification of wheat TAXI and XIP type xylanase inhibitors: a comparison of analytical techniques. Cer Chem 85(5):586–590

Georlette D, Blaise V, Collins T, D'Amico S (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42

Gessesse A, Mamo G (1998) Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp AR-135. J Ind Microbiol Biotechnol 20(3–4):210–214

Ghosh M, Nanda G (1994) Purification and some properties of a xylanase from *Aspergillus sydowii* MG49. Appl Environ Microbiol 60(12):4620–4623

Goesaert H, Gebruers K, Courtin CM, Delcour JA (2005) Purification and characterization of a XIP-type endoxylanase inhibitor from rice (*Oryza sativa*). J Enz Inhib 20:95–101

Georis J, Giannotta F, De Buyl E, Granier B, Frère J (2000) Purification and properties of three endo-β-1,4-xylanases produced by *Streptomyces* sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulps. Enzyme Microb Technol 26:178–186

Gomes DJ, Gomes J, Steiner W (1994) Factors influencing the induction of endo-xylanase by *Thermoascus aurantiacus*. J Biotechnol 33(1):87–94

Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytol 178(3):473–485

Gruber K, Klintschar G, Hayn M, Schlacher V, Steiner W, Kratky C (1998) Thermophilic xylanase from *Thermomyces lanuginosus*: high-resolution X-ray structure and modeling studies. Biochemistry 37(39):13475–13485

Guo B, Chen XL, Sun CY, Zhou BC, Zhang YZ (2009) Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo-beta-1,4-xylanase from marine *Glaciecola mesophila* KMM 241. Appl Microbiol Biotechnol 84(6):1107–1115

Gupta S, Bhushan B, Hoondal GS (2000) Isolation, purification and characterization of xylanase from *Staphylococcus* sp. SG-13 and its application in biobleaching of kraft pulp. J Appl Microbiol 88:325–334

Gupta U, Kar R (2009) Xylanase production by a thermos-tolerant *Bacillus* species under solid-state and submerged fermentation. Braz Arch Biol Technol 52:1363–1371

Hakulinen N, Tenkanen M, Rouvinen J (1998) Crystallization and preliminary X-ray diffraction studies of the catalytic core of acetyl xylan esterase from *Trichoderma reesei*. Acta Crystallogr D Biol Crystallogr 54(Pt 3):430–443

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293(3):781–788

Hessing JGM, van Rotterdam C, Verbakel JMA, Roza M, Maat J, van Gorcom RFM, van den Hondel CAMJJ (1994) Isolation and characterization of a 1,4-b-endoxylanase gene of *A. awamori*. Curr Genet 26:228–232

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315 (5813):804–807

Honda Y and Kitaoka M (2004) A family 8 glycoside hydrolase from Bacillus halodurans C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. J Biol Chem 279:55097–55103

Hu YJ, Smith DC, Cheng KJ, Foresberg CW (1991) Cloning of a xylanase gene from *Fibrobacter succinogenes* 135 and its expression in *Escherichia coli*. Can J Microbiol 37(7):554–561

Huang J, Wang G, Xiao L (2006) Cloning, sequencing and expression of the xylanase gene from a Bacillus subtilis strain B10 in Escherichia coli, Bioresour Technol 97:802–808

Humphry DR, GeorgeA Black GW, Cummings SP (2001) *Flavobacterium frigidarium* sp. an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. Int J Syst Evol Microbiol 51:1235–1243

Hung KS, Liu SM, Fang TY, Tzou WS, Lin FP, Sun KH, Tang SJ (2011) Characterization of a salt-tolerant xylanase from *Thermoanaerobacterium saccharolyticum* NTOU1. Biotechnol Lett 33(7):1441–1447

Igawa T, Tokai T, Kudo T, Yamaguchi I, Kimura M (2005) A wheat xylanase inhibitor gene, Xip-I, but not Taxi-I, is significantly induced by biotic and abiotic signals that trigger plant defense. Biosci Biotechnol Biochem 69(5):1058–1063

Ingelbrecht JA, Moers K, Abecassis J, Rouau X, Delcour JA (2001) Influence of arabinoxylans and endoxylanases on pasta processing and quality. Production of high-quality pasta with increased levels of soluble fiber. Cereal Chem 78:721–729

Ito K, Ikemasu T, Ishikawa T (1992) Cloning and sequencing of the xynA gene encoding xylanase A of *Aspergillus kawachii*. Biosci Biotechnol Biochem 56(6):906–912

Jeong MY, Kim S, Yun CW, Choi YJ, Cho SG (2007) Engineering a de novo internal disulfide bridge to improve the thermal stability of xylanase from *Bacillus stearothermophilus* No. 236. J Biotechnol 127(2):300–309

Joo JC, Pack SP, Kim YH, Yoo YJ (2011) Thermostabilization of *Bacillus circulans* xylanase: computational optimization of unstable residues based on thermal fluctuation analysis. J Biotechnol 151(1):56–65

Joshi MD, Hedberg A, McIntosh LP (1997) Complete measurement of the pKa values of the carboxyl and imidazole groups in *Bacillus circulans* xylanase. Protein Sci 6(12):2667–2670

Jun H, Bing Y, Zhang K, Ding X, Daiwen C (2008) Expression of a *Trichoderma reesei* B-xylanase gene in *Escherichia coli* and activity of the enzyme on fiber-bound substrates. Protein Expr Purif 67:1–6

Kapoor M, Nair LM, Kuhad RC (2008) Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. Biochem Eng J 38:88–97

Karlsson EN, BartonekRoxa E, Holst O (1997) Cloning and sequence of a thermostable multidomain xylanase from the bacterium *Rhodothermus marinus*. Biophys Acta Gene Struct Expr 1353(2):118–124

Karlsson EN, Bartonek Roxa E, Holst O (1998) Evidence for substrate binding of a recombinant thermostable xylanase originating from *Rhodothermus marinus*. FEMS Microbiol Lett 168 (1):1–7

Karlsson EN, Holst O, Tocaj A (1999) Efficient production of truncated thermostable xylanases from Rhodothermus marinus in Escherichia coli fed-batch cultures. J Biosci Bioeng 87:598–606

Katapodis P, Christakopoulos P (2008) Enzymatic production of feruloyl xylo-oligosaccharides from corn cobs by a family 10 xylanase from *Thermoascus aurantiacus*. LWT Food Sci Technol 41(7):1239–1243

Khandeparkar R, Bhosle NB (2006) Purification and characterization of thermoalkalophilic xylanase isolated from the *Enterobacter* sp. MTCC 5112. Res Microbiol 157(4):315–325

Khasin A, Alchanati I, Shoham Y (1993) Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. Appl Environ Microbiol 59(6):1725–1730

Kimura T, Kitamoto N, Kito Y, Karita S, Sakka K, Ohmiya K (1998) Molecular cloning of xylanase gene xynG1 from *Aspergillus oryzae* KBN 616, a shoyu koji mold, and analysis of its expression. J Ferm Bioeng 85(1):10–16

Kimura T, Ito J, Kawano A, Makino T, Kondo H, Karita S, Sakka K, Ohmiya K (2000) Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. Biosci Biotechnol Biochem 64(6):1230–1237

Kitamoto N, Yoshino S, Ohmiya K, Tsukagoshi N (1999) Purification and characterization of the over expressed *Aspergillus oryzae* xylanase, XynF1. Biosci Biotechnol Biochem 63 (10):1791–1794

Ko EP, Akatsuka H, Moriyama H, Shinmyo A, Hata Y, Katsube Y, Urabe I, Okada H (1992) Site-directed mutagenesis at aspartate and glutamate residues of xylanase from *Bacillus pumilus*. Biochem J 288(Pt 1):117–121

Ko CH, Tsai CH, Tu J, Yang BY, Hsieh DL, Jane WN, Shih TL (2011) Identification of *Paenibacillus* sp. 2S-6 and application of its xylanase on biobleaching. Int Biodeterior Biodegrad 65:334–339

Kohli U, Nigam P, Singh D, Chaudhary K (2001) Thermostable, alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalophilus* subgroup C. Enzyme Microb Technol 28(7–8):606–610

Kolenova K, Vrsanska M, Biely P (2006) Mode of action of endo-beta-1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. J Biotechnol 121:338–345

Kormelink FJ, Gruppen H, Vietor RJ, Voragen AG (1993) Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans. Carbohydr Res 249(2):355–367

Krengel U, Dijkstra BW (1996) Three-dimensional structure of Endo-1,4-beta-xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. J Mol Biol 263(1):70–78

Kudo T, Ohkoshi A, Horikoshi K (1985) Molecular cloning and expression of a xylanase gene of alkalophilic *Aeromonas* sp. no. 212 in *Escherichia coli*. J Gen Microbiol 131(10):2825–2830

Kui H, Luo H, Shi P, Bai Y, Yuan T, Wang Y, Yang P, Dong S, Yao B (2010) Gene cloning, expression, and characterization of a thermostable xylanase from *Nesterenkonia xinjiangensis* CCTCC AA001025. Appl Biochem Biotechnol 162(4):953–965

Kulkarni N, Lakshmikumaran M, Rao M (1999) Xylanase II from an alkaliphilic thermophilic *Bacillus* with a distinctly different structure from other xylanases: evolutionary relationship to alkaliphilic xylanases. Biochem Biophys Res Commun 263(3):640–645

Kumar KS, Manimaran A, Permaul K, Singh S (2009) Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. J Biosci Bioeng 107(5):494–498

Kumar PR, Eswaramoorthy S, Vithayathil PJ, Viswamitra MA (2000) The tertiary structure at 1.59 Å resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *Paecilomyces varioti* Bainier. J Mol Biol 295(3):581–593

Kumar V, Satyanarayana T (2011) Applicability of thermo-alkali-stable and cellulase-free xylanase from a novel thermo-halo-alkaliphilic *Bacillus haloduransin* producing xylooligosaccharides. Biotechnol Lett 33:2279–2285

La Grange DC, Claeyssens M, Pretorius IS, Van Zyl WH (2000) Coexpression of the *Bacillus pumilus* beta-xylosidase (xynB) gene with the *Trichoderma reesei* beta xylanase 2 (xyn2) gene in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 54(2):195–200

Lagaert S, Van Campenhout S, Pollet A, Bourgois TM, Delcour JA, Courtin CM, Volckaert G (2007) Recombinant expression and characterization of a reducing-end xylose-releasing exo-oligoxylanase from *Bifidobacterium adolescentis*. Appl Environ Microbiol 73:5374–5377

Lama L, Calandrelli V, Gambacorta A, Nicolaus B (2004) Purification and characterization of thermostable xylanase and beta-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus*. Res Microbiol 155(4):283–289

Lamed R, Setter E, Bayer EA (1983) Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. J Bacteriol 156(2):828–836

Lappalainen A, Siika-aho M, Kalkkinen N, Fagerstrom R, Tenkanen M (2000) Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points. Biotechnol Appl Biochem 31:61e68

Larson SB, Day J, Barba de la Rosa AP, Keen NT, McPherson A (2003) First crystallographic structure of a xylanase from glycoside hydrolase family 5: implications for catalysis. Biochemistry 42(28):8411–8422

Lee J (1997) Biological conversion of lignocellulosic biomass to ethanol. J Biotechnol 56(1):1–24

Lee JM, Hu Y, Zhu H, Cheng KJ, Krell PJ, Forsberg CW (1993) Cloning of a xylanase gene from the ruminal fungus *Neocallimastix patriciarum* 27 and its expression in *Escherichia coli*. Can J Microbiol 39(1):134–139

Lee TH, Lim PO, Lee YE (2007) Cloning, characterization, and expression of xylanase A gene from *Paenibacillus* sp. DG-22 in *Escherichia coli*. J Microbiol Biotechnol 17(1):29–36

Levasseur A, Asther M, Record E (2005) Overproduction and characterization of xylanase B from *Aspergillus niger*. Can J Microbiol 51(2):177–183

Li XL, Ljungdahl LG (1996) Expression of *Aureobasidium pullulans* xynA in, and secretion of the xylanase from *Saccharomyces cerevisiae*. Appl Environ Microbiol 62(1):209–213

Li JH, Wang LS (2011) Why substituting the asparagine at position 35 in *Bacillus circulans* xylanase with an aspartic acid remarkably improves the enzymatic catalytic activity? A quantum chemistry-based calculation study. Polym Degrad Stab 96(5):1009–1014

Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101(13):4900–4906

Li XL, Skory CD, Ximenes EA, Jordan DB, Dien BS, Hughes SR, Cotta MA (2007) Expression of an AT-rich xylanase gene from the anaerobic fungus Orpinomyces sp. strain PC-2 in and secretion of the heterologous enzyme by Hypocrea jecorina. Appl Microbiol Biotechnol 74:1264–1275

Liu JR, Duan CH, Zhao X, Tzen JT, Cheng KJ, Pai CK (2008) Cloning of a rumen fungal xylanase gene and purification of the recombinant enzyme via artificial oil bodies. Appl Microbiol Biotechnol 79(2):225–233

Liu MQ, Weng XY, Sun JY (2006) Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan. Protein Expr Purif 48(2):292–299

Lo YC, Lu WC, Chen CY, Chen WM, Chang JS (2010) Characterization and high-level production of xylanase from an indigenous cellulolytic bacterium *Acinetobacter junii* F6-02 from southern Taiwan soil. Biochem Eng J 53(1):77–84

Lundgren KR, Bergkvist L, Hogman S, Joves H, Eriksson G, Bartfai T, Vanderlaan J, Rosenberg E, Shoham Y (1994) TCF mill trial on softwood pulp with korsnas thermostable and alkaline stable xylanase T6. Fems Microbiology Reviews 13(2–3):365–368

Luthi E, Love DR, McAnulty J, Wallace C, Caughey PA, Saul D, Bergquist PL (1990) Cloning, sequence analysis, and expression of genes encoding xylan-degrading enzymes from the thermophile *Caldocellum saccharolyticum*. Appl Environ Microbiol 56(4):1017–1024

Lynd LR, Cushman JH, Nichols RJ, Wyman CE (1991) Fuel ethanol from cellulosic biomass. Science 251(4999):1318–1323

Mach-Aigner A, Pucher ME, Mach RL (2010) D-Xylose as a repressor or inducer of xylanase expression in *Hypocrea jecorina* (*Trichoderma reesei*). Appl Environ Microbiol 76:1770–1776

Mamo G, Hatti-Kaul R, Mattiasson B (2006) A thermostable alkaline active endo-b-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterization. Enzyme Microb Technol 39:1492–1498

Mamo G, Hatti-Kaul R, Mattiasson B (2007) Fusion of carbohydrate binding modules from *Thermotoga neapolitana* with a family 10 xylanase from *Bacillus halodurans* S7. Extremophiles 11:169–177

Mannarelli BM, Evans S, Lee D (1990) Cloning, sequencing, and expression of a xylanase gene from the anaerobic ruminal bacterium *Butyrivibrio fibrisolvens*. J Bacteriol 172(8):4247–4254

Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009) New improvements for lignocellulosic ethanol. Curr Opin Biotechnol 20:372–380

Marrone L, McAllister KA, Clarke AJ (2000) Characterization of function and activity of domains A, B and C of xylanase C from *Fibrobacter succinogenes* S85. Protein Eng 13(8):593–601

McCarthy AA, Morris DD, Bergquist PL, Baker EN (2000) Structure of XynB, a highly thermostable beta 1,4-xylanase from *Dictyoglomus thermophilum* Rt46B.1, at 1.8 Å resolution. Acta Crystallogr D56:1367–1375

Mirande C, Mosoni P, Bera-Maillet C, Bernalier-Donadille A, Forano E (2010) Characterization of Xyn10A, a highly active xylanase from the human gut bacterium *Bacteroides xylanisolvens* XB1A. Appl Microbiol Biotechnol 87(6):2097–2105

Moreau A, Durand S, Morosoli R (1992) Secretion of a *Cryptococcus albidus* xylanase in *Saccharomyces cerevisiae*. Gene 116(1):109–113

Morris DD, Gibbs MD, Bergquist PL (1996) Cloning of a family G xylanase gene (XYNB) from the extremely thermophilic bacterium *Dictyoglomus thermophilum* and action of the gene product on kraft pulp. Am Chem Soc 211:234

Muilu J, Torronen A, Perakyla M, Rouvinen J (1998) Functional conformational changes of endo-1,4-xylanase II from *Trichoderma reesei*: a molecular dynamics study. Proteins 31:434–444

Mullai P, Fathima NSA, Rene ER (2010) Statistical analysis of main and interaction effects to optimize xylanase production under submerged cultivation conditions. J Agric Sci 2 (1):144–153

Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993) Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp strain 41 m-1. World J Microbiol Biotechnol 9(2):221–224

Narang S, Satyanarayana T (2001) Thermostable alpha-amylase production by an extreme thermophile *Bacillus thermooleovorans*. Lett Appl Microbiol 32:31–35

Nascimento RP, Coelho RRR, Marques S, Alves L, Girio FM, Bon EPS, Amaral-Collaco MT (2002) Production and partial characterisation of xylanase from *Streptomyces* sp strain AMT-3 isolated from Brazilian cerrado soil. Enzyme Microb Technol 31(4):549–555

Natesh R, Bhanumoorthy P, Vithayathil PJ, Sekar K, Ramakumar S, Viswamitra MA (1999) Crystal structure at 1.8 angstrom resolution and proposed amino acid sequence of a thermostable xylanase from *Thermoascus aurantiacus*. J Mol Biol 288(5):999–1012

Ogasawara W, Shida Y, Furukawa T, Shimada R, Nakagawa S, Kawamura M, Yagyu T, Kosuge A, Xu J, Nogawa M, Okada H, Morikawa Y (2006) Cloning, functional expression and promoter analysis of xylanase III gene from *Trichoderma reesei*. Appl Microbiol Biotechnol 72(5):995–1003

Ohta K, Moriyama S, Tanaka H, Shige T, Akimoto H (2001) Purification and characterization of an acidophilic xylanase from *Aureobasidium pullulans* var. *melanigenum* and sequence analysis of the encoding gene. J Biosci Bioeng 92(3):262–270

Okeke BC, Obi SKC (1994) Lignocellulose and sugar compositions of some agro-waste materials. Bioresour Technol 47(3):283–284

Olfa E, Mondher M, Issam S, Ferid L, Nejib NM (2007) Induction, properties and application of xylanase activity from *Sclerotinia sclerotiorum* S2 fungus. J Food Chem 31:96–107

Paës G, Berrin J-G, Beaugrand J (2012) GH11 xylanases: structure/function/properties relationships and applications. Biotechnol Adv 30:564–592

Paës G, O'Donohue MJJ (2006) Engineering increased thermostability in the thermostable GH-11 xylanase from *Thermobacillus xylanilyticus*. J Biotechnol 125:338–350

Payan F, Leone P, Porciero S, Furniss C, Tahir T, Williamson G, Durand A, Manzanares P, Gilbert HJ, Juge N, Roussel A (2004) The dual nature of the wheat xylanase protein inhibitor XIP-I: structural basis for the inhibition of family 10 and family 11 xylanases. J Biol Chem 279 (34):36029–36037

Payan F, Flatman R, Porciero S, Williamson G, Juge N, Roussel A (2003) Structural analysis of xylanase inhibitor protein I (XIP-I), a proteinaceous xylanase inhibitor from wheat (*Triticum aestivum* var *soisson*). Biochem J 372(Pt 2):399–405

Petrescu I, Lamotte-Brasseur J, Chessa JP, Ntarima P, Claeyssens M, Devreese B, Marino G, Gerday C (2000) Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles 4(3):137–144

Pokhrel S, Joo JC, Yoo YJ (2013) Shifting the optimum pH of *Bacillus circuulans* xylanase towards acidic side by introducing arginine. Biotechnol Bioprocess Eng 18:35–42

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591

Pollet A, Schoepe J, Dornez E, Strelkov SV, Delcour JA, Courtin CM (2010) Functional analysis of glycosidehydrolase family 8 xylanases shows narrow but distinct substrate specificities and biotechnological potential. Appl Microbiol Biotechnol 87:2125–2135

Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13 (1):101–132

Puchkaev AV, Koo LS, Ortiz de Montellano PR (2003) Aromatic stacking as a determinant of the thermal stability of CYP119 from *Sulfolobus solfataricus*. Arch Biochem Biophys 409:52–58

Puls J, Tenkanen M, Korte HE, Poutanen K (1991) Products of hydrolysis of beechwood acetyl-4-O-methylglucuronoxylan by a xylanase and an acetyl xylan esterase. Enzyme Microb Technol 13(6):483–486

Qureshy AF, Khan LA, Khanna S (2002) Cloning, regulation and purification of cellulase-free xylanase from *Bacillus circulans* Teri-42. Ind J Microbiol 42:35–41

Raedschelders G, Debefve C, Goesaert H, Delcour JA, Volckaert G, Van Campenhout S (2004) Molecular identification and chromosomal localization of genes encoding *Triticum aestivum* xylanase inhibitor I-like proteins in cereals. Theor Appl Genet 109(1):112–121

Ratto M, Poutanen K, Viikari L (1992) Production of xylanolytic enzymes by an alkalitolernt *Bacillus circulans* stain. Appl Microbiol Biotechnol 37:470–473

Rizzatti ACS, Jorge JA, Terenzi HF, Rechia CGV, Polizeli MLTM (2001) Purification and properties of a thermostable extracellular β-xylosidase produced by a thermotolerant *Aspergillus phoenicis*. J Ind Microbiol Biotechnol 26:156–160

Rose SH, van Zyl WH (2002) Constitutive expression of the *Trichoderma reesei* beta-1,4-xylanase gene (xyn2) and the beta-1,4-endoglucanase gene (egl) in *Aspergillus niger* in molasses and defined glucose media. Appl Microbiol Biotechnol 58(4):461–468

Rouau X (1993) Investigations into the effects of an enzyme preparation for baking on what flour dough pentosans. J Cereal Sic 18:145–157

Rouau X, Daviet S, Tahir T, Cherel B, Saulnier L (2006) Effect of the proteinaceous wheat xylanase inhibitor XIP-I on the performances of an *Aspergillus niger* xylanase in bread making. J Sci Food Agric 86(11):1604–1609

Ruanglek V, Sriprang R, Ratanaphan N, Tirawongsaroj P, Chantasigh D, Tanapongpipat S, Pootanakit K, Eurwilaichitr L (2007) Cloning, expression, characterization, and high cell-density production of recombinant endo-1,4-beta-xylanase from *Aspergillus niger* in *Pichia pastoris*. Enzyme Microb Technol 41(1–2):19–25

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30(5):279–291

Sakka K, Maeda Y, Hakamada Y, Takahashi N, Shimada K (1991) Purification and some properties of xylanase from *Clostridium stercorarium* strain Hx-1. Agric Biol Chem 55 (1):247–248

Salles BC, Cunha RB, Fontes W, Sousa MV, Filho EXF (2000) Purification and characterization of a new xylanase from *Acrophialophora nainiana*. J Biotechnol 81(2–3):199–204

Salles BC, Te'o VS, Gibbs MD, Bergquist PL, Filho EX, Ximenes EA, Nevalainen KM (2007) Identification of two novel xylanase-encoding genes (xyn5 and xyn6) from *Acrophialophora nainiana* and heterologous expression of xyn6 in *Trichoderma reesei*. Biotechnol Lett 29 (8):1195–1201

Sanghi A, Garg N, Kuhar K, Kuhad RC, Gupta VK (2009) Enhanced production of cellulase-free xylanase by alkalophilic *Bacillus subtilis* ASH and its application in biobleaching of kraft pulp. BioResources 4:1109–1129

Sandhu JS, Kennedy JF (1984) Molecular cloning of Bacillus polymyxa (1–4)-P-D-xylanase gene in Escherichia coli. Enzyme Microb Technol 6:271–274

Sapre MP, Jha H, Patil MB (2005) Purification and characterization of a thermoalkalophilic xylanase from *Bacillus* sp. World J Microbiol Biotechnol 21(5):649–654

Schlacher A, Holzmann K, Hayn M, Steiner W, Schwab H (1996) Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus*. J Biotechnol 49 (1–3):211–218

Schulze E (1891) Amphibia Europaea, annual report and treatises society of natural scientists in Magdeburg 1890:163–178

Shallom D, Shoham Y (2003) Microbial hemicellulases. Curr Opin Microbiol 6(3):219–228

Shao W, Wiegel J (1995) Purification and characterization of two thermostable acetyl xylan esterases from *Thermoanaerobacterium* sp. strain JW/SL-YS485. Appl Environ Microbiol 61 (2):729–733

Sharma A, Adahikari S, Styanarayana T (2007) Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile *Geobacillus thermoleovorans*. World J Microbiol Biotechnol 23:483–490

Shatalov AA, Pereira AA (2007) Xylanase pre-treatment of giant reed organosolv pulps: direct bleaching effect and bleach boosting. Ind Crops Prod 25:248–256

Shibuya H, Kaneko S, Hayanshi K (2000) Enhancement of thermostability and hydrolytic activity of xylananse by random gene shuffling. Biochem J 349:651–656

Shrinivas D, Savitha G, Raviranjan K, Naik GR (2010) A highly thermostable alkaline cellulase-free xylanase from thermoalkalophilic *Bacillus* sp. JB 99 suitable for paper and pulp industry: purification and characterization. Appl Biochem Biotechnol 162(7):2049–2057

Sidhu G, Withers SG, Nguyen NT, McIntosh LP, Ziser L, Brayer GD (1999) Sugar ring distortion in the glycosyl-enzyme intermediate of a family G/11 xylanase. Biochemistry 38 (17):5346–5354

Silva CHC, Puls J, de Sousa MV, Ferreira EX (1999) Purification and characterization of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. Rev Microbiol 30(2):114–119

Simpson HD, Haufler UR, Daniel RM (1991) An extremely thermostable xylanase from the thermophilic bacterium Thermotoga. Biochem J 277(2):413–417

Simpson PJ, Bolam DN, Cooper A, Ciruela A, Hazlewood GP, Gilbert HJ, Williamson MP (1999) A family IIb xylan-binding domain has a similar secondary structure to a homologous family IIa cellulose-binding domain but different ligand specificity. Structure 7(1999):853–864

Singh S, Pillay B, Dilsook V, Prior BA (2000) Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. J Appl Microbiol 88:975–982

Sorensen JF, Sibbesen O (2006) Mapping of residues involved in the interaction between the *Bacillus subtilis* xylanase A and proteinaceous wheat xylanase inhibitors. Protein Eng Des Sel 19(5):205–210

Sriprang R, Asano K, Gobsuk J, Tanapongpipat S, Champreda V, Eurwilaichitr L (2006) Improvement of thermostability of fungal xylanase by using site-directed mutagenesis. J Biotechnol 126(4):454–462

Srivastava R, Srivastava AK (1993) Characterization of a bacterial xylanase resistant to repression by glucose and xylose. Biotechnol Lett 15(8):847–852

Sriyapai T, Somyoonsap P, Matsui K, Kawai F, Chansiri K (2011) Cloning of a thermostable xylanase from *Actinomadura* sp. S14 and its expression in *Escherichia coli* and *Pichia pastoris*. J Biosci Bioeng 111(5):528–536

St John FJ, Godwin DK, Preston JF, Pozharski E, Hurlbert JC (2009) Crystallization and crystallographic analysis of *Bacillus subtilis* xylanase C. Acta Crystallogr Sect F Struct Biol Crystallogr Commun 65(Pt 5):499–503

Strauss J, Mach RL, Zeilinger S, Hartler G, Stoffler G, Wolschek M, Kubicek CP (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. FEBS Lett 376:103–107

Stephens DE, Singh S, Permaul K (2009) Error-prone PCR of a fungal xylanase for improvement of its alkaline and thermal stability. FEMS Microbiol Lett 293(1):42–47

Subramaniyan S (2000) Studies on the production of bacterial xylanases. Ph.D. Thesis, Cochin University of Science and Technology, India

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and application. Crit Rev Biotechnol 22:33–46

Sun JY, Liu MQ, Weng XY, Qian LC, Gu SH (2007) Expression of recombinant *Thermomonospora fusca* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. Food Chem 104(3):1055–1064

Sunna A, Prowe SG, Stoffregen T, Antranikian G (1997) Characterization of the xylanases from the new isolated thermophilic xylan-degrading *Bacillus thermoleovorans* strain K-3d and *Bacillus flavothermus* strain LB3A. FEMS Microbiol Lett 148(2):209–216

Suzuki T, Ibata K, Hatsu M, Takamizawa K, Kawai K (1997) Cloning and expression of a 58-kDa xylanase VI gene (xynD) of *Aeromonas caviae* ME-1 in *Escherichia coli* which is not categorized as a family F or family G xylanase. J Ferm Bioeng 84(1):86–89

Takahashi-Ando N, Inaba M, Ohsato S, Igawa T, Usami R, Kimura M (2007) Identification of multiple highly similar XIP-type xylanase inhibitor genes in hexaploid wheat. Biochem Biophys Res Commun 360(4):880–884

Tanaka H, Okuno T, Moriyama S, Muguruma M, Ohta K (2004) Acidophilic xylanase from *Aureobasidium pullulans*: efficient expression and secretion in *Pichia pastoris* and mutational analysis. J Biosci Bioeng 98(5):338–343

Taneja K, Gupta S, Kuhad RC (2002) Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresour Technol 85 (1):39–42

Teng C, Jia H, Yan Q, Zhou P, Jiang Z (2011) High-level expression of extracellular secretion of a β-Xylosidase gene from Paecilomyces thermophila in Escherichia coli. Bioresour Technol 102:1822–1830

Tomme P, Gilkes NR, Miller RC, Warren AJ, Kilburn DG (1994) An internal cellulose-binding domain mediates adsorption of an engineered bifunctional xylanase/cellulase. Protein Eng 7 (1):117–123

Torronen A, Harkki A, Rouvinen J (1994) 3-Dimensional structure of endo-1,4-beta-xylanase-II from *Trichoderma reesei*-2 conformational states in the active-site. EMBO J 13 (11):2493–2501

Torronen A and Rouvinen J (1997) Structural and functional properties of low molecular weight endo-1,4-beta-xylanases. J Biotechnol 57:137–149

Trudel J, Grenier J, Potvin C, Asselin A (1998) Several thaumatin-like proteins bind to beta-1,3-glucans. Plant Physiology 118:1431–1438

Tsai CT, Huang CT (2008) Overexpression of the *Neocallimastix frontalis* xylanase gene in the methylotrophic yeasts *Pichia pastoris* and *Pichia methanolica*. Enzyme Microb Technol 42 (6):459–465

Turunen O, Etuaho K, Fenel F, Vehmaanpera J, Wu XY, Rouvinen J, Leisola M (2001) A combination of weakly stabilizing mutations with a disulfide bridge in the alpha-helix region of *Trichoderma reesei* endo-1,4-beta-xylanase II increases the thermal stability through synergism. J Biotechnol 88(1):37–46

Valenzuela SV, Diaz P, Javier Pastor FI (2010) Recombinant expression of an alkali stable GH10 xylanase from *Paenibacillus barcinonensis*. J Agric Food Chem 58(8):4814–4818

Van Campenhout S, Pollet A, Bourgois TM, Rombouts S, Beaugrand J, Gebruers K, De Backer E, Courtin CM, Delcour JA, Volckaert G (2007) Unprocessed barley aleurone endo-beta-1,4-xylanase X-I is an active enzyme. Biochem Biophys Res Commun 356 (3):799–804

Van Der Borght A, Goesaert H, Veraverbeke WS, Delcour JA (2005) Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. J Cereal Sci 41:221–237

Van Peij NN, Brinkmann J, Vrsanska M, Visser J, de Graaff LH (1997) Beta-xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. Eur J Biochem 245(1):164–173

Van Petegem F, Collins T, Meuwis MA, Gerday C, Feller G, Van Beeumen J (2003) The structure of a cold-adapted family 8 xylanase at 1.3 Å resolution. Structural adaptations to cold and investigation of the active site. J Biol Chem 278(9):7531–7539

Vandeplas S, Dauphin RD, Thonart P, Thewis A, Beckers Y (2010) Effect of the bacterial or fungal origin of exogenous xylanases supplemented to a wheat-based diet on performance of broiler chickens and nutrient digestibility of the diet. Can J Anim Sci 90:221–228

Vardakou M, Flint J, Christakopoulos P, Lewis RJ, Gilbert HJ, Murray JW (2005) A family 10 *Thermoascus aurantiacus* xylanase utilizes arabinose decorations of xylan as significant substrate specificity determinants. J Mol Biol 352:1060–1067

Verjans P, Dornez E, Delcour JA, Courtin CM (2010) Selectivity for water-unextractable arabinoxylan and inhibition sensitivity govern the strong bread improving potential of an acidophilic GH11 *Aureobasidium pullulans* xylanase. Food Chem 123:331–337

Verma D, Satyanarayana T (2012) Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Bioresour Technol 107:333–338

Wang JS, Bai YG, Yang PL, Shi PJ, Luo HY, Meng K, Huang HQ, Yin J, Yao B (2010) A new xylanase from thermoalkaline *Anoxybacillus* sp E2 with high activity and stability over a broad pH range. World J Microbiol Biotechnol 26(5):917–924

Wang Q, Xia T (2008) Enhancement of the activity and alkaline pH stability of *Thermobifida fusca* xylanase A by directed evolution. Biotechnol Lett 30(5):937–944

Whitehead TR, Hespell RB (1989) Cloning and expression in *Escherichia coli* of a xylanase gene from *Bacteroides ruminicola* 23. Appl Environ Microbiol 55(4):893–896

Winterhalter C, Liebl W (1995) Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl Environ Microbiol 61:1810–1815

Wong KK, Tan LU, Saddler JN (1988) Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52(3):305–317

Wu SJ, Liu B, Zhang XB (2006) Characterization of a recombinant thermostable xylanase from deep-sea thermophilic *Geobacillus* sp MT-1 in East Pacific. Appl Microbiol Biotechnol 72 (6):1210–1216

Xiong H, Turunen O, Pastinen O, Leisola M, von Weymarn N (2004) Improved xylanase production by *Trichoderma reesei* grown on L-arabinose and lactose or D-glucose mixtures. Appl Microbiol Biotechnol 64:353–358

Xiong H, Weymarn N, Turunen O, Leisola M, Pastinen O (2005) Xylanase production by *Trichoderma reesei* Rut C-30 grown on L-arabinose-rich plant hydrolysates. Bioresour Technol 96:753–759

Xue GP, Gobius KS, Orpin CG (1992) A novel polysaccharide hydrolase cDNA (celD) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities. J Gen Microbiol 138(11):2397–2403

Yang HJ, Xie CY (2010) Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn stalks in *in vitro* enzymatic and ruminal batch cultures. Anim Feed Scie Technol 159:110–121

Yang JH, Park JY, Kim SH, Yoo YJ (2008) Shifting pH optimum of *Bacillus circulans* xylanase based on molecular modeling. J Biotechnol 133(3):294–300

Yang RC, MacKenzie CR, Narang SA (1988) Nucleotide sequence of a *Bacillus circulans* xylanase gene. Nucleic Acids Res 16(14B):7187

Yamura I, Koga T, Matsumoto T, Kato T (1997) Purification and some properties of endo-1,4-[5-D-xylanase from a fresh water mollusc, *Pomacea insularus* (de Ordigny). Biosci Biotechol Biochem 61(4):615–620

Yin LJ, Lin HH, Chiang YI, Jiang ST (2010) Bioproperties and purification of xylanase from *Bacillus* sp. YJ6. J Agric Food Chem 58(1):557–562

You C, Yuan H, Huang Q, Lu H (2010) Substrate molecule enhances the thermostability of a mutant of a family 11 xylanase from Neocallimastix patriciarum. African J Biotechnol 9:1288–1294

Yuan Q, Wang J, Zhang H, Qian Z (2005) Effect of temperature shift on production of xylanase by *Aspergillus niger*. Process Biochem 40:3255–3257

Zhao Y, Chany CJ, Sims PF, Sinnott ML (1997) Definition of the substrate specificity of the 'sensing' xylanase of *Streptomyces cyaneus* using xylooligosaccharide and cellooligosaccharide glycosides of 3,4-dinitrophenol. J Biotechnol 57(1–3):181–190

Zhou CY, Bai JY, Deng SS, Wang J, Zhu J, Wu MC, Wang W (2008a) Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. Bioresour Technol 99:831–838

Zhou CY, Li DF, Wu MC, Wang W (2008b) Optimized expression of an acid xylanase from *Aspergillus usamii* in *Pichia pastoris* and its biochemical characterization. World J Microbiol Biotechnol 24(8):1393–1401

Zhu H, Paradis FW, Krell PJ, Phillips JP, Forsberg CW (1994) Enzymatic specificities and modes of action of the two catalytic domains of the XynC xylanase from *Fibrobacter succinogenes* S85. J Bacteriol 176(13):3885–3894

Chapter 8
Mannanase

Suttipun Keawsompong

Abstract β-Mannanases (endo-1,4-β-D-mannanase) is endohydrolase that catalyze the random hydrolysis of the β-1,4-D-mannopyranosyl linkage within the main chain of various mannan-based polysaccharides to yield mannooligosaccharides products. β-Mannanase have been isolated and characterized from different sources including bacteria, fungi, higher plants, and animals. However, microbial mannanases are wildly used in the industrial application. β-Mannanases was classified based on the amino acid sequence similarity into glycoside hydrolase (GH) families 5 and 26 and a few member of family 113. These enzymes from different organisms have different properties such as enzyme activity, optimal pH, and optimal temperature. So, β-mannanases with high specific activity and remarkable enzymatic properties are required for the application. β-Mannanase is very useful enzyme that has been used in several industrial applications including food, feed, pulp, and paper industries. This enzyme can be used to improve the bleaching of pulp by facilitating the release of lignin from paper pulp leading to the reduction of chemical reagents. It can be used to reduce the viscosity of instant coffee and to clarify fruit juices and wines in food industry. β-Mannanase also have been used as animal feed additive enzyme to increase the nutritional value of animal feed components. Moreover, there is increasing interest in using β-mannanase to produce mannooligosaccharides (MOS) which have the prebiotic properties from natural mannan-based substrates. This enzyme is also used for the pretreatment of biomass in the bioethanol production.

S. Keawsompong (✉)
Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University,
Bangkok, Thailand
e-mail: suttipun.k@ku.ac.th

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_8

8.1 Mannans

Mannans and heteromannans are widely distributed in nature as part of the hemicellulose fraction in hardwoods and softwoods, seeds of leguminous plants (Handford et al. 2003; Buckeridge et al. 2000), and in beans (Lundqvist et al. 2003). Hemicelluloses are copolymers of both hexose and pentose sugars. The branched structure allows hemicellulose to exist in an amorphous form that is more susceptible to hydrolysis. Within biomass, mannans or the hemicelluloses are situated between the lignin and the collection of cellulose fibers underneath. Consistent with their structure and side group substitutions, mannans seem to be interspersed and covalently linked with lignins at various points while producing a coat around underlying cellulose strands via hydrogen bonds, but as few H-bonds are involved they are much more easily broken down than cellulose. The mannan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose maybe important in maintaining the integrity of the cellulose in situ and in helping to protect the fibers against degradation to cellulases (Puls and Schuseil 1993).

Mannan is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms, but is the minor hemicellulose in hardwood from angiosperms (Puls and Schuseil 1993). Unsubstituted β-1,4-mannan, composed of a main chain of β-mannose residues (Fig. 8.1), is an important structural component of some marine algae and terrestrial plants such as ivory nut (Chanzy et al. 2004) and coffee bean (Nunes et al. 2006). It resembles cellulose in the conformation of the individual polysaccharide chains, and is water insoluble.

The galactomannans are mainly found in the seeds of the family of Leguminoseae and are located in the endospermic part of the seeds (Dea and Morrison 1975). Galactomannans consist of water-soluble 1,4-linked β-D-mannopyranosyl residues with side chains of single 1,6-linked α-D-galactopyranosyl groups (Shobha et al. 2005).

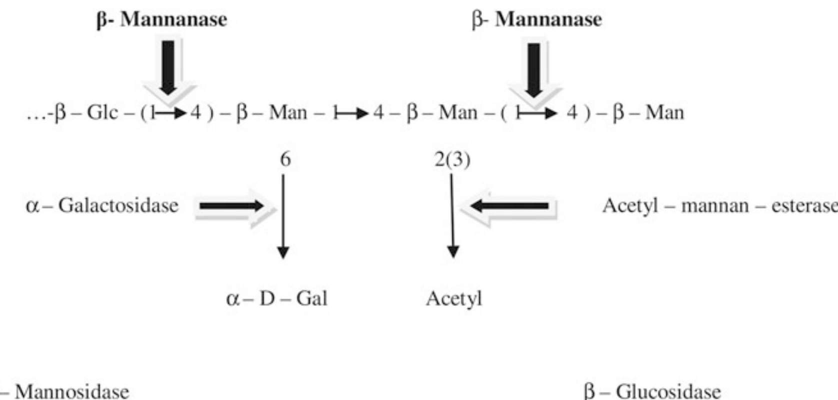
Glucomannan is a water-soluble polysaccharide that is considered a dietary fiber. It is a hemicellulose component in the cell walls of some plant species (Gonzáles et al. 2004). Glucomannan is a food additive used as an emulsifier and thickener. The polysaccharide consists of glucose and mannose in a proportion of 1:3 joined by β-(1,4) linkages (Popa and Spiridon 1998). Hardwoods consist of glucomannan with a glucose/mannose ratio of 1:1.5–2 (Hongshu et al. 2002).

Galactoglucomannan is a water-soluble hemicellulose, consisting of galactose, glucose, and mannose. Many softwood species, e.g., Norway spruce are rich of galactoglucomannans and can contain it up to 10–20 %. Galactoglucomannan consists of a backbone of randomly distributed (1 → 4)-linked mannose and glucose units with (1 → 6)-linked galactose units attached to mannose units. The hydroxyl groups in locations C2 and C3 in mannose are partially substituted by acetyl groups (Willför et al. 2007).

Fig. 8.1 Structure of **a** mannan, **b** galactomannan, **c** glucomannan, and **d** galactoglucomannan. *Source*: Dhawan and Kaur (2007)

8.2 Mannan-Degrading Enzymes

The mannan-degrading enzymes, a variety of hydrolytic enzymes involves in the degradation of mannan polysaccharides, are consisted of β-mannanase, β-mannosidase, β-glucosidase, and other addition enzymes such as acetyl mannan esterase and α-galactosidase (Moreira and Filho 2008). The overall mechanism of mannan-degrading enzymes is shown in Fig. 8.2. β-Mannanase, endo acting enzyme, randomly hydrolyzes the β-1,4-linked internal linkage of the mannan backbone to produce mannooligosacharides and new chain ends. β-Mannosidase, exo acting



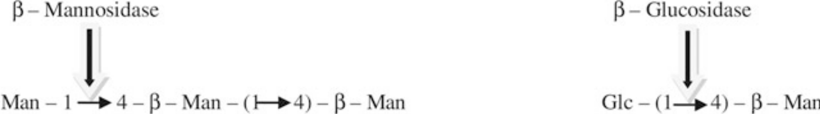


Fig. 8.2 Mechanism of mannan-degrading enzymes in the galactoglucomannan degradation. *Source* Puls and Schuseil (1993)

enzyme, cleaves β-1,4-linked mannosides and releases mannose from the nonreducing ends of mannan and mannooligosacharides. β-Glucosidases, exo acting enzyme, hydrolyzes β-1,4-glucopyranose at the nonreducing ends of oligosaccharides that released from glucomannan and galactoglucomannan degradation. In addition, the side chain sugars that attached at various points on mannan are removed by α-galactosidase and acetyl mannan esterase. α-Galactosidase hydrolyzes α-1,6-linked D-galactopyranose at side chain of galactomannan and galactoglucomannan. Acetyl mannan esterase releases acetyl groups from galactoglucomannan.

However, mannan degradation by these enzymes is affected by the degree and pattern of substitution of glucose and glucose residues in backbone of these polysaccharides (Van Zyl et al. 2010). In addition, pattern of distribution of *O*-acetyl group in glucomannan also affects the hydrolysis by the enzymes.

8.2.1 β-Mannanase

β-Mannanase or endo-1,4-β-D-mannanases (EC 3.2.1.78) is the endo hydrolase enzyme that catalyze the random hydrolysis of internal β-1,4-mannosidic linkages in backbone chain of mannan polysaccharides, producing various size of mannooligosacharides (Puls 1997). β-Mannanase requires at least four sugar residues of backbone chain for enzyme binding and efficient hydrolysis (Davies et al. 1997). β-Mannanase hydrolyzes mannooligosaccharides up to a degree of polymerization (DP) of 4 (Chauhan et al. 2012).

β-Mannanase is classified into glycoside hydrolases (GH) based on the sequence similarity. *Glycoside hydrolase* is a group of enzymes that hydrolyze the glycosidic bond between molecules in polysaccharides. Based on the amino acid sequence similarity, glycoside hydrolases are classified into 131 families. This classification is available on The Carbohydrate-Active Enzymes database (CAZy). Moreover, each glycoside hydrolase families can be classified into clan based on the catalytic domains and the protein structure. The different glycoside hydrolase families have been found to have different folds but, some enzymes from different families have related folds. Based on these criteria, β-mannanase is classified into glycoside hydrolase family 5, 26, and a few members in 113 (Dhawan and Kaur 2007; Zhang et al. 2008). Both of these families are classified into clan A which have a typical structure of $(\beta/\alpha)_8$-barrel fold (TIM barrel fold). The crystal structure of β-mannanases from both of GH family 5 and 26 showed that glutamic acid is conserved catalytic modules (nucleophiles and acid/base) that are positioned on the C-terminal of β4 and β7 strands, respectively. In addition, β-mannanase also has an open cleft with at least four subsites on the active site (Hogg et al. 2003; Zhang et al. 2008; Chauhan et al. 2012).

Glycosyl hydrolases consist of two hydrolysis mechanisms, retaining and inverting, leading to either overall retention or inversion of the anomeric configuration at the hydrolysis site (Henrissat et al. 1995). β-Mannanase hydrolyzes the mannan substrates by retaining mechanism by double displacement reaction (Fig. 8.3). For the retaining mechanism, two carboxylic acid residues on the active

Fig. 8.3 The retaining mechanism of β-mannanase. *Source* Chauhan et al. (2012)

site of enzyme involve in the hydrolysis as nucleophiles and acid/base catalytic module. In the double displacement reaction, β-mannanase enzyme attacks by a nucleophilic carboxylate on the anomeric carbon with concomitant releases of the aglycon, resulting in a covalent intermediate. Then, the covalent intermediate is attacked by a nucleophilic water lead to releasing of the glycoside from the enzyme (Chauhan et al. 2012).

Although, β-mannanases have the similar structure including active site, but there are difference in the presence and position of catalytic carbohydrate binding modules (CBMs) that may contain in the structure (Ximenes et al. 2005). CBMs is use for enhance β-mannanase activity for degrading of cellulose-conjugated mannan polysaccharide (Benech et al. 2007). β-Mannanases from GH family 5 contain CBMs that bind crystalline polysaccharides. For example, β-mannanase from *Cellulomonas fimi*, GH family 5, has a mannan-binding module that binds in a reversible way to soluble mannan, cellulose, chitin, and xylan (Stoll et al. 2000). Moreover, overexpression of mannanase gene that contains CBMs in *Escherichia coli* showed the activity of enzyme for several cellulose substrates (Ximenes et al. 2005). But, β-mannanases from GH family 26 lack of CBMs, so there are no enzyme activity in crystalline polysaccharides substrate (Hogg et al. 2003). So, this refers to the different substrate target of β-mannanase in GH family 5 and 26 in nature.

β-Mannanse enzymes have been isolated and characterized from several different sources such as bacteria, fungi, plants, and animals. Although the β-mannanases are widely distributed in microorganisms, higher plants, and animals but the microbial mannanases are regarded to be useful for their applications. Numerous bacterial and fungal β-mannanases have been identified and characterized from various strains in 20 different genuses. Among bacteria, most of β-mannanases producing strains are gram positive bacteria such as *Bacillus* species and *Clostridia* species (Dhawan and Kaur 2007). However, some gram negative bacterial strains have been reported to produce β-mannanases such as *Klebiella* sp. (Titapoka et al. 2008) and *Vibrio* sp. (Tamamura et al. 1995). The most of β-mannanases producing strains among fungi are in the genus *Aspergillus* while *Penicillium* sp. and *Trichoderma* sp. have also been reported (Chauhan et al. 2012). Moreover, some actinomycetes such as *Streptomyces* sp. and *Cellulomonas* sp. have been reported to be β-mannanases producing strains (Stoll et al. 2000; Bhoria et al. 2009).

Microbial manannanase are extracellular enzyme which could be induced by mannan polysaccharide (Moreira and Filho 2008; Chauhan et al. 2012). Many nutritional and physiochemical factors involve and influence in the β-mannanase production such as carbon and nitrogen sources, inorganic salts, temperature, pH, time, and dissolved oxygen concentration (Moreira and Filho 2008; Chauhan et al. 2012). There are differences in the incubation time and optimum temperature and pH for microbial β-mannanase production. For the incubation time, it ranges from 24 to 96 h in bacteria and 3–11 days in fungi. The optimum temperature for β-mannanase production is in the mesophilic range that corresponds with temperature for microbial growth. For the optimum pH, neutral to alkaline pH is the best

condition for bacterial growth, and production and acidic pH for fungi (Chauhan et al. 2012). However, bacterial β-mannanases are more thermostable than fungal β-mannanases that are important for industrial application, such as β-mannanases from *Bacillus* species have the advantages of high activity and convenient isolation and thus have been used in research and industry (Araujo and Ward 1990).

8.3 Application of β-Mannanase

β-Mannanase has been used in several industrial applications because of its broad substrate specificity.

8.3.1 Pulp and Paper Industry

β-Mannanase can be used in the enzymatic bleaching of softwood pulps to digest the mannan component without affecting the cellulose component. The use of β-mannanase with other enzymes is the alternative method that can equally facilitate lignin removal in pulp bleaching and give results comparable to alkaline pretreatment without the environmental pollution problems (Dhawan and Kaur 2007).

8.3.2 Detergent Industry

Alkaline β-mannanase with stable in detergents has been used in laundry segments as stain removal boosters. Mannans are generally used as thickening agents in several products such as hair gel, shampoo, conditioner, and toothpaste. The stains containing mannan are difficult to remove so this enzyme can cleave into smaller carbohydrate fragments that can reduce the stain cleaning process and remove during the washing. Moreover, β-mannanase can be formulated as sanitization products, contact lens cleanser and hard surface cleansers (Chauhan et al. 2012).

8.3.3 Food Industry

β-Mannanase has been used in the viscosity reduction of coffee bean extracts by hydrolyzing the mannan component in the coffee extract (Chauhan et al. 2012). It has been used in the maceration of fruit and vegetable materials and clarification of

fruit juices (Moreira and Filho 2008). In addition, β-mannanase can be used in enzymatic oil extraction of coconut. The enzymatic process can eliminate the problems of alfatoxin contamination and oxidative rancidity of products (Chauhan et al. 2012).

8.3.4 Feed Industry

β-Mannanase can be used to improve the nutritional value of animal feed especially poultry. Mannan polysaccharides are commonly found in feed ingredients such as soyabean meal, guar meal, copra meal, palm kernel meal, and sesame meal. All these meals have some common properties such as high fiber content, low palatability, lack of several essential amino acids, and high viscosity coupled with several anti-nutritional factors such as mannan, galactomannan, xylan, and arabinoxylan that limit the utilization in the animal intestine (Chauhan et al. 2012). Moreover, they have been found to be highly deleterious to animal performance, severely compromising weight gain and feed conversion as well as glucose and water absorption (Dhawan and Kaur 2007). Therefore, incorporation of β-mannanase in feed can help to cleave mannan and release of nutrients results in increased villus height in duodenum and jejunum that leads to increase in surface area and adsorption and decreased intestinal viscosity. So, it can improve both the weight gain and feed conversion efficiency (Adibmoradi and Mehri 2007). Hemicell supplied by ChemGen, USA is a fermentation product of *B. lentus* containing high amount of β mannanases that degrade mannan in feed (Daskiran et al. 2004).

8.3.5 Mannooligosaccharide Production

Mannooligosaccharide (MOS) is non-oligosaccharides digestibles (NODs) that can be produced from mannan polysaccharides. MOS is widely used in nutrition as a natural additive which can improve gastrointestinal health and also overall health by supporting the microflora in the digestive system.

MOS affects bacterial attachment in the intestinal tract leading to the reduction in the prevalence and concentration of different strains of *Salmonella*, as well as *E. coli*. Moreover, it effects on promoting beneficial bacteria, such as *Lactobacillus and Bifidobacteria* (Spring et al. 2000). MOS helps to limit the pathogenic microorganisms bacteria by blocking the colonization on the intestinal mucosa lead so they cannot physically reach or adhere to the intestinal cells and are eliminated from body.

Large surface area is a key for optimal digestive function of small intestine so the surface of the small intestine should be covered with long healthy villi. Several studies of MOS in poultry have looked at the intestinal structure and discovered

longer villi and a more shallow crypt (Yang et al. 2008; Baurhoo et al. 2009). These studies showed the results that MOS could increase the energy of digestion and production of digestive enzymes such as alkaline phosphatase, maltase, and leucine aminopeptidase. Moreover, MOS could increase the goblet cells, mucus-producing cells so the villi and intestinal surface could be more protected.

8.3.6 Pharmaceutical Applications

Mannose has been used as a component of medicine because of its properties such as fast dissolving and structure forming (Chauhan et al. 2012). Mannose also has been used as a remedy for urinary tract infection (Van Zyl et al. 2010). There is a significant increase in using this sugar, so β-mannanase and other enzymes can be used for the economical production of mannose from low-cost mannan substrates such as palm kernel cake and copra meal. Guar gum has the positive effects on some physiological functions like reducing plasma cholesterol and body fat without reducing protein utilization and increased fecal excretion volume (Takeno et al. 1990). Therefore, a partially hydrolyzed guar gum (PHGG) with β-mannanase is used in beverage form for treatment of several diseases such as irritable bowel syndrome (IBS). PHGG can increase stool weight and decrease colon transit time by providing non-digestible bulk, retaining water, and serving as a substrate for microbial growth in the colon (Parisi et al. 2002). In addition, PHGG supplemented with oral rehydration solution is also used for the treatment of acute diarrhea in children by providing short chain fatty acids in large intestine and maintaining the balance of salt and water (Alam et al. 2000).

8.3.7 Pretreatment of Biomass in the Bioethanol Production

The molecules in wood and soft wood are linked together in complexes known as cellulose and hemicelluloses and together can represent up to 75 % of dried wood weight. Moreover, sugar and nutrient is found in wood tissue and soft wood. Getting access to these complexes is not easy. Surrounding the cellulose and hemicelluloses is lignin which serves as the glue and protects the polysaccharides from enzyme and microbial deconstruction. The lignin, cellulose, and hemicellulose form strong bonds and their combined structure is named lignocellulose. The process to overcome lignocellulosic recalcitrance and expose the cellulose and hemicellulose, so that individual sugars can be released, is called pretreatment shown in Fig. 8.4.

Pretreatment represents the necessary steps to convert the raw substrate into a suitable form and includes size reduction by grinding, physical, chemical, or enzymatic hydrolysis to increase substrate availability to release nutrient and sugar.

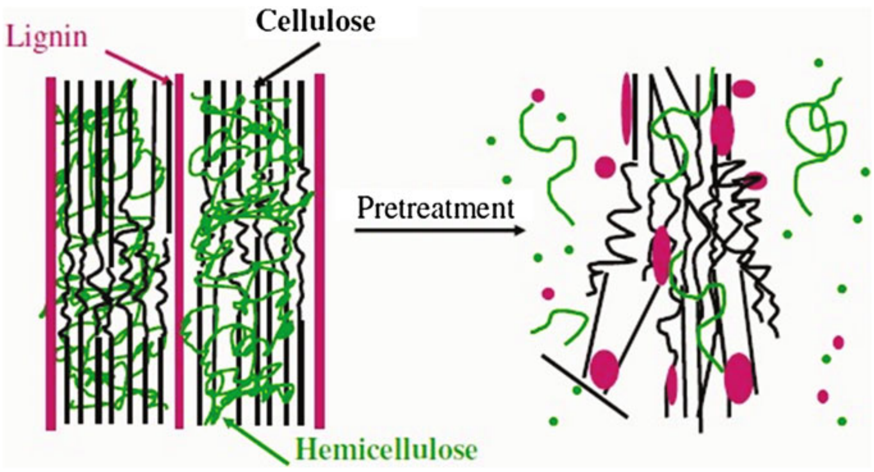


Fig. 8.4 Effect of pretreatment on lignocellulose. *Source* Mosier et al. 2005

8.3.7.1 Chemical Treatment

Acid Pretreatment

Concentrated acid hydrolysis disrupts the hydrogen bonding of the cellulose chains, converting the cellulose from a crystalline state to an amorphous state with little glucose liberation (Kosaric and Vardar-Sukan 2001; Orozco et al. 2007). In 1965, Oshima showed that crystalline cellulose achieves complete solubility at 72 % H_2SO_4 or 42 % HCl at the relatively low temperatures (10–45 °C) (Oshima 1965; Kosaric and Vardar-Sukan 2001). In the process of concentrated acidic hydrolysis, dilute acid is used initially in the prehydrolysis digestion column for hemicellulose removal prior to passing to the secondary counter current column to contact a strong acidic solution With the concentrated acid method, glucose yields greater than 90 % have been achieved with a rapid production rates whereas the increased acid concentration of HCl, a reduction of xylose yield has been showed by the work of Orozco et al. (2007).

Alkaline Hydrolysis

Alkali is seen as a swelling agent where the alkali acts indirectly with water being the lysis agent (Kosaric and Vardar-Sukan 2001). By swelling the biomass, the surface area is increased opening up the structure for water to migrate into the material. Once inside the biomass, the water disrupts the hydrogen bonding between the hemicellulose and the lignin carbohydrates (Balat et al. 2008). The effect is the decrease in crystal and lignin disruption. The advantages of using alkali over acidic methods are the removal of the lignin fraction without the degradation

of the other major constituents. The compromise is the increased length of reaction times [hours or days in comparison to minutes for other processing methods (Balat et al. 2008)].

Balat et al. (2008) pretreated corn stover with NaOH (2 %) and combined with irradiation (500 gGy). They found that the glucose yields to increase from 20 % for NaOH pretreatment to 43 % in combination (Balat et al. 2008). Although NaOH is the dominant alkali approach, lime [calcium hydroxide, $Ca(OH)_2$] can also be employed as an alternative method. $Ca(OH)_2$ has been used in the pretreatment of several feedstocks in pilot scale applications, such as wheat straw (358 K for 3 h and corn stover (373 K for 13 h). The attraction of lime/alkali pretreatment is the removal of acetyl and various uronic acid substitutions on hemicellulose that lowers the accessibility of the enzymes to the hemicellulose and cellulose surface (Sun and Cheng 2002; Balat et al. 2008).

8.3.7.2 Physical Treatment

Steam Pretreatment

Steam pretreatment, also known as "steam explosion," has been extensively investigated and tested in several pilot plants and demo plants worldwide (Galbe and Zacchi 2012). An additional acid catalyst can be used to increase the effectiveness of the steam pretreatment, in which case hemicellulose recovery and the enzymatic hydrolysis of the solids both increase (Galbe et al. 2005). The effects of initial moisture content on steam consumption, mechanism and rate of heat transfer, pentosan solubilization, and subsequent glucose yield were summarized. Treatment at 190 °C with complete bleed-down (no explosion), when compared with that at 240 °C with explosion from full pressure, showed at least as good solubilization of pentosan and enzymatic hydrolysis but showed greater pentosan destruction for the same degree of pentosan removal. Neither the explosion nor the high temperatures (above 190 °C) are necessary (Brownell and Saddle 1987).

8.3.7.3 Enzymatic Hydrolysis

During hydrolysis, the polymers of hemicellulose are degraded into their monomeric subunits. Complete hemicellulose hydrolysis results in several hexoses and pentoses. Main sugar in hemicellulose derived from soft wood is mannose (Taherzadeh and Karimi 2007). The enzyme hydrolysis is catalyzed by hemicellulose enzymes; without any pretreatment the conversion of native hemicellulose to sugar is extremely slow, since hemicellulose is well protected by the lignin. Therefore, pretreatment of these materials is necessary to increase the rate of hydrolysis of hemicellulose (Galbe et al. 2007).

Efficiently releasing all sugars in biomass therefore requires the combined action of a large number of different enzymes. Furthermore, some of these enzymes could

work synergistically and increase overall amount of monosaccharides released. Cervero et al. (2010) suggested that binary mixtures of commercial enzymes, 1:1 mixture of Mannaway and Gammanase, were used to increase the conversion of cell-wall material present in biomass to obtain monosaccharides. The binary mixtures of commercial enzymes showed good synergistic effect releasing 30 % more mannose than the sum obtained using these enzymes individually.

8.3.8 Other Applications

β-Mannanase can use as slime control agent in water purification system, waste water treatment, and cooling water treatment system. It has been used in the enzymatic hydrolysis of galactomannan to enhance the flow of oil and gas in drilling operation in the oil and gas industries (Chauhan et al. 2012).

8.4 Conclusions

β-Mannanase (endo-1,4-β-D-mannanase) is an endohydrolase that catalyzes the random hydrolysis of the β-1,4-D-mannopyranosyl linkage within the main chain of mannans and various mannan-based polysaccharides to yield mannooligosaccharides products. It can effectively remove mannan (hemicellulose) from biomass structure in pretreatment, resulting in better digestion of cellulosic materials. β-mannanases enzyme has been isolated and characterized from several different sources including bacteria, fungi, higher plants, and animals. It can be widely applied in industry, especially in the pretreatment of biomass conversion to biosugar for fermentation.

References

Adibmoradi M, Mehri M (2007) Effects of β-mannanase on broiler performance and gut morphology. In: 16th European symposium on poultry nutrition, Stasburg, France, pp 471–47

Alam NH, Meier R, Schneider H, Sarker SA, Bardhan PK, Mahalanabis D, Fuchs GJ, Gyr N (2000) Partially hydrolyzed guar gum supplemented oral rehydration solution in the treatment of acute diarrhea in children. J Pediatr Gastroenterol Nutr 31:503–507

Araujo A, Ward OP (1990) Hemicellulases of Bacillus species: preliminary comparative studies on production and properties of mannanases and galactanases. J Appl Bacteriol 8:253–261

Balat M, Balat H, Öz C (2008) Progress in bioethanol processing. Prog Energy Combust Sci 34 (5):551–573

Baurhoo B, Ferket PR, Zhao X (2009) Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult Sci 88 (11):2262–2272

Benech RO, Li X, Patton D, Powlowski J, Storms R, Bourbonnais R, Paice M, Tsang A (2007) Recombinant expression, characterization, and pulp prebleaching property of a *Phanerochaete chrysosporium* endo-β-1,4-mannanase. Enzyme Microb Technol 41:740–747

Bhoria P, Singh G, Hoondal GS (2009) Optimization of mannanase production from *Streptomyces* sp. PG-08-03 in submerged fermentation. Bioresources 4(3):1130–1138

Brownell HH, Saddle JN (1987) Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnol Bioeng 29(2):228–235

Buckeridge MS, Dietrich SMC, Lima DU (2000) Galactomannans as the reserve carbohydrate of legume seeds. In: Gupta AK, Kaur N (eds) Developments in crop science, vol 26. Elsevier Science B.V., Amsterdam, pp283–316

Cerveró JM, Skovgaard PA, Hanne CF, Sørensenc R, Jørgensen H (2010) Enzymatic hydrolysis and fermentation of palm kernel press cake for production of bioethanol. Enzyme Microbial Technol 46:177–184

Chanzy H, Dube M, Marchessault RH (2004) Single crystals and oriented crystallization of ivory nut mannan. Biopolymers 18:887–898

Chauhan PS, Puri N, Sharma P, Gupta N (2012) Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol 93:1817–1830

Daskiran MRG, Teeter DW, Fodge D, Hsiao HY (2004) An evaluation of endo-ß-D-mannanase (Hemicell) effects on broiler performance and energy use in diets varying in β-mannan content. Poult Sci 83:662–668

Davies GJKS, Wilson KS, Henrissat B (1997) Nomenclature for sugar binding subsites in glycosyl hydrolases. Biochemistry 321:557–559

Dea ICM, Morrison A (1975) Chemistry and interactions of seed galactomannans. Adv Carbohydr Chem Bio-Chem 31:241–242

Dhawan S, Kaur J (2007) Microbial mannanases: an overview of production and applications. Crit Rev Biotechnol 27:197–216

Galbe M, Zacchi G (2012) Pretreatment: the key to efficient utilization of lignocellulosic materials. Biomass Bioenergy 70:70–78

Galbe M, Liden G, Zacchi G (2005) Production of ethanol from biomass-research in Sweden. J Sci Ind Res 64:905–919

Galbe M, Sassner P, Wingren A, Zacchi G (2007) Process engineering economics of bioethanol production. Adv Biochem Eng Biotechnol 108:303–327

Gonzáles CA, Fernández MN, Sahagún AM, García VJJ, Díez LMJ, Calle PAP, Castro RLJ, Sierra VM (2004) Glucomannan: properties and therapeutic applications. Nutr Hosp 19 (1):45–50

Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu X, Dupree P (2003) Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. Planta 218:27–36

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon J, Davie G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc Natl Acad Sci 92:7090–7094

Hogg D, Pell G, Dupree P, Goubet F, Martin-oure SM, Armand S, Gilbert HJ (2003) The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. Biochem J 371:1027–1043

Hongshu Z, Jinggan Y, Yan Z (2002) The glucomannan from ramie. Carbohydr Polym 47:83–86

Kosaric N, Vardar-Sukan F (2001) Potential source of energy and chemical products. The biotechnology of ethanol. M. Roehr. Wiley, Weinheim

Lundqvist J, Jacobs A, Palm M, Zacchi G, Dahlman O, Stalbrand H (2003) Characterization of glactoglucomannan extracted from spruce (*Picea abies*) by heatfractionation at different conditions. Carbohydr Polym 51:203–211

Moreira LRS, Filho EXF (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol 79:165–178

Mosier NS, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686

Nunes MDS, Santos RAM, Ferreira SM, Vieira J, Vieira CP (2006) Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen SFB gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. New Phytol 172:577–587

Orozco A, Ahmad M, Rooney D, Walker G (2007) Dilute acid hydrolysis of cellulose and cellulosic bio-waste using a microwave reactor system. Process Saf Environ Prot 85:446–449

Oshima M (1965) Wood chemistry process engineering apsects. Noyes Development Corporation, New York

Parisi GC, Zilli M, Miani MP, Carrara M, Bottona E, Verdianelli G, Battaglia G, Desideri S, Faedo A, Marzolino C, Tonon A, Ermani M, Leandro G (2002) High-fibre diet supplementation in patients with irritable bowel syndrome (IBS): a multicenter, randomized, open trial comparison between wheat bran diet and partially hydrolyzed guar gum (PHGG). Dig Dis Sci 47(8):1697–1704

Popa VI, Spiridon J (1998) Hemicelluloses: structure, properties, in polysaccharides. In: Dumitriu S (ed) Polysaccharides: structural diversity and functional versality. Marcel Dekker, New York, pp 297–311

Puls J (1997) Chemistry and biochemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis. Macromol Symp 120:183–196

Puls J, Schuseil J (1993) Chemistry of hemicellulose: relationship between hemicellulose structure and enzyme required for hydrolysis. In Coughlan MP, Hazlewood GP (eds) Hemicellulose and hemicellulases. Portland, London, pp 1–27

Shobha MS, Kumar ABV, Tharanathan RN, Koka R, Gaonkar AK (2005) Modification of guar galactomannan with the aid of *Aspergillus niger* pectinase. Carbohydr Polym 62:267–273

Spring PC, Wenk KA, Dawson KA, Newman KE (2000) The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poult Sci 79(2):205–211

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83(1):1–11

Stoll D, Boraston A, Stålbrand H, McLean BW, Kilburn DG, Warren AJ (2000) Mannanase Man26A from *Cellulomonas fimi* has a mannan-binding module. FEMS Microbiol Lett 183:265–269

Taherzadeh MJ, Karimi K (2007) Bioethanol review. Bioresources 2(3):472–499

Takeno F, Yamada H, Sekiya K, Fujitani B, Ohtsu K (1990) Effect of partially decomposed guar gum on high-cholesterol-fed rats and non-dietary fiber-fed rats. J Jpn Soc Nutr Food Sci. 43:421–425

Tamamura Y, Araki T, Amagoi H, Mori H, Morishita T (1995) Purification and characterization of an extracellular beta-1,4-mannanase from a marine bacterium, *Vibrio* sp. strain MA-138. Appl Environ Microbiol 61(12):4454–4458

Titapoka S, Keawsompong S, Haltrich D, Nitisinprasert S (2008) Selection and characterization of mannanase-producing bacteria useful for the formation of prebiotic manno-oligosaccharides from copra meal. World J Microbiol Biotechnol 24:1425–1433

Van Zyl WH, Rose SH, Trollope K, Gorgens JF (2010) Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. Process Biochem 45:203–1213

Willför S, Sundberg K, Tenkanen M, Holmbom B (2007) Spruce-derived mannans—a potential raw material for hydrocolloids and novel advanced natural materials. Carbohydr Polym 72 (2):197–210

Ximenes EA, Chen H, Kataeva IA, Cotta MA, Felix CR, Ljungdahl LG, Li XL (2005) A mannanase, ManA, of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2 has carbohydrate binding and docking modules. Can J Microbiol 51:559–568

Yang Y, Iji PA, Kocher A, Mikkelsen LL, Choct M (2008) Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic *Escherichia coli* challenge. Br Poult Sci 49(5):550–559

Zhang Y, Ju J, Peng H, Gao F, Zhou C, Zeng Y, Xue Y, Li Y, Henrissat B, Gao GF, Ma Y (2008) Biochemical and structural characterization of the intracellular mannanase AaManA of *Alicyclobacillus acidocaldarius* reveals a novel glycoside hydrolase family belonging to clan GH-A. J Biol Chem 283(46):31551–31558

Chapter 9
The Role and Applications of Xyloglucan Hydrolase in Biomass Degradation/Bioconversion

M. Saritha, Anju Arora, Jairam Choudhary, Vijaya Rani, Surender Singh, Anamika Sharma, Shalley Sharma and Lata Nain

Abstract Lignocellulosic biomass is currently the most promising alternative energy source for realizing sustainable demands of agrarian economies. Its natural recalcitrance to degradation necessitates a detailed study on the complex biochemistry involved in bioconversion of this lignin–carbohydrate complex. A comprehension of the enzymology and role of principal and accessory glycosyl hydrolases involved in biomass degradation are, hence, noteworthy in this context and the xyloglucan-active hydrolases warrant special mention. These are enzymes which carry out hydrolysis and transglucosylation of xyloglucan, the major hemicellulosic polysaccharide in plant biomass. The structurally complex xyloglucans cover and cross-link the cellulosic microfibrils in plant cell walls and make cellulose inaccessible to saccharification by cellulases. Solubilisation of biomass polysaccharides and release of sugars are central to the biomass-to-bioethanol process. Complete conversion of biomass carbohydrates requires a suite of hydrolytic enzymes, which may be designed specifically to accommodate the predominant and subsidiary biomass-cleaving enzymes. Xyloglucan hydrolases which are known to act synergistically with cellulases and xylanases in loosening the plant cell wall are vital enzymes to be deployed for successful bioconversion processes. This chapter is an insight into the capacity of these accessory, but indispensable, hydrolytic enzymes in unlocking the inaccessible biomass polysaccharides for increased sugar recovery and thereby, in drafting the fuels of future.

9.1 Introduction

Biofuel production employs renewable agricultural waste and dedicated energy crops, which also provide opportunity for alleviation of green house gas release. Various plants and parts of plants are used for biofuel production such as grains for

M. Saritha · A. Arora · J. Choudhary · V. Rani · S. Singh · A. Sharma · S. Sharma · L. Nain (✉)
Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: latarajat@yahoo.co.in

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*, Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_9

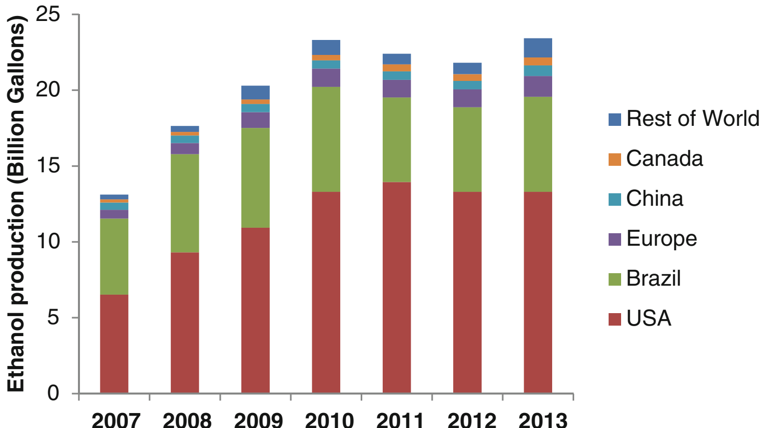


Fig. 9.1 Country-wise global ethanol production (2007–2013). Adapted from www.afdc.energy.gov/data/

the first-generation and lignocellulosic biomass for the second-generation of biofuels. The United States is the world's largest producer of bioethanol, having produced over 13 billion gallons in 2013 alone (Ethanol Industry Outlook Report 2013). Together, the U.S. and Brazil produce 84 % of the world's ethanol, which amounted to 23,429 million gallons in 2013 (Fig. 9.1). Driven by the growth in use of biofuels and natural gas, nonpetroleum energy makes up the highest percentage of total fuel consumption for transport since 1954. According to a new report of the U.S. Energy Information Administration (EIA), 8.5 % of the fuel used by transportation sector in 2014 came from nonpetroleum sources (AEO 2015). Today, most of the gasoline available throughout the United States is a blend of 90 % gasoline and up to 10 % ethanol, known as 'E10'.

The vast majority of the U.S. ethanol is produced from corn, while Brazil primarily uses sugarcane. The current production of first-generation bioethanol from sugars or starch has, but raised a worldwide "food vs fuel" debate putting a question mark on food security. The diversion of food crops for biofuel production also leads to food price hikes. Advanced generation biofuels are, therefore, imperative as they are based on inexpensive and plentiful resources which do not compete with food crops and do not threaten food security.

As a result, the center of attention in development of technology for ethanol production has shifted toward the use of nonedible residual lignocellulosic materials as well as to lower the cost of production. Lignocellulosic biomass is the most abundant source of renewable energy on the planet. The sugars present in the lignocellulosic biomass can be converted to biofuel molecules such as ethanol and butanol. Production of biofuels from lignocellulosic biomass requires the efficient deconstruction of carbohydrate polymers to fermentable sugars by a cocktail of

enzymes containing cellulases, xylanases, and other glycosyl hydrolases. The economic feasibility of production of bioethanol from lignocellulose and its competition with petrol is, but, a main challenge. The main bottlenecks in this technology are low efficiency due to the natural recalcitrance of lignocellulose to deconstruction and high cost of enzymatic conversion (Mohanram et al. 2013). To address these, significant research has to be directed toward the identification of efficient enzyme systems and process conditions for the improved enzymatic utilization of biomass feedstocks. This warrants a detailed understanding of the enzymology involved in biomass bioconversion.

9.2 Polysaccharides of Lignocellulosic Biomass

Lignocellulose is the main structural constituent of woody plants, herbs such as grasses and crop biomass. It is the most abundant biopolymer on the planet and comprises about 50 % of world's biomass (Claassen et al. 1999). About 686 MT of crop residues are generated every year in India, as predicted by The Ministry of Agriculture, Govt. of India (Hiloidhari et al. 2014), out of which 234 MT can be used as substrate for production of many value-added products like biofuel. Lignocellulose consists of 40–60 % cellulose, 20–40 % hemicellulose, and 10–25 % lignin (Wyman 1996). Lignocellulosic biomass is a substrate of massive biotechnological value because of the chemical attributes of its components (Malherbe and Cloete 2003). The average composition of some lignocellulosic materials that can be used as substrates for biofuel production is given in Table 9.1.

Plant biomass is mainly composed of the polysaccharides, cellulose, and hemicellulose with pentose and hexose sugars as the main building blocks. Cellulose is a D-glucose polymer with organized microfibrillar forms, with each microfibril containing up to 36 glucan chains having thousands of glucose residues joined by β-1,4-glycosidic bonds. Hemicelluloses are complex homopolysaccharides (glucan, xylan, mannan) or heteropolysaccharides (xyloglucan, galactoglucomannans, arabinoxylan, glucomannan) with both hexose (glucose, mannose, galactose) and pentose sugars (arabinose, xylose) along with their uronic acid derivatives (glucouronic acid, galatouronic acid, mannuronic acid) (Mohanram et al. 2013).

Thus, plant cell walls are built from a mixture of polysaccharides, proteins, and lignin, whereas the noncrystalline matrix of plant cell walls is built of one or more of the different types of hemicelluloses such as galactoglucomannan, glucomannan, arabinoglucuronoxylan, glucuronoxylan, and xyloglucan (Timell 1967). Some of the common hemicelluloses of plant origin are given in Table 9.2.

Among the hemicelluloses, xyloglucans are widely distributed throughout the plant kingdom as a major structural and storage polysaccharide. The Type I plant cell wall, predominant in dicotyledonous plants and non-commelinoid monocotyledons, is characterized by equal proportions of cellulose and xyloglucans. On

Table 9.1 Average composition of biomass expressed as percentage on dry weight basis

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Barley wood	40	20	15
Bermuda grass	25	35.7	6.4
Birch wood	40	33	21
Corn cobs	42	39	14
Corn stalks	35	15	19
Corn stover	38	26	19
Cotton seed hair	80–95	5–20	0–5
Flax sheaves	35	24	22
Forage sorghum	34	17	16
Grasses	25–40	35–50	10–30
Groundnut shells	38	36	16
Hardwood stem	40–55	34–40	18–25
Leaves	15–20	80–85	0–5
Miscanthus	43	24	19
Oat straw	41	16	11
Paper	85–99	0–5	0–15
Pine	41	10	27
Rice straw	32	24	13
Rice husk	36	15	19
Rye straw	31	25	7
Saw dust	55	14	21
Sorghum straw	33	18	15
Soybean stalks	34	25	20
Sugarcane	33	30	29
Sugarcane bagasse	42	25	20
Sweet sorghum	23	14	11
Switch grass	37	29	19
Salix	41.5	22–25	25
Softwood stem	45–50	25–35	25–35
Spruce	45	26	28
Wheat straw	30	24	18

Adapted from Jørgensen et al. (2007) and Kumar et al. (2009)

the other hand, Type II cell wall, present in commelinoid monocots like rice, contains less of xyloglucans and shows predominance of glucuronoarabinoxylan along with cellulose microfibrils (Yokoyama et al. 2004). Whatever the plant type, the considerable amount of xyloglucan present is always around 20–25 % of dry weight in dicots and 2–5 % in grasses (Hayashi 1989; Benko et al. 2008).

Table 9.2 Common hemicelluloses of plant origin

Polysaccharide type	Biological origin	Amount (%)	Type of linkage between monomeric units
Glucuronoxylans	Hardwood	15–35	β-D-xylopyranosyl units linked by β-(1,4) glycosidic bonds
Galactoglucomannans	Softwood	20–25	β-D-glucopyranosyl and β-D-mannopyranosyl units, linked by β-(1,4) glycosidic bonds
Arabinoglucuronoxylan	Agricultural crops (nonwoody material)	5–10	β-(1,4)-D-xylopyranose backbone containing 4-*O*-methyl-α-D-glucopyranosyluronic acid and α-L-arabinofuranosyl linked by α-(1,2) and α-(1,3) glycosidic bonds
Xyloglucan	Hardwood (mainly dicotyledons)	2–25	β-1,4-linked D-glucose backbone with 75 % of these residues substituted at O-6 with D-xylose. L-arabinose and D-galactose residues can be attached to the xylose residues forming di-, or triglycosyl side chains.
Glucomannan	Softwood and hard woods	2–5	β-(1 → 4)-linked D-mannose and D-glucose
Galactoglucomannan	Softwood	10–25	β-(1 → 4)-linked mannose and glucose units with α-(1 → 6)-linked galactose units attached to mannose units
Arabinoxylan	Cereal grain cell walls	0.15–30	β-(1,4)-D-xylopyranose backbone is substituted by a-L-arabinofuranosyl units in the positions 2-*O* and/or 3-*O* and by α-D-glucopyranosyl uronic unit or its 4-*O*-methyl derivative in the position 2-*O*

Adapted from Girio et al. (2010)

9.2.1 Xyloglucan: A Predominant Cell Wall Hemicellulose

Xyloglucan is one of the most widespread hemicellulosic polysaccharides in a lignocellulosic biomass. Xyloglucans form a sheath around the cellulose microfibrils preventing them from direct aggregation and cross-link different cellulose microfibrils via hydrogen bonds and other non-covalent interactions, thereby providing structural strength to the plant cell wall (Fry 1989; Hayashi 1989; Baumann 2007a). These polymers consist of a linear backbone of β-1,4-glucan linkages and are structurally related to cellulose, but are distinguished by having up to 75 % of β-D-glucopyranose (β-D-Glc*p*) residues covalently linked to α-D-xylopyranose (α-D-Xyl*p*) at the O-6 position (Carpita and McCann 2000). Some of the xylosyl residues are further substituted by galactosyl, arabinosyl, or fucosyl residues, forming a complex structure (Enkhbaatar et al. 2012; Damásio et al. 2012). Different xyloglucan side chains are depicted using a single-letter nomenclature, as

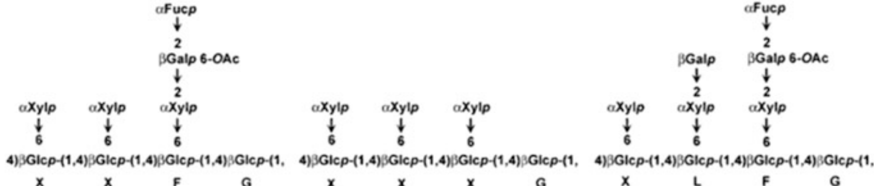


Fig. 9.2 The typical structures of the subunits in XXXG-type xyloglucans. Adapted from http://www.ccrc.uga.edu/

proposed by Fry et al. (1993), in order to overcome the complexity in their structural description. According to it, the unsubstituted and substituted Glc*p* residues are represented as G and X, respectively. The substituted Glc*p* residue may be further denoted as L/F/U/S, depending upon the substituted saccharide residue. For example, F represents a Glc*p* residue that is substituted with a fucose-containing trisaccharide. Most xyloglucans consist of repeating units of either XXXG (XXXG-type), or XXGG (XXGG-type) (Vincken et al. 1997). The primary walls of a wide range of dicots, non-graminaceous monocots, and gymnosperms contain fucosylated xyloglucans with an XXXG-type structure, the typical subunit structures of which are shown in the Fig. 9.2.

The side chains confer several properties on the polymer. The high solubility of xyloglucan in water and its rheological properties make it a useful food additive, thickener, stabilizer, and gelling agent (Yamatoya and Shirakawa 2003; Baumann 2007b). Xyloglucan can also replace starch and galactomanan used in papermaking (Shankaracharya 1998). However, it cannot form ordered crystalline microfibrils like cellulose (Fry 1989). Pauly et al. (1999) described three structural domains of xyloglucans: the xyloglucan cross-links that are susceptible to enzymatic cleavage, a xyloglucan fraction that is tightly bound to the microfibril surface and a third component that is trapped within the microfibril periphery. This structural complexity suggests the importance of understanding the mechanism of xyloglucan metabolism and biochemical characterization of the enzymes involved.

9.3 Enzymology of the Biomass Bioconversion Process

Due to high carbohydrate content, lignocellulose biomass like agricultural waste and plant residues, dedicated energy crops, municipal, and industrial wastes hold great potential for large scale production of bioethanol (Farrell et al. 2006). The cellulose and hemicellulose components of biomass can be converted into sugar monomers from which a range of biomaterials viz. ethanol or other important chemical intermediates can be synthesized via fermentation or other processes.

The classical model for hydrolysis of cellulose to glucose involves the synergistic action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21).

Endocellulases cleave glucan chains internally in a random fashion, which results in a faster decrease in length of polymer and a gradual increase in the reducing sugar concentration. Exocellulases hydrolyze cellulose mainly by removing cellobiose either from the reducing or non reducing ends, which leads to a rapid release of reducing sugars but a small change in length of polymer. By cooperative action of endocellulases and exocellulases on cellulose, cello-oligosaccharides and cellobiose are produced, which are further hydrolyzed by β-glucosidase to glucose (Mohanram et al. 2013). Though cellulases are the most important hydrolytic enzymes for saccharification of complex polymers, they can only unlock the sugars entrapped in cellulose leaving behind the unused hemicellulose. Thus, the hemicellulose portion of biomass is the largest polysaccharide fraction that gets wasted in most lignocellulosic biomass-associated ethanol production processes (Girio et al. 2010). As the cellulose polymers are entangled with hemicellulose which gives them structural strength, the accessibility of the cellulose polymers to hydrolytic enzymes is hindered, which thereby interferes with the saccharification process.

Hemicelluloses are cleaved by enzymes like carbohydrate esterases, glycoside hydrolases, polysaccharide lyases, xyloglucan hydrolases, endohemicellulases, and others, the concerted action of which hydrolyze ester bonds, glycosidic bonds, and remove the chain substituents or side chains (van den Brink and de Vries 2011; Sweeney and Xu 2012). The various hydrolytic enzymes which target the linkages present in the hemicellulose polymers are detailed in Table 9.3.

Table 9.3 List of hemicellulases that can be supplemented to cellulase enzyme to enhance saccharification efficiency of lignocellulosic biomass

Enzyme	GH family	Enzyme classification number (EC)	Site of action
β-Xylanase	GH 10, GH 11	EC 3.2.1.8	Hydrolyses β-1,4 glycosidic bond in xylan
β-Xylosidase	GH 52	EC 3.2.1.37	Hydrolyses xylobioses
Glucanase	GH 16	EC 3.2.1.-	Hydrolyses β-1,3; β-1,6; α-1,4; α-1,6 glucans
Mannanase	GH 26	EC 3.2.1.78	Hydrolyses β-1,4-mannosidic linkage in the main chain of mannan, galactomannan, glucomannan and galactoglucomannan
Xyloglucan hydrolase	GH 12, GH 16	EC 3.2.1.150,151,155	Hydrolyses xyloglucan (β-1,4 glucan with α-1,6 linked xylose)
Arabinofuranosidase	GH 43, GH 51, GH 54, GH 127	EC 3.2.1.55	Removes arabinose substituents from α-arabinofuranoside, arabinoxylans, arabinogalactans
Glucouronidase	GH 67, GH 79	EC 3.2.1.139	Removes α-1,2 linked glucuronoyl or its methyl ester in xylan
Acetyl xylan esterase	CE 1, CE 5, CE 16	EC 3.1.1.72	Removes acetyl group from xylans or other xylooligosaccharides
Feruloyl esterase	CE 10	EC 3.1.1.73	Hydrolyses feruloyl esters
Glucuronoyl esterase	CE 15	EC 3.1.1.-	Demethylates methyl glucuronoyl α-1,2 linked to backbone xylose

Adapted from Sweeney and Xu (2012)

Depending upon the type and abundance of hemicellulose polymer present in the biomass used as substrate, different combinations of hydrolytic enzymes listed in Table 9.3 can be supplemented to cellulases. Presence of hemicellulases in hydrolytic enzyme cocktail has been reported to enhance the saccharification yield by releasing the sugars entrapped in hemicelluloses and also by increasing the accessibility of the cellulase enzyme to the cellulose complex (Singh et al. 2014).

9.4 Xyloglucan-Active Enzymes in Modification of Cell Wall Polysaccharides

The hydrogen bonding present between cellulose and xyloglucan makes the extraction of latter difficult. In order to access and modify the cellulose fibers embedded and cross-linked by xyloglucans in the plant cell walls, remodeling of xyloglucan is required. In nature, the enzymatic hydrolysis of xyloglucan and the resulting xyloglucan solubilization is important in controlling wall strength during cell growth (Takahashi et al. 2015). The enzymatic machinery comprising of specific xyloglucanases that can digest xyloglucans to xyloglucan-oligosaccharides is, thus, of utmost importance in improving accessibility of cellulose to hydrolytic enzymes and in enhancing the conversion rate of polymers to sugars in order to economize the saccharification process. In contrast to the cell wall degrading enzymes, they cause cell wall modifications that are usually associated with the weakening of extracellular barriers.

Xyloglucan depolymerization was earlier thought to be due to the action of endoglucanases and other undefined hydrolases. Later, xyloglucan endotransglycolases (XETs) and xyloglucan-specific endo-β-1,4-glucanase were identified from germinating *Nasturtium* seeds, where it catalyzed the seed storage xyloglucan depolymerization (Rose et al. 2003). Subsequently, several proteins belonging to the same class and with sufficient sequence homology were discovered. Presently, xyloglucan hydrolases which carry out hydrolysis of xyloglucan are classified in the xyloglucan endotransglucosylase (XET)/hydrolase (XEH) superfamily, known as the XTH superfamily (Rose et al. 2003). The modes of action of these enzymes are illustrated in the Fig. 9.3.

Several xyloglucan-active degrading enzymes of the XTH superfamily have been reported by various researchers. These include xyloglucan-specific endo-β-1,4-glucanase (EC 3.2.1.151), xyloglucan-specific exo-β-1,4-glucanase (EC 3.2.1.155), oligoxyloglucan reducing-end-specific cellobiohydrolase (EC 3.2.1.150), xyloglucan endotransferase (EC 2.4.1.207), xyloglucan 4-glucosyltransferase (EC 2.4.1.168), xyloglucan 6-xylosyltransferase (EC 2.4.2.39), and xyloglucan 2-galactose transferase (EC 2.4.1.-), belonging to several glycosyl hydrolase (GH) families: GH5, GH12, GH16, GH 26, GH44, and GH74 (Hayashi et al. 1980; Fry et al. 1992; Pauly et al. 1999; Faik et al. 2002; Madson et al. 2003; Grishutin et al.

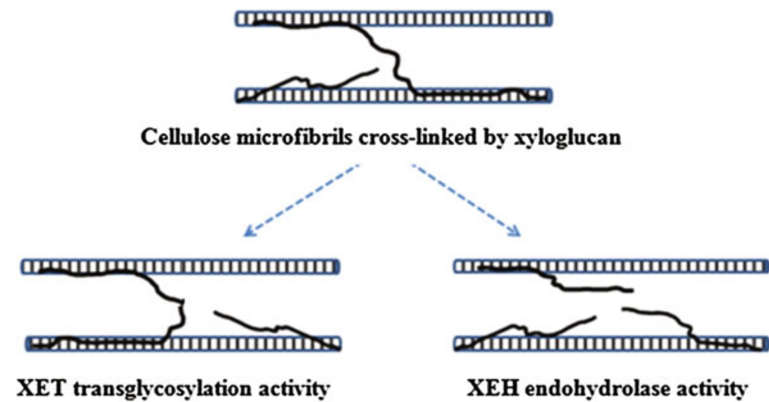


Fig. 9.3 Modes of xyloglucan depolymerization by the XTH family of enzymes

Table 9.4 Nomenclature and enzyme characteristics of xyloglucan hydrolase

Particulars	Xyloglucan hydrolase	
	Oligoxyloglucan β-glycosidase	Xyloglucosyl transferase
EC number	3.2.1.120	2.4.1.207
Systemic name	Oligoxyloglucanxyloglucohydrolase	Xyloglucan: Xyloglucosyltransferase
Reaction type	Hydrolysis of *O*-glycosyl bond	Transglycosylation
pH optima[a]	4.5–5.0	5.0–6.0
Temp. optima[a]	55–60 °C	25–30 °C
K_m value	*Varies according to substrate* Tamarind xyloglucan: 0.0024 Xyoglucan oligosaccharide: 0.966	1.4–2.2 (xyloglucan)
Thermostability[a]	Stable below 35 °C	Stable below 35 °C
Inhibitors	Cu^{2+},Fe^{2+},Fe^{3+}, Hg^{2+}, SDS	Ammonium sulfate, 4-*O*-methyl glucuronoxylan, Ag^{+}, $AlCl_3$, alginate, ATP, $CaCl_2$, La^{3+}, NaN_3, etc.

[a] Varies according to the organism from which enzyme is produced
Adapted from BRENDA enzyme database (http://www.brenda-enzymes.org/)

2004; Yaoi et al. 2004; Baumann 2007b). The particulars of a few enzymes involved in xyloglucan hydrolysis are given in Table 9.4.

As stated previously, xyloglucan hydrolases are believed to be cell wall-loosening enzymes that are responsible for cell elongation by continuous splicing of existing polymers and linking of new xyloglucan residues (Fry et al. 1992; Miedes et al. 2011). High expression of the enzyme in growing parts like elongating tissues in *Cicer arientinum* (Romo et al., 2005), hypocotyls in pine (Lorences 2004), germinating *Nasturtium* seeds (Stratilova et al. 2010), fiber elongation in cotton (Michailidis et al. 2009), and even ripening of tomatoes

(Miedes and Lorences 2009) have been reported. Enhanced expression of the enzyme in plants has also been reported on contact with pathogenic or beneficial microorganisms. Mycorrhizal association in *Medicago truncatula* caused enhanced expression of enzyme in roots both intimately and distantly associated with the fungal partner (Maldonado-Mendoza et al. 2005) and *Botryosphaeria dothidea* caused enhanced expression of the enzyme in apple tree (Bai et al. 2015).

However, due to their role in maintaining the cell wall structure, xyloglucan-active enzymes may also be considered potential hemicellulose-repairing enzymes. The XTHs produced during infection of apple fruit by *Penicillium expansum* has a dual role of integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan and restructuring existing cell wall material by catalyzing transglucosylation between previously wall-bound xyloglucan molecules (Muñoz-Bertomeu and Lorences 2014). Their activity gets inhibited as the infection progresses, leading to hemicellulose degradation.

9.4.1 Mechanism of Action of Xyloglucan Hydrolases

All glycosyl hydrolase enzymes act by causing splits in glycosidic bonds and presumably, utilize either the inverting or the retaining mechanism. The inverting mechanism is a direct displacement mechanism, resulting in the inverted configuration on the anomeric carbon. Enzymes employing this reaction mechanism belong to either GH 44 or GH 74 family and generally exhibit a distance of approximately 11 Å between the catalytic residues (Baumann 2007b). The cellulosomes of *Clostridium thermocellum* are found to produce extracellular cellulases, along with an inverting xyloglucanase CtXGH74A (Martinez-Fleites et al. 2006). Retaining xyloglucan hydrolases utilize the double displacement mechanism for catalysis. The cell wall active xyloglucan hydrolases belonging to the GH 16 family employ this mechanism that involves the formation of a covalent glycosyl-enzyme intermediate (Baumann 2007b). The only xyloglucan hydrolyzing enzyme from GH16 characterized in detail is NXG1, originally isolated from *Nasturtium* cotyledons (Edwards et al. 1985). Recently, close homologs of NXG1 analyzed by expression profiling have been shown to be expressed during different stages of plant development. These include the OsXTH19 from rice leaves (Yokoyama et al. 2004) and the SlXTH6 from tomato shoot, seeds, and roots (Saladie et al. 2006). The GH16 transglycosylases which catalyze endotransglycosylation of xyloglucan that enables cell expansion also employ the retaining mechanism (Sinnott 1990). The modes of action of xyloglucanases have also been variously classified into the endo-mode involving cleavage at multiple sites along the backbone, and the exo-mode involving reducing end-specific cleavage (Feng et al. 2014).

9.4.2 Role of Xyloglucan-Active Enzymes in Biomass Saccharification for Biofuel Production

Xyloglucan hydrolase is a ubiquitous enzyme produced by both plants and microorganisms. Plants, being more complex systems, express a great diversity of these hydrolytic enzymes, but cannot be exploited for their commercial production. Microorganisms offer more congenial culturing conditions for greater production in less space, while allowing for manipulation of culture conditions or gene expression to a greater extent.

The first described xyloglucanase of fungal origin was the xyloglucan-specific endo-β-1,4-glucanase of *Aspergillus aculeatus* (XEG; 34 kDa, pI 3.4, GH12) (Pauly et al. 1999). Since then, several enzymes involved in the transformation of xyloglucan have been identified from microbial sources. Table 9.5 gives a list of various microorganisms known to produce xyloglucan-hydrolysing enzymes.

There have also been several reports on gene cloning of xyloglucanases from bacteria and fungi. Two GH74 genes from *Streptomyces avermitilis* (*sav*_1856 and *sav*_2574 genes) have been cloned and expressed using a *Streptomyces* expression system (Ichinose et al. 2012). The C-terminal of *sav*_1856 gene product (SaGH74A) was found to be similar to CBM2, which showed similarity with the sequences of CBMs (Carbohydrate Binding Molecules) from *Cellulomonas fimi* and *Clostridium cellulovorans*, while the *sav*_2574 gene product (SaGH74B), in contrast showed relatively low similarity with the other GH74 enzymes. Song et al. (2013) cloned two GH family 12 xyloglucanase genes (designated *RmXEG12A* and *RmXEG12B*) from the thermophilic fungus *Rhizomucor miehei* and expressed in *Escherichia coli*. The xyloglucanases had the ability to hydrolyze tamarind xyloglucan into oligomers, mainly XXXG, XXLG or XLXG, and XLLG, and showed no activity toward other linear polymers. In another study, *Aspergillus nidulans* A773 (*pyrG*^{89}) recombinant secretion of a xyloglucan-specific endo-β-1,4-glucanohydrolase (XegA) cloned from *Aspergillus niveus* was studied (Damásio et al. 2012). XegA was found to generate a xyloglucan-oligosaccharides pattern similar to that observed for cellulases from family GH12, demonstrating that its mode of action includes hydrolysis of the glycosidic linkages between glucosyl residues that are not branched with xylose.

Various endoglucanases (non-xyloglucan-specific endo-β-1,4-glucanases) have been reported to hydrolyze xyloglucan as a substrate analogue (Vlasenko et al. 2010). The *Trichoderma reesei* endoglucanase (Cel12) hydrolyzes 1,4-β-glucans such as cellulose, 1,3-1,4-β-glucan and xyloglucan (Yuan et al. 2001). However, xyloglucan-specific endo-β-1,4-glucanases which display high activity toward xyloglucan, with little or no activity toward cellulose or cellulose derivatives, have also been reported (Song et al. 2013). Also, enzymes annotated as 1,4-β-D-glucan glucohydrolases or β-glucosidases of GH3 family have greater activities toward xyloglucan oligosaccharide than toward cello-oligosaccharide (Yaoi and Miyazaki 2012). Xyloglucanases from the GH74 family have been frequently reported to have high specificity toward xyloglucan (Enkhbaatar et al. 2012; Feng et al. 2014).

Table 9.5 Characteristics of xyloglucan hydrolases produced by different microorganisms

Microorganism	Molecular weight (kDa)	Isoelectric point (pI)	Optimum temperature	Optimum pH	K_m for xyloglucan (mg/ml)	References
Bacteria						
Ruminococcus flavefaciens	78	–	25	5.0	2.79	Warner et al. (2011)
Bacillus licheniformis	26	–	–	–	–	Gloster et al. (2007)
Paenibacillus pabuli	40	–	–	–	–	Gloster et al. (2007)
Fungi						
Aspergillus japonicus	32	2.8	–	5.0	0.67	Grishutin et al. (2004)
Aspergillus niger	–	–	–	–	0.54	Powlowski et al. (2009)
Chrysosporium lucknowense	78	3.8	–	6.0	0.31	Grishutin et al. (2004)
Trichoderma reesei	75–105	4.1–4.3	–	5.3	0.30	Grishutin et al. (2004)
Geotrichum sp.	80	4.8	55 °C	5.5	–	Yaoi and Mitsuishi (2004)

These cleave the 'X–X' and 'X–G' motifs and have been characterized as having endo-processive modes of action, i.e., the enzymes act by sliding along the backbone during the catalytic action (Matsuzawa et al. 2014). These include the endo-xyloglucanase (XcXGHA) from *Xanthomonas citri* pv. *mangiferaeindicae* (Feng et al. 2014), and SaGH74A and SaGH74B from *Streptomyces avermitilis* (Ichinose et al. 2012). In contrast, the OXG-RCBH from *Geotrichum* sp. M128 recognizes the reducing end of various xyloglucan-derived oligosaccharides and releases two glycosyl residues from this site in the exo-mode (Ichinose et al. 2012).

A growing interest in xyloglucanases is mainly due to the possibility of their application in a number of biotechnological processes, such as conversion of plant waste, modification of xyloglucans for use in food and feed industries as well as pulp and paper industry, production of novel surfactants from oligoxyloglucans, and thermally reversible xyloglucan gels for drug delivery (Sinitsyna et al. 2010; Damásio et al. 2012). During the conversion of biomass to value-added products, a suitable cocktail of various hydrolytic enzymes is required to release all the sugars present in the biomass. Enzymatic saccharification has renewed and centralized the focus on different aspects of hemicellulases as they play an important role in improving the economics of the overall process. The barriers, which include development of robust enzyme formulations containing all accessory enzymes in commercial cellulases, have to be alleviated for successful commercialization of biofuels. An economically feasible enzyme technology for complete hydrolysis of biomass polysaccharides into sugar monomers is very important for cost-effective biofuel production. To achieve this, there is need to search for organisms with hypercellulase activity for developing better quality cellulase formulations with superior attributes such as higher efficiencies, thermostability, and lowered feedback inhibition and tolerance to inhibition by toxic byproducts from pretreatments, using sophisticated biotechnological tools (Bon and Ferrara 2007).

Another feasible option is to supplement cellulase preparations with hemicellulases and other accessory enzymes to enhance the sugar recovery from pretreated lignocellulosic biomass. It has been reported that *Trichoderma reesei*, a widely known cellulolytic filamentous fungus and the main industrial source of commercially available cellulases has only one GH74 gene that codes for xyloglucanase, while several other fungi have more than two genes for the same (Ichinose et al. 2012). In this context, microbial xyloglucan hydrolase preparations may be important for designing enzyme cocktails for industrial biomass conversion, because the degradation of xyloglucan could enhance cellulase accessibility to cellulose (Kaida et al. 2009). There are several reports on improvement in sugar yields during biomass bioconversion when cellulases were supplemented with xyloglucan hydrolase. Supplementation of α xylosidase with commercial cellulases enhanced the release of free glucose (82–88 %) and xylose (55–60 %) from hydrogen peroxide pretreated corn stover (Jabbour et al. 2013). Xyloglucanase addition has also been shown to enhance the hydrolysability of various lignocellulosic substrates when added to a cellulase mixture (Nishitani 1995; Benko et al. 2008). Hydrolysis of glycosidic linkage of xyloglucans resulted in swelling of cellulose microfibrils which enhanced cellulose accessibility and, subsequently,

efficiency of enzymatic hydrolysis (Vincken et al. 1995; Chanliaud et al. 2004). Hydrolysis of various steam pretreated lignocellulosic substrates was greatly enhanced by the supplementation of family 10 and 11 endo-xylanases (GH10 EX and GH11 EX) and family 5 xyloglucanase (GH5 XG) with cellulose monocomponents (Hu 2014). However, the extent of enhancement caused by xyloglucan hydrolase depends on the relative concentration of hemicellulose in the biomass (Benko et al. 2008). In other words, the accessibility of polymers to hydrolytic enzymes is affected by the method of pretreatment adopted in the conversion process. Higher conversion rate is observed when large amount of hemicellulose remains in the raw material after pretreatment signifying the need of xyloglucan hydrolases to loosen the complex network of sugars (Benko et al. 2008). Supplementations of other hemicellulases like xylanase (Choudhary et al. 2014) and xylosidase (Jabbour et al. 2013) has also caused significant enhancement in sugar production, increasing the efficiency of the overall bioconversion process. It is quite evident from the above reports that supplementation of hemicellulases to enzymatic cocktails can cause significant enhancement in conversion rate of polymers to sugars but its role in economy of the overall bioconversion process cannot be clearly envisaged. It is well known that enzyme production and purification is a costly affair. It cannot be clearly said whether enhancement in sugar yield would compensate the cost involved in the production of the hydrolytic enzyme.

Development of transgenic microbes capable of producing the entire hydrolytic enzyme required for biomass conversion in appropriate ratio is one way to save the cost incurred in the process (Rani et al. 2014). Hyper-xyloglucan hydrolase producing microorganisms may be developed to produce xyloglucan-active enzyme preparation to design cellulase cocktails of the future. Another recent approach has been the creation of engineered chimeras by fusing xyloglucan-specific CBMs to xyloglucanases to assist polysaccharide recognition and binding, increasing the enzyme concentration on the substrate surface and thereby improving the hydrolysis rate (Guillen et al. 2010). Recently, the efficiency of a xyloglucan-specific endo-β-1,4-glucanase from *Aspergillus niveus* (XegA) was enhanced by fusion of the xyloglucan-specific CBM44 on XegA leading to the creation of an engineered CBM44-XegA chimera (Furtado et al. 2015). This fusion conferred superior catalytic properties and thermal stability on the XegA. Further detailed enzymatic studies at the molecular level are required to provide the fundamental understanding required to engineer these proteins toward new applications.

9.5 Conclusion

Breakdown of lignocellulosic biomass by microorganism is a highly complex process, requiring multiple types of synergistic catalytic activities concurrently acting upon a variety of both soluble and insoluble polymeric substrates. Industrial biomass utilization processes will also require a combination of hydrolytic enzymes in order to degrade lignocellulosic feedstocks. Structural complexity of

lignocellulosic biomass makes the enzymatic saccharification of polysaccharides present in biomass more difficult and costlier. As xyloglucan is a major hemicellulosic polysaccharide present in the cell wall of dicotyledonous plants which provides structural rigidity to cell wall, xyloglucan hydrolases are of special interest. Commercial cellulases in combination with accessory enzymes like xylanases and xyloglucanases enhance the yields of glucose and xylose from biomass, and may prove beneficial in industrial biofuel production. This is because cellulases interact synergistically with endo-xylanases and xyloglucanases to improve sugar recovery from various pretreated lignocellulosic substrates. The future efforts will have to focus on detailed enzyme structure–function analysis of the various xyloglucanases used by microorganisms during plant cell wall degradation to be used as accessory enzymes for effective saccharification of lignocellulosic biomass.

References

Annual Energy Outlook (AEO) (2015) U.S. Energy Information Administration, U.S. Department of Energy, Washington, DC. http://www.eia.gov/forecasts/aeo

Bai S, Dong C, Zhu J, Zhang Y, Dai H (2015) Identification of a xyloglucan-specific endo-(1-4)-beta-D-glucanase inhibitor protein from apple (Malus × domestica Borkh.) as a potential defense gene against *Botryosphaeria dothidea*. Plant Sci 231:11–19

Baumann MJ (2007a) Structural evidence for the evolution of xyloglucanase activity from xyloglucanendo-transglycosylases: biological implications for cell wall metabolism. Plant Cell 19:1947–1963

Baumann MJ (2007b) Xyloglucan-active enzymes: properties, structures and applications. Doctoral thesis submitted to School of Biotechnology, Royal Institute of Technology, Stockholm, pp 10–32

Benko Z, Siika-aho M, Viikari L, Reczey K (2008) Evaluation of the role of xyloglucanase in the enzymatic hydrolysis of lignocellulosic substrates. Enzyme Microb Technol 43:109–114

Bon EPS, Ferrara MA (2007) Bioethanol production via enzymatic hydrolysis of cellulosic biomass. In: The role of agricultural biotechnologies for production of bioenergy in developing countries. FAO. http://www.fao.org/biotech/seminaroct2007.html

Carpita NC, McCann M (2000) The cell wall. In: Buchanan B, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, pp 52–108

Chanliaud E, de Silva J, Strongitharm B, Jeronimidis G, Gidley M (2004) Mechanical effects of plant cell wall enzymes on cellulose/xyloglucan composites RID A-7266-2011. Plant J 38:27–37

Choudhary J, Saritha M, Nain L, Arora A (2014) Enhanced saccharification of steam-pretreated rice straw by commercial cellulases supplemented with xylanase. J Bioprocess Biotech 4 (7):188–194

Claassen PAM, van Lier JB, Contreras LAM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52:741–755

Damásio ARL, Ribeiro LFC, Ribeiro LF, Furtado GP, Segato F, Almeida FBR, Crivellari AC, Buckeridge MS, Souza TACB, Murakamie MT, Ward RJ, Prade RA, Polizeli MLTM (2012) Functional characterization and oligomerization of a recombinant xyloglucan-specific endo-β-1,4-glucanase (GH12) from *Aspergillus niveus*. Biochim Biophys Acta 1824:461–467

Edwards M, Dea ICM, Bulpin PV, Reid JSG (1985) Xyloglucan (amyloid) mobilization in the cotyledons of *Tropaeolum majus* L seeds following germination. Planta 163:133–140

Enkhbaatar B, Temuujin U, Lim J-H, Chi W-J, Chang Y-K, Hong S-K (2012) Identification and characterization of a xyloglucan-specific family 74 glycosyl hydrolase from *Streptomyces coelicolor* A3(2). Appl Environ Microbiol 78(2):607–611

Ethanol Industry Outlook Report-Battling for the Barrel (2013) Renewable Fuels Association. http://www.ethanolrfa.org

Faik A, Price NJ, Raikhel NV, Keegstra K (2002) An *Arabidopsis* gene encoding an α-xylosyltransferase involved in xyloglucan biosynthesis. Proc Natl Acad Sci USA 99:7797–7802

Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 113:506–508

Feng T, Yan K-P, Mikkelsen MD, Meyer AS, Schols HA, Westereng B, Mikkelsen JD (2014) Characterisation of a novelendo-xyloglucanase (XcXGHA) from *Xanthomonas* that accommodates a xylosyl-substituted glucose at subsite-1. Appl Microbiol Biotechnol 98:9667–9679

Fry SC (1989) The structure and functions of xyloglucan. J Exp Bot 40:1–11

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ (1992) Xyloglucanendotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem J 282:821–828

Fry SC, York WS, Albersheim P, Darvill A, Hayashi T, Joseleau JP, Kato Y, Lorences EP, Maclachlan GA, McNeil M, Mort AJ, Reid JSG, Seitz HU, Selvendran RR, Voragen AGJ, White AR (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. Physiol Plant 89:1–3

Furtado GP, Santos CR, Cordeiro RL, Ribeiro LF, de Moraes LAB, Damásio ARL, Polizeli MLTM, Lourenzoni MR, Murakami MT, Ward RJ (2015) Enhanced xyloglucan-specific endo-β-1,4-glucanase efficiency in an engineered CBM44-XegA chimera. Appl Microbiol Biotechnol 99(12):5095–5107

Girio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101:4775–4800

Gloster TM, Ibatullin FM, Macauley K, Eklof JM, Roberts S, Turkenburg JP, Bjornvad K, Jorgensen PL, Danielsen S, Joha KS, Borchert TV, Wilson KS, Brumer H, Davies GJ (2007) Characterization and three-dimensional structures of two Distinct bacterial xyloglucanases from Families GH5 and GH12. J Biol Chem 282(26):19177–19189

Grishutin SG, Gusakov AV, Markov AV, Ustinov BB, Semenova MV, Sinitsyn AP (2004) Specific xyloglucanases as a new class of polysaccharide-degrading enzymes. Biochim Biophys Acta 1674:268–281

Guillen D, Sanchez S, Rodriguez-Sanoja R (2010) Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol 85(5):1241–1249

Hayashi T (1989) Xyloglucans in the primary-cell wall. Annu Rev Plant Physiol Plant Molec Biol 40:139–168

Hayashi T, Kato Y, Matsuda K (1980) Biosynthesis of xyloglucan in suspension-cultured soybean cells. Plant Cell Physiol 21:1405–1418

Hiloidhari M, Das D, Baruah DC (2014) Bioenergy potential from crop residue biomass in India. Renew Sust Energ Rev 32:504–512

Hu J (2014) The role of accessory enzymes in enhancing the effective hydrolysis of the cellulosic component of pretreated biomass. Thesis submitted to The University of British Columbia, Vancouver, pp 98–106

Ichinose H, Araki Y, Michikawa M, Harazono K, Yaoi K, Karita S, Kaneko S (2012) Characterization of an endo-processive-type xyloglucanase having a β-1,4-glucan-binding module and an endo-type xyloglucanase from *Streptomyces avermitilis*. Appl Environ Microbiol 78(22):7939–7945

Jabbour D, Borrusch MS, Banerjee G, Walton JD (2013) Enhancement of fermentable sugar yields by α-xylosidase supplementation of commercial cellulases. Biotechnol Biofuels 6:58–66

Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod Bior 1:119–134

Kaida R, Kaku T, Baba M, Oyadomari M, Watanabe T, Nishida K, Kanaya T, Shani Z, Shoseyov O, Hayashi T (2009) Loosening xyloglucan accelerates the enzymatic degradation of cellulose in wood. Mol Plant 2(5):904–909

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48(8):3713–3729

Lorences EP (2004) Cell wall xyloglucan incorporation by xyloglucanendotransglucosylase/hydrolase in pine hypocotyls. Plant Sci 166(5):1269–1274

Madson M, Dunand C, Li XM, Verma R, Vanzin GF, Calplan J, Shoue DA, Carpita NC, Reiter WD (2003) The MUR3 gene of *Arabidopsis* encodes a xyloglucan galactosyl transferase that is evolutionarily related to animal exostosins. Plant Cell 15:1662–1670

Maldonado-Mendoza I, Dewbre GR, Blaylock L, Harrison MJ (2005) Expression of a xyloglucanendotransglucosylase/hydrolase gene, Mt-XTH1, from *Medicagotruncatula* is induced systemically in mycorrhizal roots. Gene 345(2):191–197

Malherbe S, Cloete TE (2003) Lignocellulosic biodegradation: fundamentals and applications: a review. Rev Environ Sci Biotechnol 1:105–114

Martinez-Fleites C, Guerreiro CIPD, Baumann MJ, Taylor EJ, Prates JAM, Ferreira LMA, Fontes CMGA, Brumer H, Davies GJ (2006) Crystal structures of *Clostridium thermocellum* xyloglucanase, XGH74A, reveal the structural basis for xyloglucan recognition and degradation. J Biol Chem 281:24922–24933

Matsuzawa T, Saito Y, Yaoi K (2014) Key amino acid residues for the endo-processive activity of GH74 xyloglucanase. FEBS Lett 588:1731–1738

Michailidis G, Argiriou A, Darzentas N, Tsaftaris A (2009) Analysis of xyloglucanendotransglycosylase/hydrolase (XTH) genes from allotetraploid (*Gossypium hirsutum*) cotton and its diploid progenitors expressed during fiber elongation. J Plant Physiol 166(4):403–416

Miedes E, Lorences EP (2009) Xyloglucanendotransglucosylase/hydrolases (XTHs) during tomato fruit growth and ripening. J Plant Physiol 166(5):489–498

Miedes E, Zarra I, Hoson T, Herbers K, Sonnewald U, Lorences EP (2011) Xyloglucanendotransglucosylase and cell wall extensibility. J Plant Physiol 168(3):196–203

Mohanram S, Amat D, Choudhary J, Arora A, Nain L (2013) Novel perspectives for evolving enzyme cocktails for lignocellulose hydrolysis in biorefineries. Sustain Chem Process 1:15

Muñoz-Bertomeu J, Lorences EP (2014) Changes in xyloglucan endotransglucosylase/hydrolase (XTHs) expression and XET activity during apple fruit infection by *Penicillium expansum* Link. A. Eur J Plant Pathol 138:273–282

Nishitani K (1995) Endo-xyloglucan transferase, a new class of transferase involved in cell-wall construction. J Plant Res 108:137–148

Pauly M, Albersheim P, Darvill A, York WS (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. Plant J 20:629–639

Powlowski J, Mahajan S, Schapira M, Master ER (2009) Substrate recognition and hydrolysis by a fungal xyloglucan-specific family 12 hydrolase. Carbohydr Res 344(10):1175–1179

Rani V, Saritha M, Tiwari R, Nain L, Arora A (2014) Beta-glucosidase: key enzyme in determining efficiency of cellulase and biomass hydrolysis. J Bioprocess Biotech 5(1):197–205

Romo S, Jimenez T, Labrador E, Dopico B (2005) The gene for a xyloglucanendotransglucosylase/hydrolase from *Cicer arietinum* is strongly expressed in elongating tissues. Plant Physiol Biochem 43(2):169–176

Rose J, Catala C, Gonzalez-Carrana Z, Roberts J (2003) Cell wall disassembly. In: Rose JK (ed) The plant cell wall. Annual Plant Reviews 8. CRC Press, Boca Raton, pp 265–324

Saladie M, Rose JKC, Cosgrove DJ, Catala C (2006) Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. Plant J 47:282–295

Shankaracharya NB (1998) Tamarind-chemistry, technology and uses—a critical appraisal. J Food Sci Technol 35:193–208

Singh S, Pranaw K, Singh B, Tiwari R, Nain L (2014) Production, optimization and evaluation of multicomponent holocellulase produced by *Streptomyces* sp. ssr-198. J Taiwan Inst Chem E 45:2379–2386

Sinitsyna OA, Fedorova EA, Pravilnikov AG, Rozhkova AM, Skomarovsky AA, Matys VYu, Bubnova TM, Okunev ON, Vinetsky YuP, Sinitsyn AP (2010) Isolation and properties of xyloglucanases of *Penicillium* sp. Biochemistry (Moscow) 75(1):41–49

Sinnott ML (1990) Catalytic mechanisms of enzymic glycosyl transfer. Chem Rev 90:1171–1202

Song S, Tang Y, Yang S, Yan Q, Zhou P, Jiang Z (2013) Characterization of two novel family 12 xyloglucanases from the thermophilic *Rhizomucor miehei*. Appl Microbiol Biotechnol 97 (23):10013–10024

Stratilova E, Ait-Mohand F, Rehulka P, Garajova S, Flodrova D, Rehulkova H, Farkas V (2010) Xyloglucanendotransglycosylases (XETs) from germinating nasturtium (*Tropaeolum majus*) seeds: isolation and characterization of the major form. Plant Physiol Biochem 48(4):207–215

Sweeney MD, Xu F (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2:244–263

Takahashi M, Yamamoto R, Sakurai N, Nakano Y, Takeda T (2015) Fungal hemicellulose-degrading enzymes cause physical property changes concomitant with solubilization of cell wall polysaccharides. Planta 241:359–370

Timell TE (1967) Recent progress in the chemistry of wood hemicelluloses. Wood Sci Technol 1:45–70

van den Brink J, de Vries RP (2011) Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 6:1477–1492

Vincken JP, Dekeizer A, Beldman G, Voragen AGJ (1995) Fractionation of xyloglucan fragments and their interaction with cellulose. Plant Physiol 108:1579–1585

Vincken J-P, York WS, Beldman C, Voragen AGJ (1997) Two general branching patterns of xyloglucan, XXXG and XXGC. Plant Physiol 114:9–13

Vlasenko E, Schülein M, Cherry J, Xu F (2010) Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour Technol 101:2405–2411

Warner CD, Go RM, García-Salinas C, Ford C, Reilly PJ (2011) Kinetic characterization of a glycoside hydrolase family 44 xyloglucanase/endoglucanase from *Ruminococcus flavefaciens* FD-1. Enzyme Microb Technol 48(1):27–32

Wyman CE (1996) Handbook on bioethanol: production and utilization. Taylor Francis, Washington, p 417

Yamatoya K, Shirakawa M (2003) Xyloglucan: structure, rheological properties, biological functions and enzymatic modification. Current Trends Polym Sci 8:27–72

Yaoi K, Kondo H, Noro N, Suzuki M, Tsuda S, Mitsuishi Y (2004) Tandem repeat of a seven-bladed β-propeller domain in oligoxyloglucan reducing-end-specific cellobiohydrolase. Structure 12:1209–1217

Yaoi K, Mitsuishi Y (2004) Purification, characterization, cDNA cloning, and expression of a xyloglucan endoglucanase from *Geotrichum sp*. M128. FEBS Lett 560:45–50

Yaoi K, Miyazaki K (2012) Cloning and Expression of isoprimeverose-producing oligoxyloglucan hydrolase from actinomycetes species, *Oerskovia* sp. Y1. J Appl Glycosci 59(2):83–88

Yokoyama R, Rose JKC, Nishitani K (2004) A surprising diversity and abundance of xyloglucanendotransglucosylase/hydrolases in rice. Classification and expression analysis. Plant Physiol 134:1088–1099

Yuan S, Wu Y, Cosgrove DJ (2001) A Fungal endoglucanase with plant cell wall extension activity. Plant Physiol 127(1):324–333

Part III
Lignocellulose Oxidureductases

Chapter 10
Role of Mushroom Mn-Oxidizing Peroxidases in Biomass Conversion

Mirjana Stajić, Jelena Vukojević, Ivan Milovanović, Jasmina Ćilerdžić and Aleksandar Knežević

Abstract Intensive agricultural and industrial production followed by increased production of lignocellulosic wastes, disruption of environment, and depletion of natural resources are features of the modern society. However, these wastes present sustainable resources of fibers and energy and can be useful raw materials for many industries. Therefore, development of the optimal ways for their environmental and economical friendly biological pretreatment where the main participants will be fungi, owing to their ability to produce lignocellulolytic enzymes, preoccupies scientists. Mn-oxidizing peroxidases play crucial role in the process and based on the substrate specificity, this group is divided into Mn-dependent- and versatile peroxidases. However, delignification capacity depends on fungal species and strain, namely on their potential of lignocellulolytic enzyme production and degradation selectivity, type and composition of lignocellulosic wastes, and fermentation conditions. Species which predominantly degrade lignin and significantly weaker cellulose could have important role in processes of food, feed, paper, and biofuel production.

10.1 Introduction

Steady growth of the world population accompanied by increase of agricultural and industrial production leads to enhancement of types and amount of plant residues, on the one hand, and to disruption of environment and rapid depletion of natural energy and fiber sources, on the other hand. Dominant crops are different at the continents, for example rice is the most common in Asia, maize in America, and wheat in Europe, and result of that is accumulation of different lignocellulosic wastes. In 2010, rice residues were the most abundant, with annual production of $104,507 \times 10^4$ tons, and wheat and maize ones followed them with production of $98,104 \times 10^4$ tons and $84,031 \times 10^4$ tons, respectively (Gupta and Verma 2015).

M. Stajić (✉) · J. Vukojević · I. Milovanović · J. Ćilerdžić · A. Knežević
Faculty of Biology, University of Belgrade, Takovska 43, Belgrade 11000, Serbia
e-mail: stajicm@bio.bg.ac.rs

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_10

According to Asim et al. (2015), world production of agricultural waste in 2013 was 150 billion tons. The municipal waste also takes significant place in total annual waste production, approximately 10 %, and it also contains certain amount of lignocellulosic residues in the form of paper products, food scraps, and garden waste (Blumenthal 2011). Nowadays, management of the wastes presents real challenge because major part is improperly deposited and fewer amounts is burned producing not only CO_2 but also other pollutants. However, lignocellulosic biomass can be useful raw materials for many industries as well as potential source of renewable energy. Currently, special attention is given to regular disposal and recycling of the enormous quantity of plant wastes, energy recovering, as well as to raising awareness of the importance of these processes.

Plant cell wall is lignocellulosic in nature and composed of cellulose (40–50 %) and hemicellulose (20–30 %) impregnated with lignin (10–25 %) (Kirk and Cullen 1998; Anwar et al. 2014). Cellulose is the most abundant insoluble and resistant carbohydrate in biosphere with crystalline and amorphous regions. It is composed of chains built of (1,4)-D-glucopyranose units connected by β-1,4 linkages that have tendency of forming intra- and intermolecular hydrogen bounds and in such a way micro-fibrils (Iqbal et al. 2011). This polymer links by non-covalent linkages with second complex and heterogeneous polymer, i.e., hemicellulose. The heterogeneity originates from the presence of numerous sugars, among which xylan is the most frequent in grasses and straws and mannan in hard- and softwood (Anwar et al. 2014).

Lignin is the most recalcitrant natural aromatic compound on Earth, which provides strength and rigidity to plant cell walls, protection of structural polysaccharides from hydrolytic enzymes, and barrier against microbial attack. This heterogeneous polymer is composed of oxygenated phenylpropanoid units (coniferyl, sinapyl, and *p*-coumaryl alcohols), which are bounded by aryl–ether, aryl–aryl, and carbon–carbon bonds (Guerriero et al. 2015). However, composition, branching level, type of linkages with carbohydrates, as well as content of lignin vary from species to species and also depend on plant development stage. Thus, composition variation depending on plant species was demonstrated by Lapierre (1993) who showed that ratio among lignin units, *p*-hydroxyphenyl, guaiacyl, and syringyl, in spruce was 2:98:0, in birch 0:24:76 and in wheat straw 5:49:46.

10.2 Lignocellulose Degradation

Nowadays, special attention is given to possibility of degradation of lignin from lignocellulosic biomass and making access to cellulose and hemicellulose that can be converted into feeds, paper, biofuels, and other valued products. Regarding the complex structure, degradation of lignocellulose is very demanding process that can be realized in four ways: physical, chemical, physico-chemical, and biological, which differ one from another in efficiency and drawbacks (Yang and Wyman 2008; Asgher et al. 2013). Although special equipments and industrial processes are

needed for physical treatments, they are useful because they lead to reduction of particle size, commonly by grinding, pelleting or heating, and in such a way improve digestibility of lignocellulosic biomass (Kuijk et al. 2015). Chemical hydrolysis is based on usage of either acids (hydrochloric or sulphuric acid in high concentrations) or bases (sodium or ammonium hydroxide) or not selective chemicals (H_2O_2), and physico-chemical hydrolysis combine heat, moisture, pressure, and chemicals (Hendriks and Zeeman 2009). However, numerous studies have shown that these degradation processes are inefficient, very expensive, and hazardous for the environment. Therefore preference is given to an alternative environmental and economical friendly biological delignification, which unique participants are bacteria and fungi owing to their ability to produce lignocellulolytic enzymes. The main advantages of the process are energy saving and environmental protection (Kuijk et al. 2015). Nevertheless, the process has specific demands and some flaws. Namely, it can be realized only under aerobic conditions and effectiveness depends on lignin structure, i.e. type and ratio of its constituents, branching level, as well as lignin amount. Grabber (2005) showed that highly branched lignin is less degradable than mainly linear lignin, Skyba et al. (2013) that syringyl-rich lignin is more resistant to degradation by fungal enzymes, and Arora and Sharma (2009) that negative correlation exists between lignin amount and dry matter digestibility. Loss of dry matter by consumption of cellulose and hemicellulose by fungi, slowness, and requirement for sterile conditions are the main drawbacks of the process (Kuijk et al. 2015).

Filamentous fungi, among which Basidiomycetes stand out specially, are good degraders of lignocellulose. According to lignocellulose decay pattern, Eriksson et al. (1990) separated three groups of fungi: white rot, brown rot and soft rot, and their activities depend on environmental conditions, plant species, and cell type. White rot fungi are common in hardwood forests and include several hundred species of Basidiomycetes and some Ascomycetes, which breakdown lignin especially during the early phase of colonization, make access to the cellulose and hemicellulose, and depolimerize them in fruiting stage (Hammel 1997; Kirk and Cullen 1998; Sánchez 2009; Kuijk et al. 2015). Ginterová and Lazarová (1987) showed that ratio among degraded lignin, cellulose, and hemicellulose at the end of wheat straw colonization by *Pleurotus ostreatus* was 37.4:20:32.3 % and at the end of fructification 41.8: 62.1: 60.8 %. Some species, such as *Phanerochaete chrysosporium*, degrade lignin, cellulose, and hemicellulose simultaneously and other species, for example *Ceriporiopsis subvermispora*, attack lignin first. White rot fungi are the most efficient lignin mineralizators which have ability to cleave ether bounds in non-phenolic lignin substructures whose proportion in the woody biomass is 90 %. Contrary to these fungi, brown-rot species that are mainly present in coniferous forests only modify lignin and their main activity is cellulose and hemicellulose depolymerisation, while soft rot species degrade only cellulose under conditions of high moisture and low lignin content (Sánchez 2009).

According to Leonowicz et al. (1999) and Sánchez (2009), white rot fungi produce three groups of enzymes: (A) lignocellulolytic enzymes, which include ligninases (lignin peroxidase, Mn-oxidizing peroxidases, laccase, horseradish

peroxidase, protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase and 1,2,4-trihydroxybenzene 1,2-dioxygenase), celullases (endo-1,4-β-glucanase, cellobiohydrolase, β-glucosidase), and hemicellulases (endo-1,4-β-xylanase, xylan 1,4-β-xylosidase, endo-1,4-β-mannanase, β-mannosidase, β-glucosidase, α-galactosidase); (B) enzymes which cooperate with the first group of enzymes but never attack wood independently (superoxide dismutase and glyoxal oxidase); (C) feedback type enzymes that include glucose 1-oxidase, aryl-alcohol oxidase, pyranose 2-oxidase, cellobiose dehydrogenase, and cellobiose quinone oxidoreductase, which are involved both in lignin depolymerization (indirectly) and cellulose degradation.

Due to unique lignin properties, such as molecule size and branching level, specific bounds and insolubility in water, delignification should be extracellular, oxidative, less specific, and slow process where non-specific oxidoreductases have the main role (Hammel 1997). Essential enzymes for delignification beginning are fungal peroxidases, which produce certain radicals that can penetrate cell wall, and enzymes producing H_2O_2 (aryl-alcohol oxidases, glucose 1-oxidase, pyranose 2-oxidase, glyoxal oxidase, and cellobiose quinone oxidoreductase) that is required for peroxidase activity (Hammel and Cullen 2008). Additionally, in that stage of lignin mineralization superoxide dismutase also has important role because of its ability to catalyze conversion of superoxide anion into O_2 and H_2O_2 and in such a way protects fungal degrader from radicals.

Lignin peroxidases (EC 1.11.1.14) are H_2O_2 dependent heme-containing enzymes which oxidize aromatic compounds, such as phenols and anilines that cannot react directly with heme. These enzymes are strong oxidants because iron ion from porphyrin ring is electron-deficient, and the presence of tryptophan enables direct oxidation of non-phenolic substructures contrary to Mn-dependent peroxidases, which indirectly degrade them by production of free radicals (Hammel and Cullen 2008). Laccases (EC 1.10.3.2) are polyphenol oxidases that contain one to four copper ions (Cu^{2+}) and catalyze the one-electron oxidation of a broad number of substrates (phenols, methoxyphenols, some non-phenolic aromatic compounds, aromatic amines, some aliphatic alcohols, and lignin) using molecular oxygen as the electron acceptor. During the process, Cu^{2+} reduces to Cu^{+} and oxygen to water (Thurston 1994; Muñoz et al. 1997a, b; Medeiros et al. 1999). Three more enzymes are important for lignin degradation process. First of them is aryl-alcohol oxidases (EC 1.1.3.7), flavoenzymes that oxidize various aromatic compounds as well as aliphatic polyunsaturated alcohols providing H_2O_2 required for activity of lignin- and Mn-oxidizing peroxidases, and generates ·OH that initiates attack on lignocellulose (Evans et al. 1991; Gutiérrez et al. 1994; Varela et al. 2001). These enzymes act synergistically with laccases and prevent repolymerization of products generated during degradation of lignin and other aromatic compounds (Guillén et al. 2000). Aryl-alcohol dehydrogenases (EC 1.1.1.90), which catalyze conversion of aromatic alcohol to aromatic aldehyde, and quinone reductases (EC 1.6.5.5) that catalyze obtaining semiquinone from quinone, are also involved in delignification.

Degradation of hemocellulose requires the presence of more enzymes, known as hemicellulases, due to more complex structure of the polysaccharide. Among numerous hemicellulases, xylan 1,4-β-xylosidases and endo-1,4-β-xylanases have the main role in depolymerization. Cleavage of xylan to oligosaccharides is catalyzed by endo-1,4-β-xylanases (EC 3.2.1.8) and hemicellulose mineralization is finished by action of 1,4-β-xylosidases (EC 3.2.1.37), which hydrolyze oligosaccharides to xylose (Sánchez 2009). However, some accessory enzymes (xylan esterases, ferulic and *p*-coumaric esterases, α-1-arabinofuranosidases, and α-4-*O*-methyl glucuronosidases) are necessary for completing the reaction.

Three cellulases catalyze synchronously hydrolysis of cellulose β-1,4-glycosidic linkages. First, endo-1,4-β-glucanases (EC 3.2.1.4) randomly attacks numerous sites inside the molecule. Further, cellobiohydrolases (EC 3.2.1.91), also known as exoglucanases, hydrolyze removing monomers and dimers from the end of the chain and act synergistically with the first enzymes. At the end, β-glucosidases (EC 3.2.1.21) cleave bounds inside glucose dimers, sometimes within cellulose oligosaccharides, releasing glucose (Sánchez 2009).

10.2.1 Mn-Oxidizing Peroxidases

Wood-decaying and soil litter decomposing Basidiomycetes (members of families *Meruliaceae*, *Coriolaceae*, *Polyporaceae*, *Strophariaceae*, and *Tricholomataceae*) have ability to synthesize Mn-oxidizing peroxidases that belong to class II of superfamily of plant-fungal-prokaryotic peroxidases, and which divided into the Mn-dependent peroxidases (EC 1.11.1.13; MnPs) and versatile peroxidases (EC 1.11.1.16; VPs) based on the substrate specificity (Fernández-Fueyo et al. 2014). MnPs posses a Mn^{2+} oxidation site that binds the cations, and VPs both Mn^{2+} oxidation site and site for direct oxidation of low- and high-redox-potential lignin-related compounds (Perez-Boada et al. 2005). These enzymes are produced in numerous isoforms characterized by certain structure and properties, especially by isoelectric points and molecular weights (Martinez et al. 1996; Camarero et al. 1999; Hofrichter 2002; Stajic et al. 2006a, 2010; Lanfermann et al. 2015). Thus, *Ganoderma applanatum*, *Ceriporiopsis subvermispora*, *Phanerochaete chrysosporium*, and *Phlebia chrysocreas* synthetise 2, 4, 5, and 7 MnP isoforms, respectively, which are encoded by separate genes (Martinez et al. 2004; Salame et al. 2012; Lanfermann et al. 2015). *Pleurotus ostreatus* besides 6 MnPs also produce 3 VPs, which structures, stability, and catalytic properties were characterized by Fernández-Fueyo et al. (2014). However, expression of the genes depends on cultivation conditions, substrate composition, and nutrient availability, as well as concentration of Mn^{2+} ions (Urzúa et al. 1995; Ruiz-Dueñas et al. 1999; Stajić et al. 2006a, b; 2009; Salame et al. 2012). Thus, Urzúa et al. (1995) obtained 7 isoforms of *C. subvermispora* MnP by cultivation in salt medium and only 4 isoenzymes on wood chips. In the case of *Ph. chrysosporium*, Datta et al. (1991) noted expression of *mnp5* gene during its cultivation on wood pulp, *mnp4* gene was

transcribed in growth on wood-containing soil (Stuardo et al. 2004), while nitrogen poor medium but enriched with Mn^{2+} expressed *mnp1* and *mnp2* genes (Hammel and Cullen 2008). Letter, Salame et al. (2012) showed that Mn^{2+}-deficient medium caused increase of expression of *mnp3* and *mnp9 P. ostreatus* genes and in such a way activity of the MnP izoforms raised in 200-fold, while expression of *mnp4* gene, responsible for VP synthesis, was down-regulated in the medium enriched with this ions. Repression of VP activities in the Mn^{2+} presence, though the enzymes have ability to oxidize the ions, Salame et al. (2012) explained by the fact that VPs are expressed during the later stages of lignin degradation when majority of Mn^{2+} presented in the biomass are exhausted. However, Urzúa et al. (1995) showed that requirement for Mn^{2+} also depends on nature of the aromatic substrate.

The main environmental factors which affect Mn-oxidizing peroxidase activities are pH and temperature. According to Fernández-Fueyo et al. (2014), the most appropriate pH for wood lignin degradation by *P. ostreatus* is 3.0 due to numerous lysine and other alkaline residues that increase positive charge and stability at acidic pH values. However, pH values below 3.0 as well as pH 9.0 inactivate all peroxidases in very short time. The peroxidase instability in alkali environment the authors explained by loss of the Ca^{2+} ions. *P. ostreatus* VPs are more stable on high temperature than MnPs, and some of these enzymes can retain about 80 % of activity after incubation at 60 °C for 10 min.

10.2.1.1 Structure

Mn-oxidizing peroxidases are H_2O_2-dependent heme glycoproteins with an iron protoporphyrin IX prosthetic group in the active site, which generate Mn^{3+} that initiates delignification (Wariishi et al. 1988). Heme iron is Fe^{3+} located at an internal cavity and connects with the protein through two histidine residues which situate proximally and distally to the iron ion. The central cavity is delimited with two approximately equal domains built of 10–12 predominantly α-helices composed of conserved amino acid residues and located proximally and distally to the heme (Martinez 2002; Fernández-Fueyo et al. 2014). Heme pocket bounds with two Ca^{2+} ions (one above and one below the heme) which link with oxygen of amino acid residues and form Ca^{2+} binding sites that stabilize the protein structure. Amino acid residues in distal Ca^{2+} binding sites are conserved in all fungal Mn-oxidizing peroxidases, while proximal site differs among fungal species by two residues. Four or five disulfide bridges, composed of cysteine residues, also stabilize the protein structure (Martinez 2002). Heme connects with enzyme surface by two channels. One of them is located in front of heme propionate and delimited by three acidic residues which form Mn^{2+} binding site. When Mn^{2+} enters in the channel it oxidizes to Mn^{3+} which is released from the active site in the presence of chelators that are extracellular metabolites of white rot fungi. Second, narrow, channel is the place where H_2O_2 reacts with heme Fe^{3+} and has important role in protection of the enzyme from substrate radicals which could inactivate it (Ruiz-Dueñas et al. 2009).

Mn-oxidizing peroxidases posses multiple substrate sites at both sides of the heme pocket and could oxidize different substrates.

However, Fernández-Fueyo et al. (2014) demonstrated significant differences between *Pleurotus ostreatus* MnPs and VPs in amino acid sequence length, amino acid composition and distribution, as well as in substrate specificity and catalytic efficiency on Mn^{2+}. Thus, MnPs contains sequences composed of 331 and VPs of 339 residues mainly located in C-terminal region. In MnPs number of proline residues varies from 25 to 26 and in VPs from 30 to 31, while number of lysine residues is ranged from 7 to 10 in all isoforms except MnP4 where the number is 20. In the case of substrate and ability of Mn^{2+} oxidation, VPs have wider specificity and higher efficiency than MnPs. This could be explained by the fact that VPs, besides Mn^{2+} oxidation site composing of three conserved acidic residues near the internal heme propionate, also posses lignin oxidation site near tryptophan that is substituted by an alanine or aspartic acid in MnPs. Exceptions are *P. ostreatus* MnP1 and *P. pulmonarius* peroxidase which posses tryptophan but not activity characterized for VPs, that Fernández-Fueyo et al. (2014) explained by change of shape and charge of surface around tryptophan and in such a way substrate binding. Analyzing the structures of *P. ostreatus* MnP4 and VP1 these authors noted a few differences: (i) in domain close to the Ca^{2+} ion; (ii) in the main heme access channel, which is wider in VP1 than in MnP4 due to the presence of three substrate oxidation sites; (iii) in shape and charge of lower channel lip, as wide reservoir with negative charge in VP1 and as narrow groove of less charged surface in MnP4; (iv) in number of lysine residues, MnP4 has the minimum one unit more than VP1, which influences isoelectic points, and (v) in number of hydrogen bonds and salt bridges that are more numerous in MnP4 than in VP1.

10.2.1.2 Catalytic Properties

Mn-dependent peroxidases play a crucial role in the process of delignification and characterized by classical peroxidase cycle and inability to oxidize directly non-phenolic lignin-related structures (Hammel and Cullen 2008). H_2O_2 or organic peroxide donate one electron to MnP heme producing Compound I (Fe^{4+}-oxo-porphyrin-radical complex) and one water molecule is expelled (Fig. 10.1). Compound I is reduced to Compound II (Fe^{4+}-oxo-porphyrin complex) by peroxidase substrate, phenols or Mn^{2+}. However, efficient completion of MnP catalytic cycle, i.e. reduction of Compound II to the native enzyme requires the presence of only Mn^{2+} that is commonly presented in lignocellulose and oxidizes to Mn^{3+} with release of the second water molecule (Martinez 2002). Mn^{3+} is small quite unstable oxidizer, which is stabilized by organic acids such as oxalate, malonate, tartrate, and lactate, and can diffuse into the plant cell wall and catalyze cleavage of bounds in phenolic lignin substructures producing a phenoxyl radical intermediate (Fig. 10.1). Chelates of Mn^{3+} with the acids can also oxidize other phenolic, various non-phenolic aromatic, amino-aromatic, thiol compounds, and unsaturated fatty acids to phenoxyl, aryl, amino, thiyl, and peroxyl radicals, respectively. These

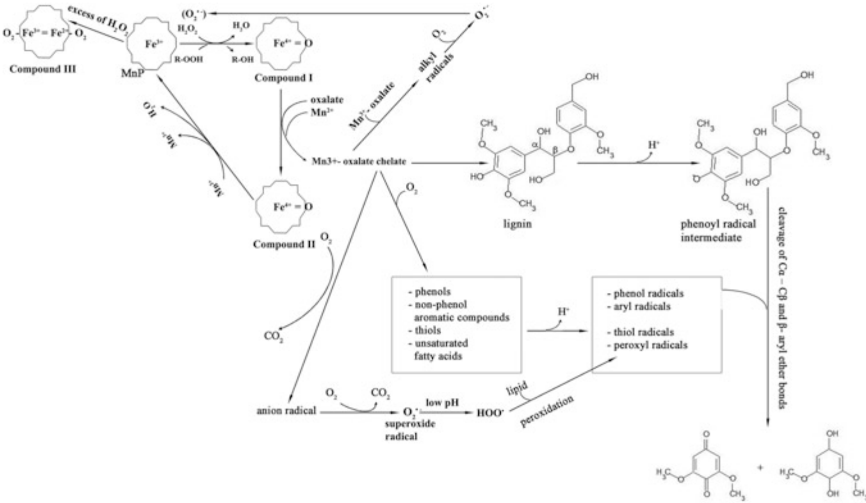


Fig. 10.1 Mechanism of action of Mn-oxidizing peroxidases

radicals can cleave Cα–Cβ and β-aryl ether bonds in abundant non-phenolic lignin substructures. Contrary to fatty acids, which are normally presented in the fungal environment, thiols are not produced by fungi and SH-groups are released in small amount by protein degradation during cell lyses (Hofrichter 2002). Likewise, Mn^{3+} can oxidize the oxalate from chelate generating CO_2 and anion radical, which in the presence of O_2 forms another CO_2 molecule and superoxide radical ($O_2^{\cdot-}$) that at low wood pH values transforms into perhydroxyl radical ($HOO^{\cdot}$) leading to lipid peroxidation and releasing of ligninolytic peroxyl radicals (Fig. 10.1). However, more efficient mechanism of mineralization of non-phenolic lignin moieties means their modification by cellobiose dehydrogenase to structures which are accessible to MnPs. Chelates of Mn^{3+} with the acids can also react with each other and convert to alkyl radicals which then react spontaneously with oxygen and form some other radicals, for example superoxide, that could be used by native enzyme in the absence of H_2O_2 (Hofrichter 2002). This author also emphasized that high concentrations of H_2O_2 or O_2 lead to formation of Compound III, catalytically inactive form, which cause reversible inactivation of MnPs.

Martinez et al. (1996) first purified and characterized two VPs after submerged cultivation of *Pleurotus eryngii* in glucose/peptone/yeast extract medium. Later Martinez (2002) showed that these enzymes can oxidize both Mn^{2+} and aromatic compounds, and that present hybrids of MnPs and lignin peroxidases. These enzymes occur naturally and until now it is shown that various species of the genera *Pleurotus* and *Bjerkandera*, *Lentinus edodes*, *Panus tigrinus*, etc. can synthesize them (Hofrichter 2002; Hammel and Cullen 2008).

10.3 Plant Residue Conversion

At the beginning of twenty-first century, humankind faces with high rate of world population growth and in such a way with food insufficient, environmental pollution, and climate change. It should be emphasized that 70 % of agricultural products are not utilized and become ballast and potential pollutant of environment. Numerous fungi have essential role in conversion of this enormous amount of lignocellulosic waste in food, feed, paper pulp, biofuels, and various chemicals. However, all of the conversions require delignification without or with minimal loss of carbohydrates, which could be achieved by selection of the most efficient fungal species and strains, the optimal lignocellulosic biomass, as well as by optimization of cultivation conditions (Kuijk et al. 2015). Nutritional value and availability are the main criteria for selection of lignocellulosic residues. However, composition of plant waste of the same origin and consequently its degradation can vary because of existence of numerous cultivars of the same crop, for example number of wheat cultivars is still grown. It was demonstrated by Arora and Sharma (2009) who noted that *Phlebia floridensis* more efficiently delignificated wheat straw from north-eastern India than straw from other climatic regions. As efficiency of fungal enzymatic system depends on substrate type, selection of degrader is conditioned by lignocellulosic residue. Thus, *Ceriporiopsis subvermispora*, *Pleurotus eryngii*, *P. ostreatus*, *Lentinus edodes*, *Hericium clathroides,* and *Trametes versicolor* are excellent selective degraders, namely they mineralize more than 20 % of lignin and less than 5 % of cellulose in wheat straw (Tuyen et al. 2012; Knežević et al. 2013a, b; Stajić et al. 2013; Kuijk et al. 2015). Numerous studies confirmed that *C. subvermispora* and *P. eryngii* are the most effective mineralizers of wheat straw lignin and showed that Mn-oxidizing peroxidases have the essential role in the process of delignification (Fernandez-Fueyo et al. 2012; Tuyen et al. 2012). However, Asiegbu et al. (1996) and Chi et al. (2007) demonstrated that combination of few fungal species can be more effective in depolymerization of lignin in some plant wastes than their monocultures. Thus, extent of spruce sawdust delignification by co-culture of *Pleurotus sajor-caju*, *T. versicolor,* and *Phanerochaete chrysosporium* was significantly higher than by each monoculture separately (16 and 0–5 %, respectively), and co-culture of *C. subvermispora* and *P. ostreatus* caused higher loss of lignin from aspen wood than in its colonization with the monocultures. However, due to divergence in genotype, significant differences in lignocellulolytic enzyme production and selectivity of lignocellulose degradation exist among strains of the same mushroom species (Simonić et al. 2010; Knežević et al. 2013b). For example, percents of degraded wheat straw lignin and cellulose by some *P. ostreatus* strains were almost the same (Salvachua et al. 2011), while other strains predominantly depolymerized lignin (Adamović et al. 1998). Solid state fermentation, small lignocellulosic particles of suitable shape, high oxygen level, moderate water content, certain inoculum type (spores or spawn) in moderate amount, and the presence of copper, manganese, linoleic acid, and veratryl alcohol

are good conditions for biological treatment of lignocellulose by white rot fungi (Kuijk et al. 2015).

10.3.1 Food

Ability of mushrooms to produce lignocellulolytic enzymes has double benefit, degradation of plant residues, and production of high valued food. Therefore, numerous species are cultivated commercially and cheap and quality food, rich in proteins, fibers, vitamins, minerals as well as biologically active compounds, is obtained. Their consumption could compensate insufficient proteins in regions where famine presents serious problem and enhance immune system and resistance to diseases.

According to enzymatic system, mushrooms are divided into two groups, primary degraders-species with strong system and high degradation ability, and secondary degraders-species with poor enzymatic system that need help of other degraders. For example, species of the genera *Pleurotus*, *Lentinus*, *Grifola*, *Auricularia*, *Flammulina,* etc. belong to the first group, while *Agaricus* species make the second group.

Frequently cultivation in the plastic bags enables usage of various lignocellulosic wastes from agriculture, forestry, and food industries for compost preparation. If it bears in mind that production of cereal residues in Europe is high (55,585 $\times$ 10^4 tons in 2010) and if wastes from sugar crops and tuber vegetables are added to the value, it comes to 61,484 $\times$ 10^4 tons and it can be concluded that amount of potential substrates for mushroom production is enormous (Gupta and Verma 2015). Nowadays, mushroom producers could obtain high biological efficiency, from 60 to 75 %, depending on species. Additionally, considering that only third of world cereal straw production is used in the processes, it can be estimated potential of mushroom production.

Nowadays, three species of the genus *Agaricus* (*A. bisporus*, *A. blazei,* and *A. bitorquis*) are produced in the highest amounts and the first of them is leader (Chang and Miles 2004). Based on annual world production, *Lentinus edodes* takes the second place and numerous members of the genus *Pleurotus* (*P. ostreatus*, *P. ostreatus* var. *florida*, *P. eryngii*, *P. eryngii* var. *nebrodensis*, *P. sajor-caju*, *P. cystidiosus*, *P. citrinopileatus*, *P. djamor*, *P. ferulae*, *P. tuber-regium,* and *P. sapidus*) take third place. Efficiently usage of wide range of plant residues (straw, sawdust, maize, sugar cane pulp, wastes from cotton production, etc.), without any treatment and enrichment, by *Pleurotus* species (they can convert 100 g of dry lignocellulosic wastes in 50–70 g of fresh fruiting bodies in only few weeks) is the result of possession of strong ligninolytic enzymes (especially Mn-oxidizing peroxidases) characterized by well selectivity (Ginterová and Lazarová 1987). However, biological efficiency depends on substrate composition and strain. Thus, depending on wheat cultivar, content of reducing sugar differs considerably, between 13.1 and 40.7 mg/g and yield of various *P. ostreatus* strains can vary

between 123 and 262 kg of fruiting bodies per ton of wheat straw (Kuijk et al. 2015).

Nowadays, number of cultivated mushroom species is relatively small, 100 species are cultivated economically, 60 commercially, and only 10 in industrial scale, but current trend is growth of world production in favor of new species, such as *Agaricus blazei*, *Agrocybe aegerita*, *Armillaria mellea*, *Auricularia fuscosuccinea*, *Coprinus comatus*, *Dictyophora duplicata*, *Lepista nuda*, *Stropharia rugosaannulata*, *Tremella cinnabarina*, *T. aurantialba,* and *Tricholoma giganteum* (Chang and Miles 2004).

10.3.2 Feed

Most agricultural and industrial wastes that are used for animal feeding are rich in low digestible fibers and poor in nutrients, especially in proteins and vitamins. Besides high nutritionally valued food, result of mushroom cultivation is also production of enormous amount of spent substrate that can be an important feed ingredient. Mushroom mycelium is rich in protein, essential amino acids, and chitin and could be important nitrogen sources, while β-glucans and other extra- and intracellular polysaccharides could be an additional glucose sources and immunostimulators for ruminants (Reis et al. 2012; Cheung 2013). Thus, protein content in wheat straw after *P. ostreatus* cultivation can be increased by 89 % that leads to overcoming of the main limitation of straw feed, i.e. nitrogen lack (Kuijk et al. 2015). The authors also emphasized that the protein increases rumen microorganism population and in such a way cellulose and hemicellulose digestibility and energy releasing. Spend mushroom compost is also rich in vitamins, minerals, antimicrobial agents, probiotics, and other biologically active compounds that modify animal metabolism, enhance growth, improve immune system, and increase resistance to various diseases (Enshasy and Hatti-Kaul 2013).

Mushroom contribution to feed production is also transformation of natural recalcitrant lignin into digestible compounds owing to well developed lignocellulolytic enzyme system, especially Mn-oxidizing peroxidases. Although ruminants posses cellulolytic microorganisms in rumen that can depolymerize cellulose and hemicellulose from plant cell wall, connection of these carbohydrates for lignin by covalent or other linkages is negatively correlated with their digestibility under rumen anaerobic conditions. Rodrigues et al. (2008) showed that cultivation of various white rot mushrooms on wheat straw could increase degradability of neutral detergent fibers in rumen in even 13 % due to their strong ligninolytic enzymes which act to lignin. Contrary to wheat straw that was not biologically treated, spent wheat straw composts of *Pleurotus ostreatus* and *P. sajor-caju* were well consumed and digested by sheep (Calzada et al. 1987; Fazaeli et al. 2006). Similar efficient consumption and decomposition of crude protein, hemicellulose, and cellulose from spend *Coprinus fimetarius* rice straw compost and spent *Ganoderma* sp. wheat straw compost by goats were reported by Rai et al. (1989) and Shrivastava et al.

(2012). However, cows are more selective and their feed can be enriched with maximum 17 % of spend *P. ostreatus* wheat straw substrate (Adamović et al. 1998).

Kuijk et al. (2015) reviewed results of numerous studies and also reported that addition of selected microelements and inducers (copper, manganese, linoleic acid, di-rhamnolipid, and veratryl alcohol) to lignocellulosic residues enhance activity of fungal Mn-oxidizing peroxidases and indirectly fiber digestibility by animals. However, some mushroom metabolites could be toxic for animals and humans and special attention has to be given to that aspect of plant waste bioconversion by fungi.

10.3.3 Paper Pulp

Since nowadays forest preservation is compulsory, plant residues that are produced in enormous amount, but not used, can be important natural sources for paper-making. Good sample for extraordinary production of some agricultural wastes was given by González Alriols et al. (2009). Namely, they noted huge quantity of unutilized *Elaeis guineensis* residues after oil extraction in Malaysia, even 15 million tons per year, which present serious ballast. In a few last decades, white rot fungi or their enzymes have the main role in the process, known as biopulping. The main reasons for that are its healthy, environmental, and economical friendly relationships contrary to traditional usage of alkaline sulfide, as well as improvement of pulp quality and quantity (Bajpai et al. 2001). Yang et al. (2008) showed that pretreatment of eucalyptus woody biomass with *Trametes hirsuta* led to increasing fiber internal bond strength in 32 %. Fungal ligninolytic enzymes, primarily peroxidases, catalyze one-electron oxidation of lignin phenolic groups producing phenoxy radicals on fiber surfaces and in such a way enhancing inter fiber adhesion (Singh and Singh 2014). Thus, this modified lignin could replace traditionally harmful adhesives that is current trend and one more possible application of fungal enzymatic system.

Besides enhancement of the bond strength and fiber adhesion, wood pretreatment with white rot fungi removes pitches rich in lipophylic compounds (resin and fatty acids and triglycerides), which can reduce paper and effluent quality and cause technical problems (Martinez-Inigo et al. 2000; Dorado et al. 2001; Gutiérrez et al. 2006; Van Beek et al. 2007). Martinez-Inigo et al. (2000) reported that *Phlebia radiate* and *Poria subvermispora* were the most active in the removal of lipophylic extractives and in such a way pitch from eucalypt wood, and Dorado et al. (2001) that *Bjerkandera* sp. and *Trametes versicolor* eliminated up to 90 % of these compounds from *Pinus sylvestris* wooden biomass. Letter Van Beek et al. (2007) showed that *T. versicolor* was also effective in removal of resin acids and triglycerides from spruce wood chips, which content was reduced in 40 and 100 %, respectively, and that *Pycnoporus cinnabarinus* caused losses of sterols, resin acids, and triglycerides from various pulp systems in 65–100 %. Further study showed

that this ability of white rot fungi is based on production of lipase, resinase, lipoxigenases, and laccases (Gutiérrez et al. 2009). As pitch control is combined with lignin removal from the pulp, white rot fungi play important role in pulp bleaching (bio-bleaching) and thus in improving paper brightness, environment protection, and energy saving (Bajpai et al. 2001; Jerusik 2010). However, the process should be controlled in order to avoid cellulose degradation.

Besides papermaking, white rot fungi and their enzymes have one more role, bioremediation of waste water. Namely, huge amount of water, approximately 45–227 × 10^3 litters, is used for production of a ton of product resulting in the releasing of the same quantity of effluent and further in zooplankton and fish dying, slime and scum formation, esthetic problems, and disruption of the environment (Pokhrel and Viraraghavan 2004). Fungi are characterized by better survival in effluents and production of strong extracellular ligninolytic enzymes, especially lignin- and Mn-oxidizing peroxidases that degrade various phenolic compounds among which colors are the most resistant. Thus, numerous studies showed high effectiveness of *Trametes pubescens*, *Phanerochaete chrysosporium*, and *Pleurotus ostreatus* in the process (Prasad and Gupta 1997; Choudhury et al. 1998; González et al. 2010; Zhang et al. 2012). Prasad and Gupta (1997), Choudhury et al. (1998), and Saxena and Gupta (1998) noted high level of color degradation, even 80–93.8 %, in effluents from paper industry treated by *T. versicolor*, *Ph. chrysosporium* (sole or in combination with *Pycnoporus sanguineus*), *Pleurotus ostreatus*, and *Heterobasidion annosum*.

10.3.4 Biofuels

Intensive world industrialization and motorization result in enormous rise of demands for fuels, and nowadays 80 % needed energy is met by use of fossil fuels that have numerous harmful effects on environment. If rapid depletion of the fuels is added to the negative effects, current trend of finding new, alternative, renewable, and sustainable energy sources is justified. One of the most environmental friendly are biofuels, the consumption of which in 2020 should be about 20 % of overall energy usage (Nigam and Singh 2011). Biofuels are classified into two groups, primary (wood, wood chips and pellets, etc.) and secondary which are produced by biomass processing (ethanol, biodiesel, etc.), and secondary ones are further classified based on sources (forest, agricultural or fishery products, municipal wastes and wastes from food industry and services) and type (solid, liquid or gaseous). The main advantages of biofuel utilization are usage of broad distributed sustainable natural bioresources that could be serious environmental ballast, as well as lower CO_2 and polycyclic aromatic hydrocarbons emissions (Demirbas 2009).

Although crops rich in sugars or compounds that can be converted into the sugars (starch or cellulose) present an essential bioresources for bioethanol production, current trend is development of techniques for its production from inedible fast-growing biomass (certain trees and grasses), for example from grass

Miscanthus sinensis, which production is 5–6 times higher than cereal one, or from dried citrus waste that amount is about 800,000 tons only in USA (Zechendorf 1999; Tabka et al. 2006). However, in order to cellulose to be broken down to the glucose and further ferment, depolymerization of recalcitrant network around cellulose fibers, built of lignin, is necessary. Thus, it comes again to white rot fungi and their peroxidases which are the main participants in the first step of bioethanol production known as pretreatment, i.e. the essential catalyzers of beginning phase of lignin degradation. This pretreatment also depends on fungal species and strain, cultivation conditions, enzyme production and activity, and oxidative mechanisms (Dias et al. 2010; Wan and Li 2010; Salvachua et al. 2011). Dias et al. (2010) reported that various white rot fungi degraded between 2 and 65 % of wheat straw lignin during its solid state fermentation and in such a way increased cellulose/lignin ratio. However, Salvachua et al. (2011) emphasized that mode of lignocellulosic mineralization is different from species to species, for example contrary to *Panus tigrinus* and *Phlebia radiata* that degraded lignin and polysaccharides simultaneously, *Pleurotus eryngii* and *Phellinus robustus* removed lignin selectively and faster. Likewise, according to these authors, delignification extent is not always in correlation with ligninolytic enzymes production, as well as with fiber digestibility and sugar production. Thus, contrary to *P. radiata* and *P. robustus*, characterized both by high Mn-oxidizing peroxidase activities and effective lignin removal, good lignin degraders *Bjerkandera adusta* and *Coriolopsis rigida* had low active enzymes. In the case of effectiveness of cellulose and hemicellulose enzymatic hydrolysis in the second step of bioethanol production, *Pycnoporus coccineus* was high effective hemicellulose and weak cellulose degrader (98 and 31 %, respectively), *Bjerkandera adusta* was almost equal depolymerizer of these polymers (43 and 54 %, respectively), *Stereum hirsutum* mineralized only significant portion of cellulose (43 %), the highest glucose yield was obtained by cultivation of *Poria subvermispora* (69 %) and *Irpex lacteus* (66 %) and the lowest by *P. eryngii* and *P. robustus*.

10.4 Future Trends

Importance of fungi and their enzymes in various biotechnological processes stimulates serious investment for study of genomes of numerous fungal species, i.e. sequencing, characterization of ligninolytic enzyme genes, their expression in appropriate organisms, improvement of product properties, and production of recombinants in significant amount under non-natural conditions. As Mn-oxidizing peroxidases are secreted under conditions characterized by high moisture level and acidic environment (pH about 3.0), which are not typical in biotechnological processes, it needs to adapt the enzymes to the conditions. According to Guerriero et al. (2015), the adaptation could be reached either by directed evolution of certain ancestral enzyme, i.e. mutation of its gene, or by enzyme engineering, and result in the both cases is similar enzyme form but with improved and desired property.

Namely, one enzyme rarely possesses all catalytic properties required for effective lignocellulosic conversion. Therefore recombination can be used for unification of several beneficial characteristics from a few enzymes into superior one with high stability and broad substrate promiscuity. Finding of appropriate organism for expression of the gene leads to obtaining of significant amount of the recombinant (Alcalde 2015). Thus, Ogawa et al. (1998) showed that expression of *mnp* genes of *Pleurotus ostreatus*, excellent Mn-oxidizing peroxidase producer, in *Coprinus cinereus*, characterized with fast growth, led to increase of lignin degradation level in only 16 days, and Pérez-Boada et al. (2002) and Salame et al. (2012) obtained active versatile peroxidase by expression of *mnp4* gene in *Escherichia coli* but in lower yield. Considering that lignocellulolytic enzymes act synergistically, genetic engineering could be used for fusion of certain enzymes and also for generation of strain deficient in some enzyme. With this aim, Chalamcherla et al. (2010) getting cellulase-deficient *P. ostreatus* strain characterized by low cellulose and high lignin degradation, as well as by high capacity of application in various biotechnological processes. Nowadays, special attention is also given to optimization of cultivation conditions for production of certain lignocellulolytic enzyme and study of enzymatic system of fungal species living in extreme environment as well as their genes encoding proteins with unknown function among which some can be lignocellulolytic enzyme (Guerriero et al. 2015). Everything mentioned show great possibilities of usage and managing of fungi and their enzymes with the aim of biomass conversion into high-valued products and environment protection.

References

Adamović M, Grubić G, Milenković I, Jovanović R, Protić R, Lj Sretenović, Lj Stoićević (1998) The biodegradation of wheat straw by *Pleurotus ostreatus* mushrooms and its use in cattle feeding. Anim Feed Sci Tech 71:357–362

Alcalde M (2015) Engineering the ligninolytic enzyme. Trends Biotechnol 33:55–162

Anwar Z, Gulfraz M, Irshad M (2014) Agro-industrial lignocellulosic biomass a key to unlack the future bio-energy: a brief review. J Radiat Res Appl Sci 7:163–173

Arora DS, Sharma RK (2009) Enhancement in *in vitro* digestibility of wheat straw obtained from different geographical regions during solid state fermentation by white rot fungi. Bioresources 4:909–920

Asgher M, Ahmed Z, Iqbal HMN (2013) Alkali and enzymatic delignification of sugarcane bagasse of expose cellulose polymers for saccharification and bio-ethanol production. Ind Crop Prod 44:488–495

Asiegbu FO, Paterson A, Smith JE (1996) The effects of co-fungal cultures and supplementation with carbohydrate adjuncts on lignin biodegradation and substrate digestibility. World J Microb Biot 12:273–279

Asim N, Emdadi Z, Mohammad M, Yarmo MA, Sopian K (2015) Agricultural solid wastes for green desiccant applications: an overview of research achievements, opportunities and perspectives. J Clean Prod 91:26–35

Bajpai P, Bajpai PK, Akhtar M, Jauhari MB (2001) Biokraft pulping of eucalyptus with selected lignin-degrading fungi. J Pulp Pap Sci 27:235–242

Blumenthal K (2011) Generation and treatment of municipal waste. Stat Focus 31:1–12

Calzada JF, Franco LF, de Arriola MC, Rolz C, Ortiz MA (1987) Acceptability, body weight changes and digestibility of spent wheat straw after harvesting of *Pleurotus sajor-caju*. Biol Waste 22:303–309

Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martinez MJ, Martinez AT (1999) Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. J Biol Chem 274:10324–10330

Chalamcherla VL, Singaracharya MA, Lakshmi MV (2010) Amino acids profile of the lignocellulosic feed treated with cellulase-free lignolytic mutants of *Pleurotus ostreatus*. Bioresources 5:259–267

Chang ST, Miles PG (2004) Mushrooms. Cultivation, nutritional value, medicinal effect, and environmental impact. CRC Press LLC, London

Cheung PCK (2013) Mini-review on edible mushrooms as source of dietary fiber: preparation and health benefits. Food Sci Hum Well 2:162–166

Chi YJ, Hatakka A, Maijala P (2007) Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? Int Biodeter Biodegr 59:32–39

Choudhury S, Sahoo N, Manthan M, Rohela RS (1998) Fungal treatment of pulp and paper mill effluents for pollution control. J Ind Pollut Control 14:1–13

Datta A, Bettermann A, Kirk TK (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl Environ Microbiol 57:1453–1460

Demirbas A (2009) Progress and recent trends in biodisel fuels. Energ Convers Manag 50:14–34

Dias AA, Freitas GS, Marques GSM, Sampaio A, Fraga IS, Rodrigues MAM, Evtuguin DV, Bezerra RMF (2010) Enzymatic saccharification of biologically pre-treated wheat straw with white-rot fungi. Bioresource Technol 101:6045–6050

Dorado J, van Beek TA, Claassen FW, Sierra-Alvarez R (2001) Degradation of lipophilic wood extractive constituents in *Pinus sylvestris* by the white rot fungi *Bjerkandera* sp. and *Trametes versicolor*. Wood Sci Technol 35:117–125

Enshasy HAE, Hatti-Kaul R (2013) Mushroom immunomodulators: unique molecules with unlimited applications. Trends Biotechnol 31:668–677

Eriksson KEL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin

Evans CS, Gallagher IM, Atkey PT, Wood DA (1991) Localisation of degradative enzymes in white-rot decay of lignocellulose. Biodegradation 2:93–106

Fazaeli H, Azizi A, Amile M (2006) Nutritive value index of treated wheat straw with *Pleurotus* fungi fed to sheep. Pakistan J Biol Sci 9:2444–2449

Fernandez-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P et al (2012) Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Nat Acad Sci USA 109:5458–5463

Fernández-Fueyo E, Ruiz-Dueñas FJ, Martínez MJ, Romero A, Hammel KE, Medrano FJ, Martínez AT (2014) Ligninolytic peroxidase genes in the oyster mushroom genome: heterologous expression, molecular structure, catalytic and stability properties, and lignin-degrading ability. Biotechnol Biofuels 7:2

Ginterová A, Lazarová A (1987) Degradation dynamics of lignocellulose materials by wood-rotting *Pleurotus* fungi. Folia Microbiol 32:434–437

González Alriols M, Tejado A, Blanco M, Mondragon I, Labidi J (2009) Agricultural palm oil tree residues as raw material for cellulose, lignin and hemicelluloses production by ethylene glycol pulping process. Chem Eng J 148:106–114

González LF, Sarria V, Sánchez OF (2010) Degradation of chlorophenols by sequential biological-advanced oxidative process using *Trametes pubescens* and TiO_2/UV. BioresourceTechnol 101:3493–3499

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 45:820–831

Guerriero G, Hausman JF, Strauss J, Ertan H (2015) Destructuring plant biomass: focus on fungal and extremophilic cell wall hydrolases. Plant Sci 234:180–193

Guillén F, Gómez-Toribio V, Martinez MJ, Martinez AT (2000) Production of hydroxyl radical by the synergistic action of fungal laccase and aryl-alcohol oxidase. Arch Biochem Biophys 383:142–147

Gupta A, Verma JP (2015) Sustainable bio-ethanol production from agro-residues: a review. Renew Sust Energ Rev 41:550–567

Gutiérrez A, Caramelo L, Prieto A, Martinez MJ, Martinez AT (1994) Anisaldehyde production and aryl-alcohol oxidase and dehydrogenase activities in ligninolytic fungi from the genus *Pleurotus*. Appl Environ Microbiol 60:1783–1788

Gutiérrez A, del Rio JC, Rencoret J, Ibarra D, Martinez AT (2006) Main lipophilic extractives in different paper pulp types can be removed using the laccase-mediator system. Appl Microbiol Biotechnol 72:845–851

Gutiérrez A, del Rio JC, Martinez AT (2009) Microbial and enzymatic control of pitch in the pulp and paper industry. Appl Microbiol Biotechnol 82:1005–1018

Hammel EK (1997) Fungal degradation of lignin. In: Cadisch G, Giller KE (eds) Driven by nature: plant litter quality and decomposition. CAB International, Wallingford, pp 33–45

Hammel KE, Cullen D (2008) Role of fungal peroxidases in biological ligninolysis. Curr Opin Plant Biol 11:349–355

Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technol 100:10–18

Hofrichter M (2002) Review: lignin conversion by manganese peroxidase (MnP). Enzyme Microb Tech 30:454–466

Iqbal HMN, Ahmed I, Zia MA, Irfan M (2011) Purification and characterization of the kinetic parameters of cellulose produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility. Adv Biosci Biotechnol 2:149–156

Jerusik RJ (2010) Fungi and paper manufacture. Fungal Biol Rev 24:68–72

Kirk TK, Cullen D (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. In: Young RA, Akhtar M (eds) Environmentally friendly technologies for the pulp and paper industry. Wiley, New York, pp 273–308

Knežević A, Milovanović I, Stajić M, Lončar N, Brčeski I, Vukojević J, Ćilerdžić J (2013a) Lignin degradation by selected fungal species. Bioresource Technol 138:117–123

Knežević A, Milovanović I, Stajić M, Vukojević J (2013b) Potential of *Trametes* species to degrade lignin. Int Biodeter Biodegr 85:52–56

Kuijk SJA, Sonnenberg ASM, Baars JJP, Hendriks WH, Cone JW (2015) Fungal treated lignocellulosic biomass as ruminant feed ingredient: a review. Biotechnol Adv 33:191–202

Lanfermann I, Linke D, Nimtz M, Berger RG (2015) Manganese peroxidases from *Ganoderma applanatum* degrade β-carotene under alkaline conditions. Appl Biochem Biotechnol 175:3800–3812

Lapierre C (1993) Application of new methods for the investigation of lignin structure. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) Forage cell wall structure and digestibility. American Society of Agronomy, Madison, pp 133–166

Leonowicz A, Matuszewska A, Luterek J, Ziegenhagen D, Wojtaś-Wasilewska M, Cho NS, Hofrichter M, Rogalski J (1999) Biodegradation of lignin by white rot fungi. Fungal Gen Biol 27:175–185

Martinez AT (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb Tech 30:425–432

Martinez MJ, Ruiz-Dueñas FJ, Guillén F, Martinez AT (1996) Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. Eur J Biochem 237:424–432

Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F et al (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700

Martinez-Inigo MJ, Gutiérrez A, del Rio JC, Martinez MJ, Martinez AT (2000) Time course of fungal removal of lipophilic extractives from *Eucalyptus globulus* wood. J Biotechnol 84:119–126

Medeiros BM, Bento VA, Nunes LLA, Oliveira CS (1999) Optimization of some variables that affect the synthesis of laccase by *Pleurotus ostreatus*. Bioprocess Eng 21:483–487
Muñoz C, Guillen F, Martínez TA, Martínez JM (1997a) Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. Cur Microbiol 34:1–5
Muñoz C, Guillen F, Martínez TA, Martínez JM (1997b) Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. Appl Environ Microbiol 63:2166–2174
Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energ Combust 37:52–68
Ogawa K, Yamazaki T, Kajiwara S, Watanabe A, Asada Y, Shishido K (1998) Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and -degradation activities using novel heterologous protein expression vectors. Appl Microbiol Biotechnol 49:285–289
Pérez-Boada M, Doyle WA, Ruiz-Dueñas FJ, Martínez MJ, Martínez AT, Smith AT (2002) Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimisation of *in vitro* folding. Enzyme Microb Tech 30:518–524
Perez-Boada M, Ruiz-Dueñas FJ, Pogni R, Basosi R, Choinowski T, Martinez MJ, Piontek K, Martinez AT (2005) Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. J Mol Biol 354:385–402
Pokhrel D, Viraraghavan T (2004) Treatment of pulp and paper mill wastewater—a review. Sci Total Environ 333:37–58
Prasad GK, Gupta RK (1997) Decolourization of pulp and paper mill effluent by two white-rot fungi. Indian J Environ Health 39:89–96
Rai SN, Walli TK, Gupta BN (1989) The chemical composition and nutritive value of rice straw after treatment with urea or *Coprinus fimetarius* in a solid state fermentation system. Anim Feed Sci Tech 26:81–92
Reis FS, Barros L, Martins A, Ferreira ICFR (2012) Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: an inter-species comparative study. Food Chem Toxicol 50:191–197
Rodrigues MAM, Pinto P, Bezerra RMF, Dias AA, Guedes CVM, Cardoso VMG et al (2008) Effect of enzyme extracts isolated from white-rot fungi on chemical composition and in vitro digestibility of wheat straw. Anim Feed Sci Technol 141:326–338
Ruiz-Dueñas FJ, Guillen F, Camarero S, Perez-Boada M, Martinez MJ, Martinez AT (1999) Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus *Pleurotus eryngii*. Appl Environ Microbiol 65:4458–4463
Ruiz-Dueñas FJ, Morales M, Garcia EMY, Martinez MJ, Martinez AT (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycetes peroxidases. J Exp Bot 60:441–452
Salame TM, Knop D, Levinson D, Mabjeesh SJ, YardenO Hadar Y (2012) Release of *Pleurotus ostreatus* versatile-peroxidase from Mn^{2+} repression enhances anthropogenic and natural substrate degradation. PLoS ONE 7:e52446
Salvachua D, Prieto A, Lopez-Abelairas M, Lu-Chau T, Martinez AT, Martinez MJ (2011) Fungal pretreatment: an alternative in second-generation ethanol from wheat straw. Bioresource Technol 102:7500–7506
Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185–194
Saxena N, Gupta RK (1998) Decolourization and delignification of pulp and paper mill effluent by white rot fungi. Indian J Exp Biol 36:1049–1051
Shrivastava B, Nandal P, Sharma A, Jain KK, Khasa YP, Das TK, Mani V, Kewalramani NJ, Kundu SS, Kuhad RC (2012) Solid state bioconversion of wheat straw into digestible and nutritive ruminant feed by *Ganoderma* sp. rckk02. Bioresource Technol 107:347–351
Simonić J, Vukojević J, Stajić M, Glamočlija J (2010) Intraspecific diversity within *Ganoderma lucidum* in laccase and Mn-dependent peroxidases production during plant residues fermentation. Appl Biochem Biotechnol 162:408–415

Singh AP, Singh T (2014) Biotechnological applications of wood-rotting fungi: a review. Biomass Bioenerg 62:198–206

Skyba O, Douglas CJ, Mansfield SD (2013) Syringyl-rich lignin renders poplars more resistant to degradation by wood decay fungi. Appl Environ Microbiol 79:2560–2571

Stajić M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E, Vukojević J (2006a) Effect of different carbon and nitrogen sources on laccase and peroxidases activity by selected *Pleurotus* species. Enzyme Microb Tech 38:65–73

Stajić M, Persky L, Hadar Y, Friesem D, Duletić-Laušević S, Wasser SP, Nevo E (2006b) Effect of copper and manganese ions on activities of laccase and peroxidases in three *Pleurotus* species grown on agricultural wastes. Appl Biochem Biotechnol 128:87–96

Stajić M, Vukojević J, Duletić-Laušević S (2009) Biology of *Pleurotus eryngii* and the role in biotechnological processes: a review. Crit Rev Biotechnol 29:55–66

Stajić M, Kukavica B, Vukojević J, Simonić J, Veljović-Jovanović S, Duletić-Laušević S (2010) Wheat straw conversion by enzymatic system of *Ganoderma lucidum*. Bioresources 5:2362–2373

Stajić M, Vukojević J, Knežević A, Milovanović I (2013) Influence of trace elements on ligninolytic enzyme activity of *Pleurotus ostreatus* and *P. pulmonarius*. Bioresources 8:3027–3037

Stuardo M, Vasquez M, Vicuna R, Gonzalez B (2004) Molecular approach for analysis of model fungal genes encoding ligninolytic peroxidases in wood-decaying soil system. Lett Appl Microbiol 38:43–49

Tabka MG, Herpoel-Gimbert I, Monod F, Asther M, Sigoillot JC (2006) Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulose xylanase and feruloyl esterase treatment. Enzyme Microb Tech 39:897–902

Thurston C (1994) The structure and function of fungal laccase. Microbiol 140:19–26

Tuyen VD, Cone JW, Baars JJP, Sonnenberg ASM, Hendriks WH (2012) Fungal strain and incubation period affect chemical composition and nutrient availability of wheat straw for rumen fermentation. Bioresource Technol 111:336–342

Urzúa U, Larrondo LF, Lobos S, Larrain J, Vicuña R (1995) Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. FEBS Lett 371:132–136

Van Beek TA, Kuster B, Chassen FW, Tienvieri T, Bertaud F, Lenon G, Petit-Conil M, Sierra-Alvarez R (2007) Fungal biotreatment of spruce wood with *Trametes versicolor* for pitch control: influence of extractive contents, pulping process parameters, paper quality and effluent toxicity. Bioresource Technol 98:302–311

Varela E, Guillén F, Martínez AT, Martínez MJ (2001) Expression of *Pleurotus eryngii* aryl-alcohol oxidase in *Aspergillus nidulans*: purification and characterization of the recombinant enzyme. Biochim Biophys Acta 1546:107–113

Wan C, Li Y (2010) Microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production. Bioresource Technol 101:6398–6403

Wariishi H, Akilswaran L, Gold MH (1988) Manganese peroxidase from the basidiomycetes *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. Biochemistry 27:5365–5370

Yang Q, Zhan H, Wang S, Fu S, Li K (2008) Modification of *Eucalyptus* CTMP fibers with white rot fungus *Trametes hirsute*—effects on fiber morphology and paper physical strengths. Bioresource Technol 99:8118–8124

Yang B, Wyman C (2008) Pre-treatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Bioref 2:26–40

Zechendorf B (1999) Sustainable development: How can biotechnology contribute? Feature 17:219–225

Zhang S, Jiang M, Zhou Z, Zhao M, Li Y (2012) Selective removal of lignin in steam-exploded rice straw by *Phanerochaete chrysosporium*. Int Biodeter Biodegr 75:89–95

Chapter 11
Role and Application of Versatile Peroxidase (VP) for Utilizing Lignocellulose in Biorefineries

Nadine Busse and Peter Czermak

Abstract Within the last few years, the relatively new heme peroxidase, versatile peroxidase (VP), attracted some attention as a model enzyme and as an industrial biocatalyst. VPs are interesting for efficient delignification processes due to their ability to degrade a broad spectrum of recalcitrant substrates without using mediators. Lignin is very complex, highly aromatic, and one of the major compounds of lignocellulose, which is an important feedstock for biorefineries, particularly when originated from wood. In utilizing lignocellulose (e.g., second generation biofuels), lignin modification, and removal must first be addressed. Moreover, lignin degradation for value-added bio-products is also of great interest. An industrial implementation of VPs would be beneficial in this context, but involves several scientific and technical challenges.

11.1 Introduction

Lignin modification and removal plays a key role in utilizing lignocellulose/lignocellulosic biomass for second generation biofuels, and other bio-based products, and therefore must be addressed first. Lignocellulose from wood is of particularly high interest for this process due to the following reasons: Wood is inexpensive, available in large amounts (Stöcker 2008), CO_2 neutral (Lange 2007), and rich in lignin (18–35 %), as well as the carbohydrates cellulose (40–55 %) and hemicellulose (24–40 %) (Sun and Cheng 2002; Howard et al. 2003).

Due to the fact that lignin is the only naturally synthesized aromatic biopolymer (Dashtban et al. 2010) its effective use and degradation to value-added macromolecules (e.g., low cost carbon fibers, polymer modifiers) and/or aromatic chemicals (e.g., phenols, vanillin, quinone, guaiacol, BTX chemicals) is of crucial

N. Busse · P. Czermak (✉)
Institute of Bioprocess Engineering and Pharmaceutical Technology,
Mittelhessen University of Applied Sciences, Wiesenstrasse 14,
35390 Giessen, Germany
e-mail: peter.czermak@kmub.thm.de; peter.czermak@ibpt.de

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_11

importance, with the latter of these being of high economic potential and therefore particularly interesting (Holladay et al. 2007; Huang and Ramaswamy 2013). However, the contributing factors affecting the probability of their successful implementation include the structural complexity of native lignin, its recalcitrance, and its uniqueness (the woody plant's fingerprint) leading to inconsistent, polydisperse product streams. These facts still constitute a major scientific and technical challenge (Holladay et al. 2007). Consequently, further fundamental research and development is needed for lignin degradation in both general and targeted usage.

Prerequisites for efficient lignin modification and degradation in biorefineries are:

(i) Conservation of important lignin related aromatic structures.
(ii) Subsequent processes like saccharification of cellulose by cellulases should not be negatively influenced.
(iii) Mild conditions.
(iv) Moderate waste streams/effluent treatment and moderate operating costs.

Ligninolytic heme peroxidases (POXs) have gained increased importance in this context, with particular attention on the use of versatile peroxidases (VPs). VPs are relatively new POXs and were first identified as MnPs in the late nineties. Within the last few years they have attracted attention "as a model enzyme and as a source of industrial/environmental biocatalyst" (Ruiz-Dueñas et al. 2009a). The main reason for this is their capability to digest/convert a broad spectrum of complex substrates, ranging from azo dyes like RB5 to (non)phenolic lignin, without the necessity of mediators (Pogni et al. 2005; Martínez et al. 2009). Nonetheless, several factors hinder their short-term commercialization and industrial implementation. This chapter outlines the main issues for industrial applications, starting first with a brief overview of the involvement of POXs in natural delignification.

11.2 Involvement of Heme Peroxidases in the Lignocellulose Degradation Mechanism

In nature, lignocellulose (LC) degradation involves several factors as follows:

(i) Lignocellulose composition (e.g., lignin content, extractables).
(ii) Environmental conditions (e.g., T, pH, moisture, N_2, O_2) (Blanchette 1991; Wong 2009).
(iii) Biological community.

Biological community includes microbial population, fungi, and insects, and determines the degradation process. In order to enable the use of LC, lignin modification and removal must first be addressed. *Basidiomycetes*, white-rot fungi, are well known to be the most efficient lignin degraders due to their extracellular ligninolytic enzymes (Kirk and Farrell 1987), the H_2O_2-dependent heme peroxidases

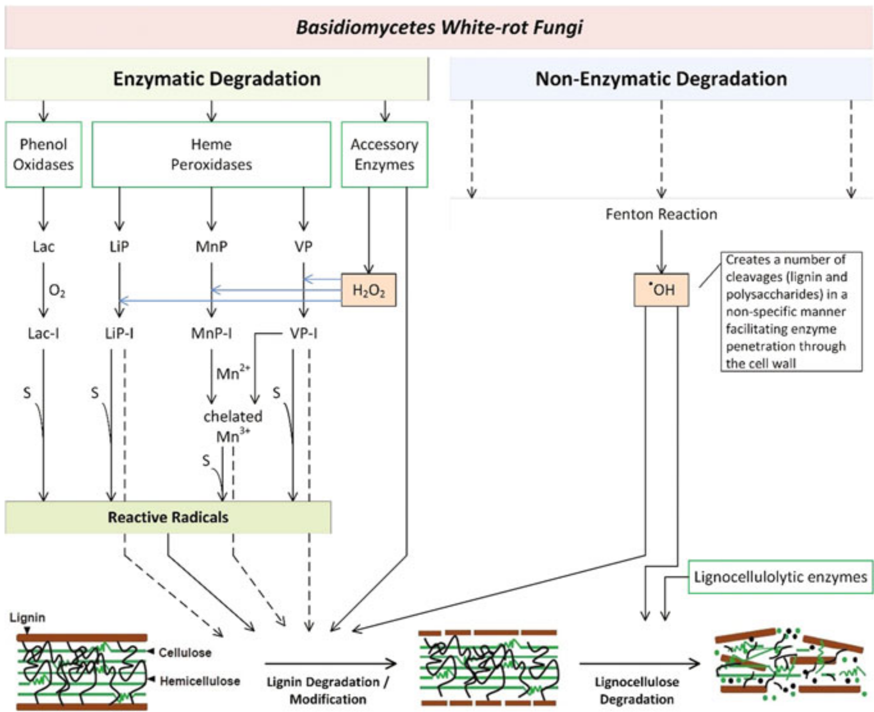


Fig. 11.1 Summary of the natural degradation process of lignocellulosic biomass and the importance of ligninolytic heme peroxidases (modified based on Dashtban et al. 2010). Here, the reactions of the first POX intermediates are demonstrated. *Lac* laccase, *LiP* lignin peroxidase, *MnP* manganese peroxidase, *VP* versatile peroxidase; Lac-I, LiP-I, MnP-I, and VP-I are corresponding protein radicals, generally known as compound I. S stands for an appropriate substrate of low molecular weight resulting in relatively reactive radicals which ultimately mediate the cleavage of lignin bonds and lignin modifications

(POX) and O_2-dependent phenol oxidases lacasses (Lac, EC 1.10.3.2) as seen in Fig. 11.1. Figure 11.1 demonstrates a part of the full enzymatic reaction mechanism for better clarity (specifically the reactions of the first enzyme intermediate). More details on this will follow in subsequent chapters. The ligninolytic enzyme system varies individually in composition. In addition, it must be further differentiated into selective and nonselective lignin degraders (Table 11.1). The former selectively remove lignin without considerable cellulose degradation, whereas the latter also attack cellulose (Blanchette 1991; Hatakka 2005; Hatakka and Hammel 2011). Some can however produce both types, such as *Heterobasidion annosum* (in Table 11.1) (Eriksson et al. 1990).

Concerning lignin degradation, two pathways must be differentiated (Fig. 11.1):

(i) Direct (black dashed lines).
(ii) Indirect (black solid lines).

Table 11.1 A selection of POX and Lac producing white-rot fungi (modified based on Hatakka (1994), Ayala et al. (2008), Sánchez (2009) and Lundell et al. (2010))

Species	Secreted enzymes
Lignin-selective	
Bjerkandera adusta	LiP, MnP, **VP**[a]
Bjerkandera species	**VP**[b]
Ceriporiopsis subvermispora	MnP, Lac
Dichomitus squalens	MnP, Lac
Heterobasidion annosum	MnP
Phanerochaete chrysosporium	LiP, MnP
Phellinus pini	LiP, MnP
Phlebia radiata	LiP, MnP, Lac
Phlebia subserialis	MnP
Phlebia tremellosus	LiP, MnP, Lac
Pleurotus eryngii	MnP, **VP**[c], Lac
Pleurotus ostreatus	MnP, **VP**[d], Lac
Pleurotus sapidus	**VP**[e]
Pycnoporus cinnabarinus	LiP, MnP, Lac
Nonselective	
Ganoderma applanatum	LiP
Heterobasidion annosum	MnP
Trametes versicolor	LiP, MnP, **VP**[f], Lac

Bold indicates VP producing white-rot fungi
[a]Wang et al. (2003) and Busse et al. (2013)
[b]Mester and Field (1998) and Moreira et al. (2006)
[c]Martínez et al. (1996) and Camarero et al. (1999)
[d]Hatakka and Hammel (2011)
[e]Zorn et al. (2005) and Schüttmann et al. (2014)
[f]Carabajal et al. (2013)

Direct degradation refers to when the ligninolytic enzyme is in its active state (characterized by an appended suffix-I) and directly attacks native lignin linkages. However, when low molecular weight (LMW) substrate (S) is present/provided, this interposes an intermediate step where enzyme action results first in substrate-related diffusible radicals mediating nonenzymatic reactions, which then cause the cleavage of lignin bonds. This is called indirect lignin degradation, because the LMW substrates serve as so-called mediators. Lac rely on phenolic mediators for promoting their oxidation capabilities "towards more recalcitrant non-phenolic lignin compounds" (Cañas and Camarero 2010). For oxidative ability, the enzyme redox potential is a limited criterion (Ayala 2010). Lac exhibits lower redox potential properties (≤ 0.8 V) than POX (> 1 V) (Bourbonnais and Paice 1990; Cañas and Camarero 2010). Lignin comprises 80–90 % non-phenolic moieties, where just 10–20 % are phenolics (Kawai et al. 1999). The cleavage of non-phenolics is thus essential for efficient lignin modification and degradation. The redox potential of non-phenolic lignin compounds is also > 1 V (Cañas and Camarero 2010). Consequently, POX have recently gained increased attention for delignification purposes (Cañas and Camarero 2010) and received higher interest as

industrial biocatalysts (Martínez 2007). Nevertheless, manganese peroxidases (MnP, EC 1.11.1.13) principally need manganese (Mn^{2+}) as a substrate and mediator to form Mn^{3+} ions. These ions will be subsequently chelated by organic acids (e.g., oxalate, malonate, lactate) to powerful oxidants. As a result, MnPs are able to oxidize certain non-phenolic aromatics (e.g., carboxylic acids, methoxybenzenes, lipids) and are not restricted to phenolics as usual. Recalcitrant non-phenolic lignin units still remain unaffected. Therefore, it is supposed that MnP activity rather causes phenoxyl radical formation (as in case of Lac) which in turn is responsible for subsequent bond cleavages in the biopolymer lignin (Hatakka 2005). Contrary to this, lignin peroxidases (LiP, EC 1.11.1.14) prefer to react with non-phenolic lignin substructures, interestingly, without the need for mediators. White-rot fungi, however, simultaneously produces the non-phenolic monomer veratryl alcohol (VA) as a second metabolite (Schoemaker and Piontek 1996). VA is well known to be an essential substrate S for LiP, elevating its activity, as well as increasing lignin degradation. Reaction with VA again causes radical formation (veratryl alcohol radicals $VA^{\bullet}$), which additionally enables phenolic compound oxidation.

Versatile peroxidases (VP, EC 1.11.1.16) exemplify catalytic properties of both LiP and MnP, and thus initiate similar reaction pathways. VPs, however, are less specific in facilitating degradation among a broad spectrum of substrates, irrespective of phenolic or non-phenolic, and do not require the presence of mediators (Pogni et al. 2005).

In conclusion, lignin degradation in nature is not performed by just one enzyme, but rather a synergistic process of an array of ligninolytic enzymes and their isozymes, accessory enzymes (e.g., H_2O_2-generating oxidases (aryl-alcohol oxidase, glyoxal oxidase)), aryl-alcohol oxidases, and quinone reductases) and non-enzymatic reactions (Fig. 11.1). The latter mainly produces hydroxyl radicals ($^{\bullet}OH$) which support lignin modification and degradation, as well as the degradation of cellulose and hemicellulose in a non-specific manner. The involvement of such radicals results in easier penetration of:

(i) POX, since they are unable to penetrate through the lignified cell wall and therefore first erosion is necessary (Hammel et al. 1993).
(ii) Lignocellulolytic enzymes, such as (hemi)cellulases for subsequent (hemi)-cellulose digestion (Dashtban et al. 2010).

11.3 Catalytic Mechanism of VP

As initially mentioned, VPs are relative new POXs and are attractive (model) biocatalysts (Ruiz-Dueñas et al. 2009a). One key advantage is their independence from mediators. For industrial application, mediators are used in high amounts. Therefore, mediators must be (i) cheap, (ii) non-toxic, (iii) readily available,

(iv) in large quantities, and/or (v) easy recyclable (Rodríguez Couto and Toca Herrera 2006; Cañas and Camarero 2010).

Research work concerning delignification processes by VPs are limited, particularly in kinetic studies with representative lignin model compounds (LMCs) like adlerol (1-(3,4-dimethoxyphenyl)-2-(2-methoxy-phenoxy)-1,3-propanediol), a non-phenolic β-O-4 lignin model dimer. The reason for this is the underlying, highly complex, POX reaction mechanism which will be demonstrated in the following, prior to focusing on mediator-independent adlerol degradation.

11.3.1 The General Peroxidase Reaction Mechanism

The catalytic peroxidase cycle is expected to follow a ping–pong mechanism. Contrary to the classical mechanism, peroxidase reaction schemes are further assumed to be irreversible, as sketched in Fig. 11.2 in simplified terms. Enzyme–substrate complex formation (e.g., ES) prior to reaction product generation can thus be neglected, since they are considered of fleeting non-detectable existence (Dunford 1991). This is valid for most heme peroxidases, (Dunford 2010) with iron protoporphyrin (P) IX and iron/heme (Fe^{3+}) as the catalytic center and the prosthetic group, respectively (de Montellano 2010).

During the catalytic cycle (Fig. 11.2), the enzyme undergoes two fundamental structural changes. First (pathway 1), the reaction is initiated by H_2O_2 through a pH-independent, two-electron oxidation (pH range is ~2–7.5 for LiP (Andrawis et al. 1988) or 3.5 and 7 for VP (Pérez-Boada et al. 2005)). As a result, the enzyme will be converted from its native resting ferric/ground state (E^0) to the so-called compound I (E^I, first intermediate) (Eq. 11.1), a protein cation radical (de Montellano 2010) of strong oxidative power (Arnao et al. 1990b).

$$E^0[Fe^{3+}] + H_2O_2 \xrightarrow{k_1} E^I[Fe^{4+} = OP^{\bullet +}] + H_2O \quad (11.1)$$

with k as the reaction rate constant and the index for describing the pathway number.

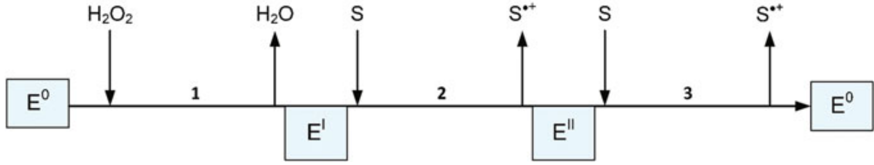


Fig. 11.2 Simplified "POX ping–pong" mechanism. E^0–E^{II} represents the three main peroxidase oxidation states. The symbols S and $S^{\bullet +}$ stand for an appropriate substrate (e.g., lignin, lignin model compounds such as adlerol) and its corresponding radical cation. The reaction will be initiated by H_2O_2 (pathway 1), and followed by two consecutive, one-electron reduction steps (pathway 2–3). Reprinted from Busse et al. (2013) with slight modification

Subsequently, E^I returns back to E^0 via a second enzyme intermediate (E^{II}, compound II) due to two consecutive one-electron reduction steps triggered by suitable reducing substrates (S, the actual electron donor "preferentially electron-rich aromatic compounds" (Lundell et al. 1993a)). Both reactions are pH-dependent (Pérez-Boada et al. 2005; Wong 2009) resulting in the release of radical cation intermediates ($S^{\bullet +}$) (Palmer et al. 1987) (pathway 2 and 3 in Fig. 11.2; Eqs. 11.2, 11.3):

$$E^I[Fe^{4+} = OP^{\bullet +}] + S \xrightarrow{k_2} E^{II}[Fe^{4+} = O] + S^{\bullet +} \tag{11.2}$$

$$E^{II}[Fe^{4+} = O] + S \xrightarrow{k_3} E^0[Fe^{3+}] + S^{\bullet +} + H_2O \tag{11.3}$$

In Eq. (11.2), substrates causing E^I reduction can also reduce E^{II} (Eq. 11.3) as described by Dunford (2010) for horseradish peroxidase (HRP). The same applies for ligninolytic enzymes, such as LiP, and phenolic substrates (Schoemaker 1990). Here, the overall net enzyme reaction can be simply expressed by Eq. (11.4).

$$2S + H_2O_2 \xrightarrow{r} 2S^{\bullet +} + 2H_2O \tag{11.4}$$

Corresponding consumption rates r for S and H_2O_2 result in Eq. (11.5).

$$r = -\frac{1}{2}\frac{d[S]}{dt} = -\frac{d[H_2O_2]}{dt} = \frac{1}{2}\frac{d[S^{\bullet +}]}{dt} \tag{11.5}$$

Assuming the POX cycle is not inhibited, and considering k_3 is rate limiting ($k_3 = k_{cat}$ because of $k_2 \gg k_3$), r results in Eq. (11.6) (Rasmussen et al. 1995).

$$r = \frac{2k_3[E_T][S_2][S_1]}{\frac{k_3}{k_1}[S_2] + [S_1]} \tag{11.6}$$

$$[E_T] = \sum_{i=0}^{II} [E^i] \tag{11.7}$$

E_T means total enzyme.

When non-phenolic substrates are involved, caution should be exercised regarding generalization. E^{II} of LiP, for instance, can be reduced just by di-methoxylated (minimum amount of alkoxy substituents), non-phenolic aromatic compounds, whereas E^I also accepts mono-methoxylated aromatic components (Schoemaker 1990).

All produced radical cation intermediates (Fig. 11.2) will further pass through diffusion-controlled, non-enzymatic reactions as schematically illustrated in Fig. 11.3. Such radical reactions yield complex product mixtures. Depending on the

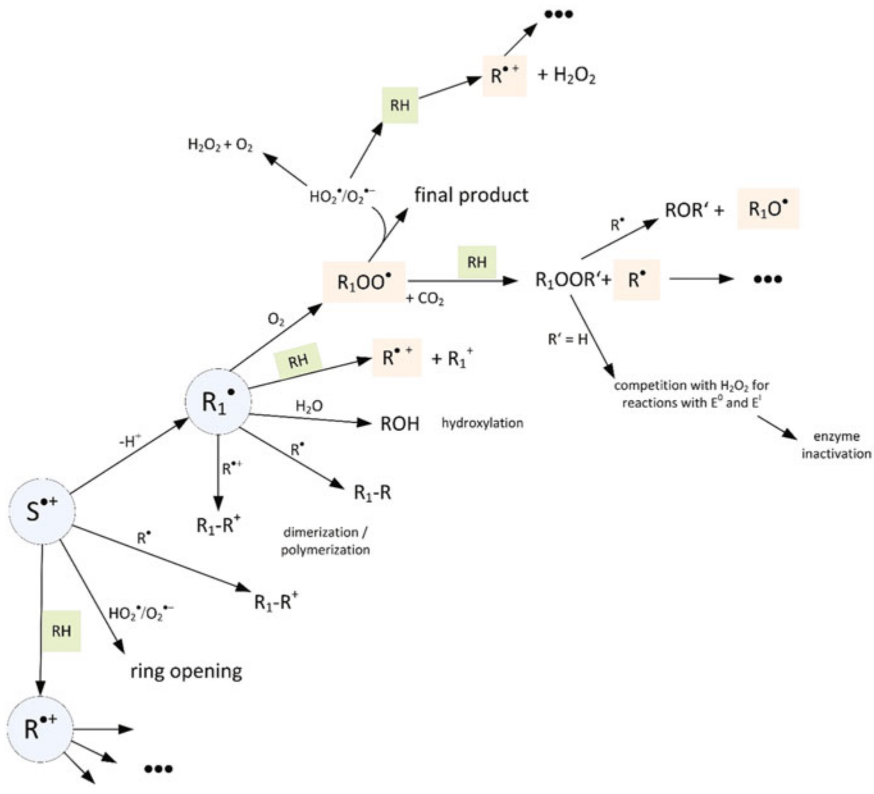


Fig. 11.3 Schematic drawing of nonenzymatic radical reactions–radical chain reactions. The reaction is initiated by the cation radical intermediate $S^{\bullet +}$, a product of enzyme activity

nature of $S^{\bullet +}$, subsequent non-enzymatic reactions are the following (recently summarized by Busse et al. (2013) based on Palmer et al. (1987) and Schoemaker (1990)):

(a) One-electron oxidation of another appropriate substrate molecule RH (R also stands for aromatic residuals, e.g., lignin or lignin derivatives), which in turn is transferred to $R^{\bullet +}$ (Fig. 11.3). $S^{\bullet +}$ is reduced back to its ground state (Palmer et al. 1987). This kind of reaction is referred to as mediator behavior.

(b) $S^{\bullet +}$ can also immediately be degraded to free radicals $R_1^{\bullet}$ (R_1 stands again for aromatic residual) (Palmer et al. 1987), which is caused by rapid deprotonation processes (Schoemaker et al. 1994a) (Fig. 11.3). Note, index 1 is just used for differentiation purposes.

(c) Side-chain cleavage, e.g., Cα–Cβ bond cleavage (Fig. 11.4, section 11.3.2), and C–H bond cleavage.

(d) Demethoxylation.

(e) Ether-bond cleavage [e.g., addition of solvent (H_2O)] (Palmer et al. 1987).
(f) Hydroxylation of benzylic methylene groups, e.g., through oxygen incorporation via O_2 or H_2O in the solvent (Fig. 11.3).
(g) Phenol formation by nucleophilic attack, e.g., the addition of a solvent such as H_2O (Palmer et al. 1987).
(h) Aromatic ring cleavage caused by reactions with perhydroxy radicals $HOO^•$/$HO_2^•$ (Fig. 11.3); details will follow in the next section (Palmer et al. 1987).

The highly reactive free-radical species (in Fig. 11.3 denoted as $R^•$ and $R_1^•$) can bind to molecular oxygen (O_2), forming final products as well as superoxide anion radicals ($O_2^{•-}$) (Schoemaker 1990) via degradation of organic peroxy radical intermediates ($ROO^•$ or $R_1OO^•$ in Fig. 11.3) (Palmer et al. 1987). $O_2^{•-}$ ultimately undergoes rapid disproportionation forming H_2O_2 and O_2 (Harman et al. 1986; Schoemaker 1990). Here, the $O_2^{•-}$ is in a pH-dependent equilibrium with its protonated counterpart $HOO^•$ (Bielski et al. 1985). At low pH conditions, $HOO^•$ predominates, existing as a powerful oxidant (Halliwell and Gutteridge 1985) and thus contributing to the substrate degradation processes. This occurs because additional bond cleavage is initiated as a consequence of the substrate (S) oxidizing to its radical cation, where $HOO^•$ is reduced to H_2O_2 (Palmer et al. 1987). Alternatively, $R_1OO^•$ can further react with substrate molecules RH, resulting in new free radical $R^•$ and organic peroxide R_1OOR' formation. If R' represents hydrogen, organic hydroperoxide (R_1OOH) will instead be produced. As shown in Fig. 11.3, R_1OOH competes with the co-substrate H_2O_2 for reaction with E^0–E^I and with S for E^I conversion, depending on their relative concentrations. Reactions of R_1OOH with E^I lead to undesired enzyme inactivations. In a low-oxygen atmosphere, R_1OOH formation diminishes (Acosta et al. 1988), but polymerization increases (Palmer et al. 1987). $R^•$ products may also attack the peroxidase, again leading to inactivation (Nicell et al. 1993). However, these radicals can also undergo dimerization and polymerization processes (e.g., reaction with other free radicals such as $R_1^•$) (Palmer et al. 1987), or can act as suitable substrates S for maintaining the catalytic cycle. The latter is, however, limited in applicability for radical cation intermediates $S^{•+}$, as demonstrated later.

With consideration of further enzymatic reactions, both $O_2^{•-}$ and $HOO^•$ (depending on pH) can additionally serve as electron donors, instead of reducing substrates (competitive reaction). Consequently, all three enzyme states, E^I, E^{II}, and E^{III} may be, for instance, reduced by $O_2^{•-}$ under O_2 production (Bielski et al. 1985; Kettle et al. 2007) according to Eqs. (11.8)–(11.11), based on Kettle et al. (2007). The reaction in Eq. (11.9) is supposed to prevent enzyme inhibition caused by poor reducing substrates, in order to maintain activity (Kettle et al. 2007).

$$E^I + O_2^{•-} \rightarrow E^{II} + O_2 \tag{11.8}$$

$$E^{II} + O_2^{•-} + H^+ \rightarrow E^0 + O_2 + H_2O \tag{11.9}$$

$$E^{III} + O_2^{\bullet -} + 2H^+ \rightarrow E^0 + H_2O_2 + O_2 \quad (11.10)$$

or

$$E^{III} + O_2^{\bullet -} + 2H^+ \rightarrow E^I + H_2O + O_2 \quad (11.11)$$

At this point, it becomes obvious that peroxidase activity at least leads to radical cation formation by reacting with a suitable substrate, which triggers several non-enzymatic reactions, similar to a "domino effect." Moreover, the complexity of the peroxidase reaction mechanism is evident, which explains why even the degradation of simple aromatic monomers (e.g., VA), except pathway I in Fig. 11.2 (Dunford 2010), is not fully understood, and that further fundamental research is still needed. Indeed, in a recent review, Busse et al. (2013) found that the mechanism is far more complex and strongly depends on (i) reaction conditions, (ii) POX type, (iii) the presence of inhibitors, (iv) the used substrate.

11.3.2 Adlerol Degradation

Adlerol is a non-phenolic lignin model dimer with a β-O-4 linkage (structure I in Fig. 11.4). Such LMCs are the most abundant units in lignin polymers, with concentrations ranging from 50 to 65 % (Adler 1977). However, the question arises if adlerol is a representative model compound, since this kind of systems does not include linkages to wood polysaccharides. Studying fungal lignin-degrading ability based on ^{14}C-lignins, by contrast, is suggested as the most reliable method (Hatakka and Hammel 2011). From this perspective, and due to the fact that natural lignin is a very complex, water-insoluble biopolymer, low molecular weight LMCs like adlerol, which is also less soluble in water, do not initially seem to appropriately mimic reality. Not even industrial/technical lignins (e.g., high polydisperse ligninsulfonates) or dimeric LMCs attached to PEG (polyethylene glycol) would ever result in realistic test scenarios. Woody biomass is exclusively the most representative substrate, which complicates the situation even more. Major drawbacks include: (i) variations in quality from batch to batch, and the fact that (ii) high enzyme loads are required just to achieve satisfactory reaction rates.

Alternatively, the choice of an appropriate model system depends on the research subject(s). Upon the examination of the fundamental issues, including studying reaction mechanisms, enzyme (deactivation) kinetics, and establishing monitoring systems for process control, it can be determined that LMCs are in fact very useful. β-O-4 cleavage, for instance, is considered as a substantial indicator for both enzymatic lignin depolymerization and modification (Tien 1987; Wong 2009), making adlerol a suitable LMC for studying delignification mechanisms. Moreover, the β-O-4 bond is stable at an acidic pH (Glasser et al. 1993) simulating the most challenging scenario, because a low pH is generally needed for optimal POX activity.

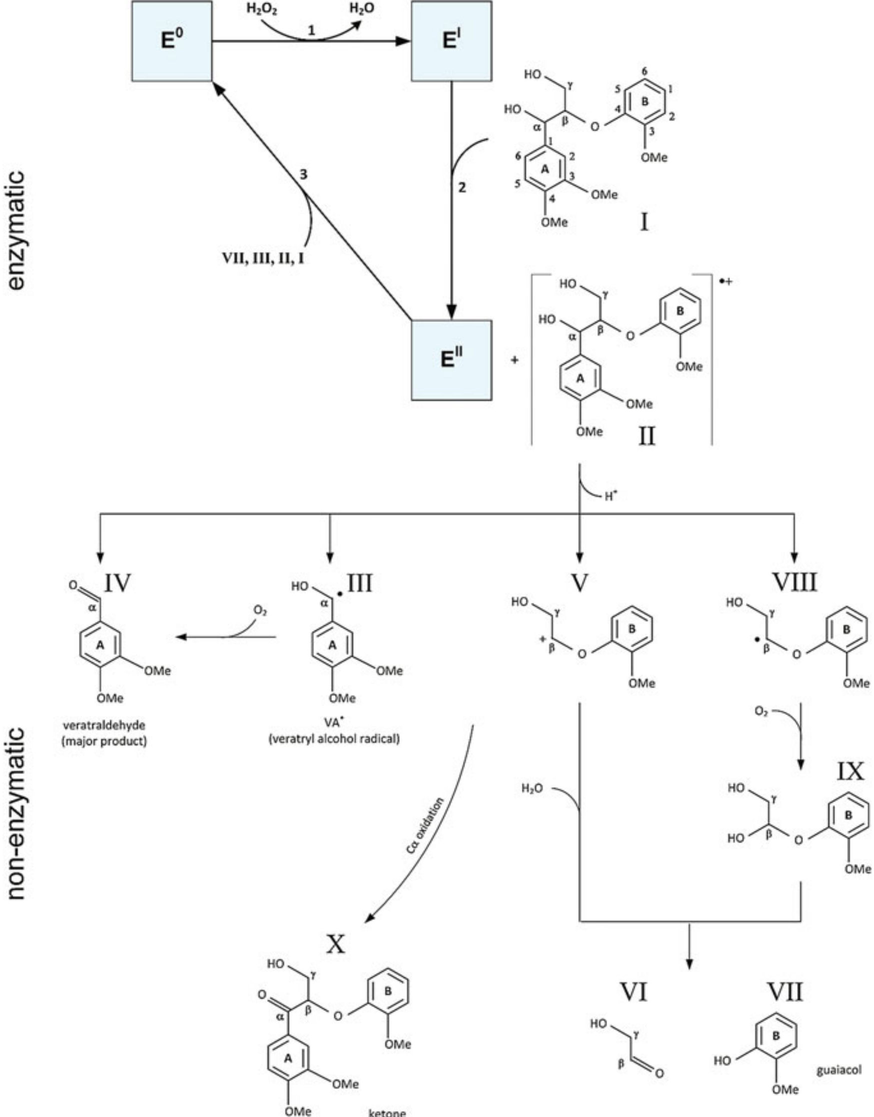


Fig. 11.4 Graphical illustration of the adlerol (β-*O*-4 model compound, structure I) biocatalysis based on LiP. Several degradation variants (partly in a summarized manner) are shown derived from proposed reaction schemes by Tien and Kirk (1984), Kirk and Farrell (1987), Lundell et al. (1993b) and Schoemaker et al. (1994b). *I* adlerol, *II* adlerol-derived radical cation, *III* veratryl alcohol radical (VA), *IV* veratraldehyde (VAld) with 60–70 % major product, *V* Cβ-cation, *VI* glycoaldehyde, *VII* guaiacol, *VIII* Cβ-centered radical, *IX* hemiacetal intermediate (unstable), *X* keton, a second major product (15 %). Reprinted from Busse et al. (2013) with slight modifications

In Fig. 11.4, the basic adlerol degradation steps, induced by (fungal) heme peroxidases, are schematically explained in conjunction to the previously described, (non)enzymatic reactions. The catalytic cycle is initiated by H_2O_2 to form E^I as usual. E^I further reacts with adlerol (structure I) to E^{II}, resulting in the production of an adlerol-derived cation radical intermediate (structure II) (Lundell et al. 1993b). Once the radical cation is generated, it will be rapidly fragmented, non-enzymatically (Hatakka et al. 1991). For this, various theories have been under consideration, as summarized in Fig. 11.4. The reason for this is most likely due to both (i) $S^{\bullet+}$ chemistry (as already mentioned above), and (ii) pH-dependent product distribution (Schoemaker 1990). Despite the non-enzymatic reaction, veratraldehyde (VAld, structure IV) is the main fission product in the end. This same procedure is repeated when another adlerol molecule is oxidized by E^{II} (refer to pathway 3, Fig. 11.4).

In case adlerol will not serve as the reducing substrate for some reason, component II ($S^{\bullet+}$), III ($VA^{\bullet}$), and VII (guaiacol) could act as substrates for driving the cycle back to E^0. The latter causes polymerization of its radical products (Chance and Maehly 1955), which are phenoxy radicals (Lundell et al. 1993b). A reduction of E^{II}–E^0 by component III also results in veratraldehyde (VAld) formation (Schoemaker 1990; Schoemaker et al. 1994b). However, this is only an occurance under low-oxygen conditions (Schoemaker et al. 1994b), since the existence of III is supposed to be short-lived by reacting with O_2 immediately. It is important here to remember that the mediator function of radical products of component III can cause co-oxidation (thus degradation) of adlerol as well. It is believed that efficient mediation can only be realized if the degrading substrate will bind close to the active site of the enzyme (Schoemaker et al. 1994b).

Under substrate (adlerol) limited conditions, Tien and Kirk (1984) demonstrated a non-stereospecifity of their used LiP, due to complete cleavage of the β-O-4 lignin model compound. This is an important feature, because lignin has a highly irregular structure (Schoemaker 1990). Conversely, the involvement of nonenzymatic radical (chain) reactions is obvious in assisting enzymatic degradation.

11.3.3 VP Reaction and Deactivation Kinetics

Up until now, the normal POX cycle has been introduced (pathway 1–3 in Figs. 11.2, 11.4). In Fig. 11.5, the supposed VP reaction mechanism is summarized. E^I and E^{II} in Fig. 11.5 are expected to be tryptophan radical ($Trp^{\bullet}$) containing proteins. Furthermore, this tryptophan radical is suggested to be located on the enzyme surface (≈10–11 Å to the heme center (Pogni et al. 2005; Ruiz-Dueñas et al. 2009a)) for substrate oxidation purposes. This means there is no direct contact between the heme (Fe^{3+}) co-factor and the reducing aromatic substrates (e.g., VA (non-phenolic monomer) or complex lignin-derived polymers (macromolecules)) avoiding steric hindrance. Interactions between substrate and heme group are

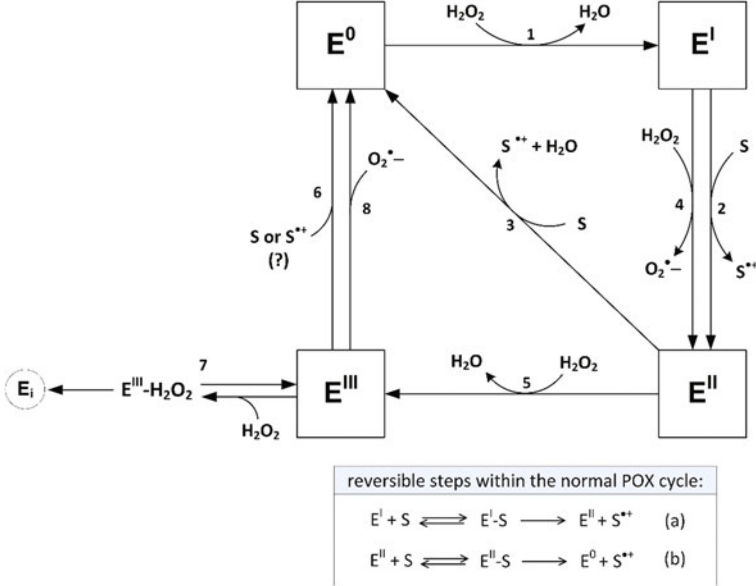


Fig. 11.5 Supposed H_2O_2-dependent reaction mechanism of the crude VP from *B. adusta* for adlerol degradation. E^0–E^{III} are enzyme intermediates. The symbols S and $S^{\bullet+}$ stand for an appropriate substrate (e.g., adlerol) and the corresponding radical cation. $O_2^{\bullet-}$ denotes superoxide radical anions. Pathway 1–3 depicts the usual POX reaction cycle with reversible steps, as described by equation (a) and (b) (Ruiz-Dueñas et al. 2009b; Busse et al. 2013). The cycle will be initiated by H_2O_2 (pathway 1). Pathway 4, 5, and 7 show important side reactions with H_2O_2. The latter is in competition with pathway 6. Enzyme deactivation (E_i: inactivated enzyme) is found to occur via E^{III}, as sketched in pathway 7. Pathway 8 illustrates a spontaneous unimolecular decay of E^{III}. Reprinted from Busse et al. (2013)

enabled via long-range electron transfer (LRET) (Ruiz-Dueñas et al. 2009a). The involvement of LRET in lignin degradation processes through ligninolytic enzymes is generally accepted (Busse et al. 2013).

Pathway 1 is the only reaction which has been understood in the past (Dunford 2010), and therefore particular evidence for pathway 3 is still pending (Busse et al. 2013). Thus, for the adlerol bioconversion by a VP type from *B. adusta*, pathway 3 in Fig. 11.5 was assumed based on transient state kinetics made through VA oxidation studies using a recombinant VP from *E. coli* (Ruiz-Dueñas et al. 2009b). Moreover, reversible binary enzyme–substrate (E–S) complex (precursor complex (Dunford 2010)) formation that occur before irreversible product release are certainly possible during the catalytic cycle of VP (concerns pathway 2 and 3, Fig. 11.5) (Ruiz-Dueñas et al. 2009b). Both E^I and E^{II} exhibit reactions described by Eqs. (11.12, 11.13) (Ruiz-Dueñas and Martínez 2009; Ruiz-Dueñas et al. 2009b).

$$E^{I}[Fe^{4+}=OTrp^{\bullet}]+S \overset{K_{m2}}{\longleftrightarrow} E^{I}-S \overset{k_2}{\longrightarrow} E^{II}[Fe^{3+}\ Trp^{\bullet}]+S^{\bullet +} \tag{11.12}$$

$$E^{II}[Fe^{3+}\ Trp^{\bullet}]+S \overset{K_{m3}}{\longleftrightarrow} E^{II}-S \overset{k_3}{\longrightarrow} E^{0}[Fe^{3+}]+S^{\bullet +} \tag{11.13}$$

K_{mi} and k_i (i = 2, 3) are the corresponding equilibrium dissociation constant and rate constant, respectively. As a result, the kinetics of VP form a two substrate ping–pong bi bi mechanism with the reaction rate r explained in Eq. (11.14) (Busse et al. 2013).

$$r=\frac{v_{max}[S_1][S_2]}{K_m^{S_1}[S_2]+K_m^{S_2}[S_1]+[S_1][S_2]} \tag{11.14}$$

v_{max}, S_1, S_2, $K_m^{S_1}$, and $K_m^{S_2}$ stand for maximum reaction velocity, co-substrate H_2O_2, substrate S (e.g., adlerol), dissociation constant for S_1 and dissociation constant for S_2.

Maintaining constant S_2 concentration, while varying S_1, Eq. (11.14) can be simplified to Eq. (11.15).

$$r=\frac{v_{max}^{app}[S_1]}{K_m^{app}+[S_1]} \tag{11.15}$$

$$v_{max}^{app}=\frac{v_{max}[S_2]}{K_m^{S_2}+[S_2]} \tag{11.16}$$

$$K_m^{app}=\frac{K_m^{S_1}[S_2]}{K_m^{S_2}+[S_2]} \tag{11.17}$$

v_{max}^{app} and K_m^{app} are the apparent kinetic parameters of maximum reaction velocity and dissociation constant, respectively. Equations (11.14)–(11.17) are valid in the absence of inhibitions/deactivation phenomena and product(s) (Bisswanger 2008; Cornish-Bowden 2012). Due to the well-known H_2O_2-sensitivity of POX, inactivation/deactivation reactions are substantial side effects of crucial importance. Figure 11.5 concerns the major reaction pathways that have a co-substrate H_2O_2 in addition to substrate S. Both substrates compete for reactions with E^{I} and E^{II} (refer to Fig. 11.5 pathway 2, 4, and 3, 5, respectively). When a certain S/H_2O_2/E ratio is unbalanced, POX deviate from their normal catalytic cycle, and instead promote reactions via compound III (E^{III}), diminishing enzyme activity (pathway 5, 7) (Arnao et al. 1990a, b; Wariishi and Gold 1990; Busse et al. 2013). E^{III} is another third enzyme intermediate and exhibits non, or sometimes barley any catalytic activity (Schoemaker 1990). Subsequently, E^{III} can react further with H_2O_2, resulting in an enzyme deactivation E_i (pathway 7 (Wariishi and Gold 1990; Goodwin et al. 1994; Busse et al. 2013)), also known as suicide inactivation (Valderrama et al. 2002). Research investigations have shown that VP deactivation

follows a time-dependent irrversible mechanism (Vlasits et al. 2010; Busse et al. 2013). Assuming first order kinetics (Eq. 11.18), and that no substrate will be present, the observed deactivation constant $k_{\mathrm{d(obs)}}$ can be determined according to Eq. (11.19) (Busse et al. 2013).

$$\mathrm{E} \xrightarrow{k_{\mathrm{d(obs)}}} \mathrm{E_i} \tag{11.18}$$

$$k_{\mathrm{d(obs)}} = \frac{k_i^{\mathrm{app}}[\mathrm{S_1}]}{K_i^{\mathrm{app}}[\mathrm{S_1}]} \tag{11.19}$$

k_i^{app} and K_i^{app} denote the apparent maximal rate of enzyme inactivation and the apparent concentration of inhibitor for reaching half-maximal rate of inactivation, respectively.

In the presence of substrate S, another competition with H_2O_2 occurs, since E^{III} may also react with S by returning back to E^0 (pathway 6, Fig. 11.5) under radical cation intermediate and H_2O_2 formation (assumed based on Yokota and Yamazaki (1965) and Acosta et al. (1988)). Alternatively, radical cation products $S^{\bullet +}$ are able to revert E^{III}–E^0. However, it does not necessarily mean that all potential substrates are suitable for such E^{III} conversion. On the contrary, for a LiP H2 isozyme, for instance, neither phenolics nor their corresponding phenoxyl radical products nor VA were capable of returning E^{III} back to the native state E^0, but $VA^{\bullet +}$ worked fine (Chung and Aust 1995). The validity of VP and adlerol and its radical cation products still needs to be clarified. Whatever the case, once an appropriate substrate like adlerol is added, H_2O_2-dependent enzyme deactivation is competitively inhibited, and Eq. (11.19) yields Eq. (11.20). This implies that the substrate does in fact possess protective properties (Busse et al. 2013). For illustration refer to Fig. 11.6.

$$k_{\mathrm{d(obs)}} = \frac{k_i^{\mathrm{app}}[\mathrm{S_1}]}{K_i^{\mathrm{app}}\left(1 + \frac{[\mathrm{S_2}]}{K_m^{\mathrm{S_2}}}\right) + [\mathrm{S_1}]} \tag{11.20}$$

Depending on the environmental circumstances, e.g., the used buffer system, the right term in Eqs. (11.19) and (11.20) must be extended by summation of k_i^0 (Copeland 2002). Thus, k_i^0 denotes the enzyme inactivation rate caused by other factors apart from H_2O_2. Furthermore, reaction pathway 5 and 7 (Fig. 11.5), and thus enzyme deactivation, are pH-dependent and will be favored by an acidic environment (Cai and Tien 1992; Busse et al. 2013). VPs, and all other POX, commonly have their reaction optimum around pH 3–4 (Wong 2009; Hofrichter et al. 2010; Liers et al. 2010; Busse et al. 2013) and are consequently quite susceptible to H_2O_2 (catalytic amounts of > 30–50 μM (Goodwin et al. 1994; Busse et al. 2013)). This is a major drawback of POX compared to Lac, particularly when attempting to reach industrial applications, as will be discussed next. Spontaneous decay of E^I (is unstable (Dunford 2010)) to E^{II} contributes to additional loss in activity (reaction path is omitted in Fig. 11.5). The unimolecular reaction described by pathway 8

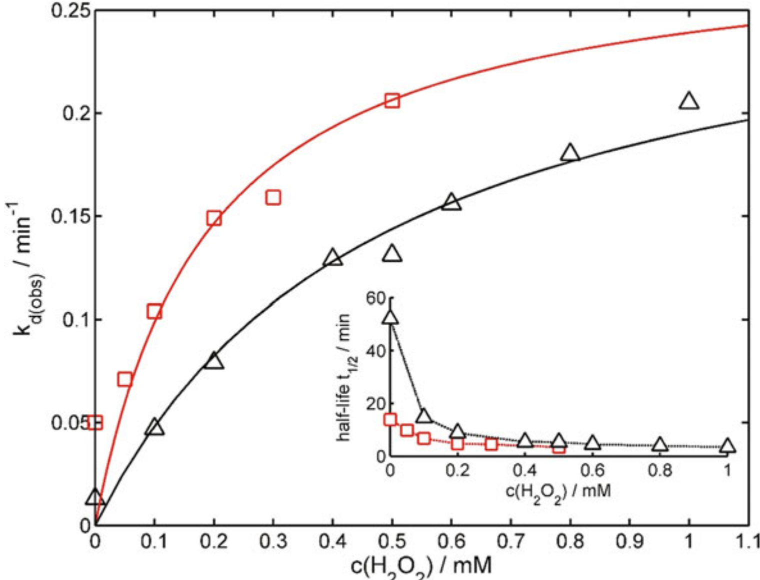


Fig. 11.6 H_2O_2-dependent deactivation kinetics in the absence of the substrate adlerol (experimental data (*squares*), computer simulation (*red solid line*)) and in the presence of 4 mM adlerol (experimental data (*triangles*), computer simulation (*black solid line*)). Reaction conditions: *T*: 30 ± 1 °C, pH: 4.0 (100 mM sodium tartrate buffer), *n*: 375 rpm, enzyme concentration: ∼0.06 mg mL^{-1}, *V*: 10 mL or 50 mL. Each examination was carried out in duplicates (parallel) with a maximal standard deviation of 10 % from the mean. The *inset* plot illustrates the corresponding half-life ($t_{1/2}$) course as a function of initial H_2O_2 concentration. Reprinted from Busse et al. (2013)

(Fig. 11.5), E^{III} decay to E^0 along with $O_2^{\bullet-}$ release, (Nakajima and Yamazaki 1987; Arnao et al. 1990b) would imply enzyme regeneration or loss of activity.

Finally, it is obvious that balancing the S/E/H_2O_2 ratio is an important task for minimizing enzyme deactivations, and thus avoiding excessive H_2O_2 concentrations. It is in fact impossible to fully eliminate enzyme deactivation, but it can be delayed. Maintaining enzyme consumption as low as possible is therefore a major tasks, since POX are currently still costly.

11.4 Technical Challenges for VP Applicability on Large Scale

11.4.1 Ensuring Cost-Effective Availability and Stability

As already stated, POX and especially VP have received a lot of attention as biocatalysts. Unfortunately, an industrial implementation has not yet be realized.

This progress is hampered by (i) low availability of active POX in sufficient amounts, (ii) H_2O_2 related instabilities (Ayala et al. 2008), and (iii) high enzyme costs (Casella et al. 2010). Therefore, major efforts are directed toward protein engineering for improving enzyme performance, particularly stability with respect to H_2O_2, catalytic activity by redox potential enhancement, and ensuring availability by efficient homologous and heterologous (over)production (Conesa et al. 2000; Ayala et al. 2008; Longoria et al. 2010). As potential expression systems, bacteria (*E. coli*), yeast (*S. cerevisiae, Pichia*) and fungi (white-rot fungi, *Aspergillus*) are all under consideration (Conesa et al. 2000; de Weert and Lokman 2010). Moreover, active POX production in insect cells using recombinant baculovirus has been examined (Pease et al. 1991; Johnson et al. 1992). However, to date, none of the investigated homologous and heterologous production approaches have matched industrial requirements (de Weert and Lokman 2010). Although homologous expression shows promising results, the most frequent cause of failure is the instability in productivity, which is not yet fully understood (Singh and Chen 2008). Further research and development is still needed. An efficient POX-specific production is dependent on (i) choice of production strain (Conesa et al. 2000; de Weert and Lokman 2010), (ii) vector system (de Weert and Lokman 2010), and (iii) culture/operating conditions (Conesa et al. 2000; Rühl et al. 2008) and inclusive production plant configuration (Rühl et al. 2007).

MnP overproduction in heterologous systems might generally be easier than for LiP and VP. It is conceivable that due to their high reactivity to a broader substrate spectrum, production of active LiP and VP, is comparatively more harmful to host organisms (Tsukihara et al. 2006). Table 11.2 lists host organisms for VP production that have been studied. Here, the homologous expression system is believed to provide a promising platform for large-scale production, resulting in recombinant enzymes exhibiting structural and kinetic properties identical to the native one (Tsukihara et al. 2006). In general, *P. ostreatus* strains are well suited for industrial production of ligninolytic enzymes (Rühl et al. 2008).

Table 11.2 Hosts for homologous and heterologous VP production (modified based on de Weert and Lokman (2010))

Organism	Host strain	Yield	References
Homologous			
P. ostreatus	*P. ostreatus* TM2-18	21 mg L^{-1} 7300 U L^{-1}	Tsukihara et al. (2006)
Heterologous			
B. adusta	*E. coli* strain BL21 (DE3)pLysS	~12 mg L^{-1} with max. 0.25 mU mg^{-1}[a]	Mohorčič et al. (2009)
P. eryngii	*Aspergillus nidulans*	466 U L^{-1}	Eibes et al. (2009)

[a]Specific activity was determined based on VA degradation

11.4.2 Supply of Lignocellulosic Biomass

In using lignocellulosic biomass as a substrate, an appropriate pretreatment is mandatory before an enzymatic bioconversion can be addressed. For reaching satisfactory reaction rates with the smallest possible enzyme amount, or in other words, for reaching high turnover numbers, wood substrates with high substrate specific surfaces (high surface to volume ratios) are favored. The minimal requirement is that native lignin must be readily accessible for enzymatic attack. Ground wood/wood flour provides the best preconditions, although a relative high energy demand is required depending on the particle size. Particle size is limiting for mass transfer (Viamajala et al. 2010). Furthermore, native lignin structures have to be conserved. Since lignin is an amorphous thermoplastic polymer (Saake and Lehnen 2012), the grinding temperature should be maintained below its glass transition temperature (T_g) (range hard-/soft-wood: 65–105 °C (Saake and Lehnen 2012)). Melting lignin would result in undesired and uncontrolled modifications (structural changes, lignin relocalization) influencing subsequent enzymatic conversion (Donohoe et al. 2008; Viamajala et al. 2010). The same is also valid for pretreating methods reaching temperatures above T_g.

Inconsistency of wood substrate, particularly lignocellulose composition, is another variable that interferes with enzyme activity and product quantity as well as quality. Hybrid poplar (e.g., made of *Populus maximowiczii* and *Populus nigra*) from short-rotation forestry is a future based raw material since it is fast-growing. Its use means variations in wood composition will be severely minimized.

11.4.3 Supply of Lignin-Containing Product Streams

Liquid lignin product streams (technical lignins) generated via chemical pretreatment of lignocellulosic biomass, for instance, is an alternative substrate for further processing and finishing by POX. The initial chemical oxidation should preferably lead to lower molecular weight and more biodegradable intermediates (Mantzavinos and Psillakis 2004). However, it has to be taken into account that such a pretreatment can also generate inhibitory products. Moreover, the pH may have to be adjusted. This might be difficult to achieve. Depending on the isolation method, the resulting technical lignin may not be soluble at an acidic pH. In general, most technical lignins are soluble in organic solvents (e.g., dimethyl sulfoxide (DMSO), dioxane, dimethylformamide, and pyridine) (Saake and Lehnen 2012). VP remains active in organic solvents like DMSO, at least in the presence of an inorganic substrate (Rodakiewicz-Nowak et al. 2006). Nonetheless, with the goal of reaching industrial application, further addition of organic solvents may be uneconomic and in fact problematic, relating to the recovery of the organic solvent as well as resulting ecological aspects.

11.4.4 Bioprocess Engineering for Delignification

For the provision of optimal operating conditions (e.g., *T*, buffer system, S/H_2O_2/E ratio, presence of additives, operation mode), the choice of an appropriate reactor design is an important issue of bioprocess engineering in order to guarantee the economic implementation of VP and POX for delignification purposes.

11.4.4.1 Reactor Design

For a rational reactor design, several aspects must be taken into account. Due to complex and rather uncontrollable radical reactions as seen above, such aspects include first and foremost an efficient maximization of catalytic turnover numbers ($k_{cat} = v_{max}/[E_T]$) in order to avoid an excess of disruptive factors like increased H_2O_2 and phenol concentrations. Phenols cause polymerization and formation of phenoxyl radicals affecting the catalytic cycle. A continuous removal of such compounds is therefore highly recommended. Moreover, reuse of the enzyme is also beneficial.

Combining the enzymatic conversion process by connecting an external cross-flow ultrafiltration (UF) unit to an enzyme membrane reactor system, as sketched in Fig. 11.7, represents a promising technological approach for delignification (Flaschel et al. 1983; Lema et al. 2010; Huang and Ramaswamy 2013). Such configurations provide several important advantages:

(i) Continuous operation mode.
(ii) Complete biocatalyst retention and recycling.
(iii) Application of free/native enzyme is possible (homogeneous catalysis), avoiding time-consuming enzyme immobilization accompanied by mass transfer limitations (Busse et al. 2016; López et al. 2007).
(iv) Replacement of fresh active enzyme depending on deactivation in order to maintain specified substrate conversion levels (Busse et al. 2016).
(v) Physical biocatalyst immobilization.
(vi) Rapid fractionation of biocatalyst and unreacted high molecular weight substrates from desired product.
(vii) Continuous removal of undesired by-products and enzyme inhibitors, shifting reaction toward product yield maximization (Busse et al. 2016; López et al. 2007).

Membrane filtration processes like tubular UF membranes are already well established for lignin recovery and separation in biorefineries (Jönsson 2013) and in the pulp and paper industry (Ebrahimi et al. 2009). This is due to the following economic aspects (Huang and Ramaswamy 2013; Jönsson 2013):

(i) Low chemical consumption.
(ii) Fractionation is controllable by pore size.
(iii) Feed volume reduction for further processing (Jönsson and Wimmerstedt 1985).
(iv) Any T and pH adjustments are superfluous.

Despite the fact that tubular UF membranes exhibit low packing densities and high energy demands (Leiknes 2009), they have “the largest foot-print” (Jönsson 2013). Moreover, these membranes require low pretreatment (Jönsson 2013), tolerate feed streams of high fouling potential and of high pollution (e.g., solids) and are easy to clean (Melin and Rautenbach 2007; Leiknes 2009). Nevertheless, proteins are serious membrane foulants (Blatt et al. 1970; Porter 1972; Field 1996), besides lignin-containing feed streams (Sridhar and Bhattacharya 1991; Pfromm and Watkins 1997; Ebrahimi et al. 2016; Busse et al. 2016). Consequently, constant enzyme addition, and/or an increased amount of lignin-derived fragments, result in secondary layer formation on the membrane surface (membrane fouling) limiting filtration performance, e.g., permeability or throughput, solute retention (Busse et al. 2016), and membrane service life. Fouling is a common issue in membrane filtration processes and is mainly affected by (i) membrane properties (e.g., selected pore size, material, configuration), (ii) hydrodynamic conditions, and (iii) feed composition (Mulder 1996; Cheryan 1998; Melin and Rautenbach 2007). Filtration performance is a crucial parameter, since it determines the efficiency of enzymatic conversion, protein load, and reactor size, along with hydraulic retention time

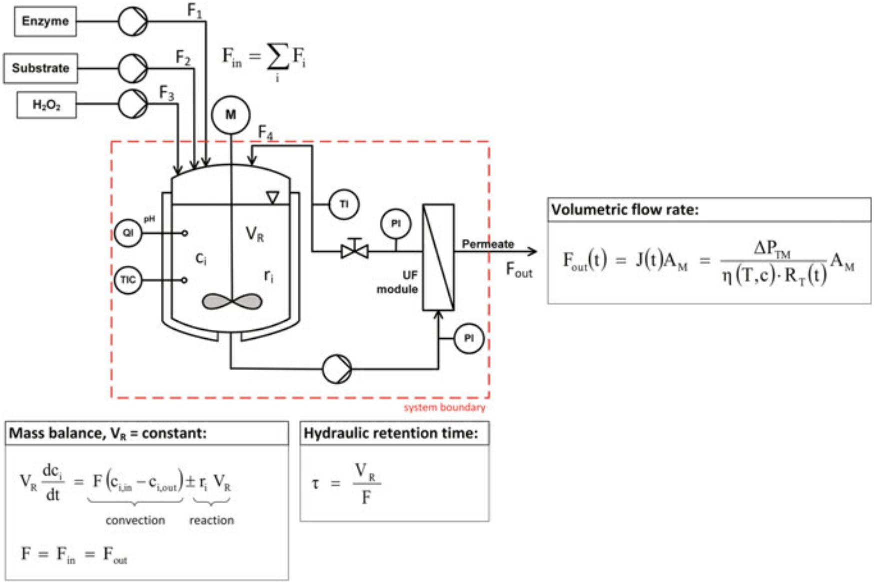


Fig. 11.7 Schematic illustration of a promising enzyme membrane reactor configuration (continuous stirred tank reactor (CSTR) equipped with an external ultrafiltration (UF) module) for delignification purposes. Enzymatic reaction can be assumed to take place primarily in the CSTR at constant reaction volume V_R. F volumetric flow rate; c, c_i concentration, concentration of component i; V_R reaction volume; t process time; r_i reaction rate; $J(t)$ time-dependent permeate flux; A_M membrane surface; ΔP_{TM} transmembrane pressure; η dynamic viscosity of the permeate depending on temperature T and concentration c; $R_T(t)$ total hydraulic resistance as a function of time t due to membrane fouling

(refer to Fig. 11.7) (Busse et al. 2016). Thus, the maintenance of constant high volumetric flow rates (or fluxes) is a major task throughout filtration processes.

Using ceramic membranes is advantageous in comparison to polymer membranes. Major aspects are:

(i) High thermal and chemical stability which is beneficial for cleaning.
(ii) High mechanical stability, particularly when filtering abrasive media.
(iii) Less susceptible to fouling and low adhesion potential for molecular organic substances (Ebrahimi et al. 2015; Fan et al. 2015).
(iv) High membrane service life/lifetime (6 years for ceramic membranes and 1.5 for polymeric) (Arkell et al. 2014).

Conversely, polymeric membranes are less expensive, easy to configure and scalable (Van der Bruggen 2009).

Several applications have already been reported using POX in membrane bioreactor systems. However, these have just been for treating xenobiotics and pollutions (e.g., azo dye Orange II by MnP, nonylphenol by VP), which use mostly polymeric UF membranes (López et al. 2007; Méndez-Hernández et al. 2015). The choice for the optimal material ultimately depends on individual needs (Arkell et al. 2014).

11.4.4.2 Process Control

Key prerequisite for implementing an effective process control, and thus ensuring optimal operation condition, is an efficient and reliable online monitoring system (except *T* and pH, since they are standard). Although H_2O_2 concentration is such a crucial parameter, the availability of commercial H_2O_2 online sensors, with sufficient sensitivity over a wide pH and *T* range, is limited. Contrarily, there are several fluorescence probes, such as some based on oxidation of *N*-acetyl-3,7-dihydroxy-phenoxazine (e.g., Amplex Red®) by HRP, for measuring H_2O_2 within a range of 10 nM to 100 μM (Gomes et al. 2005). Disadvantages of using such probes are: (i) sample preparation is required, (ii) HRP inactivation at c(H_2O_2) > 100 μM (Towne et al. 2004), thus first requiring the finding of an appropriate pre-dilution, (iii) incubation times of 15–30 min before receiving first results, and (iv) pH is also limited, ranging from 7.5 to 8.5. Similar conditions are true concerning any HRP/POX-related biosensor for H_2O_2 measurements. In the current state, amperometric sensors equipped with platinum electrodes appear rather suitable, since they are not susceptible to H_2O_2. Sample preparations are also superfluous, among other things. Particularly, the membrane protected PEROX H_2O_2 online sensors from ProMinent Dosiertechnik in Heidelberg, Germany, meet useful requirements, including aspects such as a wide pH-/*T*- and c(H_2O_2)-range with a comparatively low response time. First successful applications of this have already been reported for chloroperoxidase catalyzed oxidations (Seelbach et al. 1997; van Deurzen et al. 1997).

Another important parameter is the actual enzyme activity, which could be detected by simply monitoring the production of certain fission products and/or

substrate consumption. When complex substrates such as lignin are involved and product formation is hardly manageable, as already illustrated above, lignin degradation is usually monitored by UV absorption measurements at $\lambda = 280$ nm. Highly concentrated lignin solutions appear dark brown. The color intensity is an indirect indicator of lignin amount (Kinstre 1993). Conversely, the release of lignin-derived high molecular weight fragments from LC biomass would result in an intensification of color in the culture medium. A precise assignment of these fragments is not possible, and further analytical methods will be required which are limited in applicability, partly very costly, and not yet sufficiently standardized. An extensive overview will be given by Lin and Dence (1992). Moreover, a fundamental expertise is mandatory for process optimization and data interpretation. At present, there is no simple, standalone analytical method. Based on these facts, alternative (in)direct measurement techniques must be found. Such techniques should be (i) cheap, (ii) easy to implement, online, (iii) robust, (iv) quick and precise and (v) low maintenance. Measuring dissolved oxygen (DO), especially via optical sensors, is currently the most promising parameter for process monitoring due to pseudo-catalase activity of POX at H_2O_2 excess (or low $c(S)/c(H_2O_2)$ ratios) resulting in O_2 formation (Acosta et al. 1988; Wariishi and Gold 1989; Vlasits et al. 2010). Excessive $c(H_2O_2)$ causes considerable enzyme deactivation (Busse et al. 2013). On the contrary, O_2 consumption is an indicator for high $c(S)/c(H_2O_2)$ ratios (López et al. 2007). Thus, O_2 consumption is also indicative for substrate degradation processes as seen in Figs. 11.3 and 11.4, whereas O_2 can also be generated simultaneously concerning $HO_2^{\bullet}/O_2^{\bullet-}$ release (Figs. 11.3 and 11.5). Recent studies by López et al. (2007), treating xenobiotic compounds such as Organe II by MnP, have shown that DO could be valuable for implementing process control strategies (e.g., feedback control). However, based on the variety of radical reactions, O_2 formation/consumption is not trivial (Palmer et al. 1987). Further research is therefore needed for a better (process) understanding, particularly in the field of delignification. Moreover, the presence of substrates (e.g., VA and adlerol) capable of bond cleavage seems to be a precondition for significant O_2 consumption (Palmer et al. 1987).

11.5 Conclusion and Outlook

The high susceptibility to H_2O_2 of VP (POX in general) is an important issue which needs to be overcome, as they are currently making wild type less attractive for industrial use. In vivo relative low H_2O_2 levels are rather assumed due to H_2O_2 assimilation by the fungus avoiding unfavorable high concentrations (Hammel et al. 1993; Böckle et al. 1999). Moreover, VPs are regrettably limited in availability. Thus, numerous efforts are being carried out for recombinant VP production, in order to elevate H_2O_2 tolerance and availability. Alternatively, enzyme immobilization could be another technique to overcome premature inactivation (E_i formation). However, immobilization may not prevent E^{III} formations at H_2O_2 excess. Enzyme reconditioning ($E^{III} \rightarrow E^0$, pathway 6 in Fig. 11.5) in turn is again influenced by the

underlying reaction conditions and not by the immobilization method. Despite several studies, the proof-of-concept is still outstanding. Additionally, in the case of woody biomass, good enzyme penetration must be further guaranteed, affected by porosity and the pore size, of the substrate to be treated.

Another promising/future based strategy may be enzymes from insects and insect-associated microorganisms. Insects are the most diverse taxonomic animal class, colonizing almost every ecological niche. To succeed in these sometimes extreme niches, insects have established diverse biological and chemical systems, e.g., the production of defense molecules (Gross et al. 2008; Schlipalius et al. 2012), protein stabilization (Bale 2002), or lytic enzymes (Landureau and Jolles 1970; Mika et al. 2013). Insects also house symbiotic microorganisms as digestive helpers, fodder, or both (Scharf et al. 2011). This is possible due to the special metabolic systems, or exogenous enzymes, of the associated organisms. For instance, insects like bark beetles, ambrosia beetles and termites, are able to feed on wood, making them xylophagous, and are well known as wood pests. The function of their digestive systems is not yet completely clarified. The enzymatic apparatus for the oxidation of lignin and the hydrolysis of cellulose is only marginally understood. One reason might be the diverse sources of the key enzymes, which may be produced either by gut-inhabiting microorganisms (Morrison et al. 2009), by symbiotic fungi cultivated by the insects (Currie 2001), or by the insects themselves as endogenous enzymes (Pauchet et al. 2010). Nevertheless, a lot of work has been done in attempts to understand the lignocellulolytic system of the insects and the degree of participation by symbionts (Geib et al. 2008; Scharf et al. 2011).

Currently, POX are still costly for industrial use. Nonetheless, there is no doubt that once limitations are resolved, particularly H_2O_2 instability and efficient production, the potential for large-scale application of VPs raises significantly (Casella et al. 2010). However, many questions with respect to the enzymatic delignification mechanism still remain in place, i.e., substrate preparation/pretreatment, E^0 recovery, control of inhibitory reactions/factors besides H_2O_2, involvement of accessory factors and enzymes, etc. Moreover, for better process control, developments focusing on simple and fast online monitoring systems will also be necessary. For these purposes, further fundamental research is needed.

Acknowledgments The researchers would like to thank the Hessen State Ministry of Higher Education, Research and the Arts for the financial support within the Hessen initiative for scientific and economic excellence (LOEWE).

References

Acosta M, del Río JA, Arnao MB, Sánchez-Bravo J, Sabater F, García-Carmona F, García-Cánovas F (1988) Oxygen consumption and enzyme inactivation in the indolyl-3-acetic acid oxidation catalyzed by peroxidase. Biochim Biophys Acta (BBA) Protein Struct Mol Enzymol 955(2):194–202. doi:10.1016/0167-4838(88)90193-8

Adler E (1977) Lignin chemistry—past, present and future. Wood Sci Technol 11(3):169–218. doi:10.1007/BF00365615

Andrawis A, Johnson KA, Tien M (1988) Studies on compound I formation of the lignin peroxidase from *Phanerochaete chrysosporium*. J Biol Chem 263(3):1195–1198

Arkell A, Olsson J, Wallberg O (2014) Process performance in lignin separation from softwood black liquor by membrane filtration. Chem Eng Res Des 92(9):1792–1800. doi:10.1016/j.cherd.2013.12.018

Arnao MB, Acosta M, del Rio JA (1038) García-Cánovas F (1990a) Inactivation of peroxidase by hydrogen peroxide and its protection by a reductant agent. Biochim Biophys Acta (BBA) Protein Struct Mol Enzymol 1:85–89. doi:10.1016/0167-4838(90)90014-7

Arnao MB, Acosta M, del Río JA, Varón R (1041) García-Cánovas F (1990b) A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide. Biochim Biophys Acta Protein (BBA) Struct Mol Enzymol 1:43–47. doi:10.1016/0167-4838(90)90120-5

Ayala M (2010) Redox potential of peroxidases. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 61–77. doi:10.1007/978-3-642-12627-7_4. ISBN 978-3-642-12626-0

Ayala M, Pickard MA, Vazquez-Duhalt R (2008) Fungal enzymes for environmental purposes, a molecular biology challenge. J Mol Microbiol Biotechnol 15(2–3):172–180. doi:10.1159/000121328

Bale JS (2002) Insects and low temperatures: from molecular biology to distributions and abundance. Philos Trans B 357(1423):849–862. doi:10.1098/rstb.2002.1074

Bielski BH, Cabelli DE, Ravindra LA, Ross AB (1985) Reactivity of HO_2/O_2-radicals in aqueous solution. J Phys Chem Ref Data 14(4):1041–1100

Bisswanger H (2008) Enzyme kinetics: section 2. In: Enzyme kinetics. Wiley-VCH Verlag GmbH & Co. KGaA, pp 124–193. ISBN 9783527319572

Blanchette RA (1991) Delignification by wood-decay fungi. Annu Rev Phytopathol 29(1): 381–403. doi:10.1146/annurev.py.29.090191.002121

Blatt WF, Dravid A, Michaels AS, Nelsen L (1970) Solute polarization and cake formation in membrane ultrafiltration: causes, consequences, and control techniques. In: Flinn JE (ed) Membrane science and technology. Springer, USA, pp 47–97. doi:10.1007/978-1-4684-1851-4_4. ISBN 978-1-4684-1853-8 (print), 978-1-4684-1851-4 (online)

Böckle B, Martínez MJ, Guillén F, Martínez ÁT (1999) Mechanism of peroxidase inactivation in liquid cultures of the ligninolytic fungus *Pleurotus pulmonarius*. Appl Environ Microbiol 65 (3):923–928

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Lett 267(1):99–102. doi:10.1016/0014-5793(90)80298-W

Busse N, Wagner D, Kraume M, Czermak P (2013) Reaction kinetics of versatile peroxidase for the degradation of lignin compounds. Am J Biochem Biotechnol 9(4):365–394. doi:10.3844/ajbbsp.2013.365.394

Busse N, Fuchs F, Kraume M, Czermak P (2016) Treatment of enzyme initiated delignification reaction mixtures with ceramic ultrafiltration membranes: Experimental investigations and modeling approach. Sep Sci Technol 51(9):1546–1565. doi:10.1080/01496395.2016.1167739

Cai D, Tien M (1992) Kinetic studies on the formation and decomposition of compounds II and III. Reactions of lignin peroxidase with H_2O_2. J Biol Chem 267(16):11149–11155

Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martínez MJ, Martínez ÁT (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem 274(15):10324–10330. doi:10.1074/jbc.274.15.10324

Cañas AI, Camarero S (2010) Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28(6):694–705. doi:10.1016/j.biotechadv.2010.05.002

Carabajal M, Kellner H, Levin L, Jehmlich N, Hofrichter M, Ullrich R (2013) The secretome of Trametes versicolor grown on tomato juice medium and purification of the secreted oxidoreductases including a versatile peroxidase. J Biotechnol 168(1):15–23. doi:10.1016/j.jbiotec.2013.08.007

Casella L, Monzani E, Nicolis S (2010) Potential applications of peroxidases in the fine chemical industries. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 111–153. doi:10.1007/978-3-642-12627-7_6. ISBN 978-3-642-12626-0

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. In: Methods in enzymology, vol 2. Academic Press, London, pp 764–775. doi: 10.1016/S0076-6879(55)02300-8

Cheryan M (1998) Ultrafiltration and microfiltration handbook. CRC Press, London. ISBN 978-1-56676-598-5

Chung N, Aust SD (1995) Inactivation of lignin peroxidase by hydrogen peroxide during the oxidation of phenols. Arch Biochem Biophys 316(2):851–855. doi:10.1006/abbi.1995.1114

Conesa A, van den Hondel CAMJJ, Punt PJ (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl Environ Microbiol 66(7):3016–3023. doi:10.1128/aem.66.7.3016-3023.2000

Copeland RA (2002) Enzymes: a practical introduction to structure, mechanism, and data analysis. Wiley-VCH, London. ISBN 0-471-35929-7, 0-471-22063-9

Cornish-Bowden A (2012) Fundamentals of enzyme kinetics. Wiley-Blackwell, London. ISBN 9783527330744

Currie CR (2001) A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. Annu Rev Microbiol 55(1):357–380. doi:10.1146/annurev.micro.55.1.357

Dashtban M, Schraft H, Syed TA, Qin W (2010) Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 1(1):36–50

de Montellano PRO (2010) Catalytic mechanisms of heme peroxidases. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 79–107. doi:10.1007/978-3-642-12627-7_5. ISBN 978-3-642-12626-0

de Weert S, Lokman BC (2010) Heterologous expression of peroxidases. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 315–333. doi:10.1007/978-3-642-12627-7_12. ISBN 978-3-642-12626-0

Donohoe BS, Decker SR, Tucker MP, Himmel ME, Vinzant TB (2008) Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol Bioeng 101(5):913–925. doi:10.1002/bit.21959

Dunford HB (1991) Horsradish peroxidase: structure and kinetic properties. In: Everse J (ed) Peroxidases in chemistry and biology, vol 2. CRC Press, London, pp 1–24. ISBN 0849369649

Dunford HB (2010) Peroxidases and catalases: biochemistry, biophysics, biotechnology and physiology. Wiley, London. ISBN 9780470224762

Ebrahimi M, Kerker S, Wienold A, Neul H, Hilpert M, Mund P, Czermak P (2009) Processing and characterization of ceramic membranes for the efficient removal of COD and residual lignin from bleaching effluents. International conference and exhibition for filtration and separation technology. Filtech Wiesbaden, Germany

Ebrahimi M, Busse N, Kerker S, Schmitz O, Hilpert M, Czermak P (2016) Treatment of the bleaching effluent from sulfite pulp production by ceramic membrane filtration. Membranes 6(1). doi:10.3390/membranes6010007

Ebrahimi M, Kerker S, Daume S, Geile M, Ehlen F, Unger I, Schütz S, Czermak P (2015) Innovative ceramic hollow fiber membranes for recycling/reuse of oilfield produced water. Desalination Water Treat 55(13):3554–3567. doi:10.1080/19443994.2014.947780

Eibes GM, Lú-Chau TA, Ruiz-Dueñas FJ, Feijoo G, Martínez MJ, Martínez AT, Lema JM (2009) Effect of culture temperature on the heterologous expression of *Pleurotus eryngii* versatile peroxidase in *Aspergillus* hosts. Bioprocess Biosyst Eng 32(1):129–134. doi:10.1007/s00449-008-0231-7

Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin. ISBN 354051600X

Fan R, Ebrahimi M, Quitmann H, Czermak P (2015) Lactic acid production in a membrane bioreactor system with thermophilic *Bacillus coagulans*: fouling analysis of the used ceramic membranes. Sep Sci Technol 50(14):2177–2189. doi:10.1080/01496395.2015.1031401

Field RW (1996) Mass transport and the design of membrane systems. In: Scott K, Hughes R (eds) Industrial membrane separation technology. Springer, Netherlands, pp 67–113. doi:10.1007/978-94-011-0627-6_4
Flaschel E, Wandrey C, Kula M-R (1983) Ultrafiltration for the separation of biocatalysts. Downstream processing. Springer, Berlin, pp 73–142. doi:10.1007/978-3-662-39694-0_2
Geib SM, Filley TR, Hatcher PG, Hoover K, Carlson JE, Jimenez-Gasco MdM, Nakagawa-Izumi A, Sleighter RL, Tien M (2008) Lignin degradation in wood-feeding insects. Proc Natl Acad Sci 105(35):12932–12937. doi:10.1073/pnas.0805257105
Glasser WG, Davé V, Frazier CE (1993) Molecular weight distribution of (semi-) commercial lignin derivatives. J Wood Chem Technol 13(4):545–559. doi:10.1080/02773819308020533
Gomes A, Fernandes E, Lima JLFC (2005) Fluorescence probes used for detection of reactive oxygen species. J Biochem Biophys Methods 65(2–3):45–80. doi:10.1016/j.jbbm.2005.10.003
Goodwin DC, Barr DP, Aust SD, Grover TA (1994) The role of oxalate in lignin peroxidase-catalyzed reduction: protection from compound III accumulation. Arch Biochem Biophys 315(2):267–272. doi:10.1006/abbi.1994.1499
Gross J, Schumacher K, Schmidtberg H, Vilcinskas A (2008) Protected by fumigants: beetle perfumes in antimicrobial defense. J Chem Ecol 34(2):179–188. doi:10.1007/s10886-007-9416-9
Halliwell B, Gutteridge JMC (1985) The importance of free radicals and catalytic metal ions in human diseases. Mol Aspects Med 8(2):89–193. doi:10.1016/0098-2997(85)90001-9
Hammel KE, Jensen KA, Mozuch MD, Landucci LL, Tien M, Pease EA (1993) Ligninolysis by a purified lignin peroxidase. J Biol Chem 268(17):12274–12281
Harman LS, Carver DK, Schreiber J, Mason RP (1986) One- and two-electron oxidation of reduced glutathione by peroxidases. J Biol Chem 261(4):1642–1648
Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiol Rev 13(2–3):125–135. doi:10.1111/j.1574-6976.1994.tb00039.x
Hatakka A (2005) Biodegradation of lignin. In: Biopolymers online. Wiley-VCH Verlag GmbH & Co. KGaA. doi:10.1002/3527600035.bpol1005. ISBN 9783527600038
Hatakka A, Hammel K (2011) Fungal biodegradation of lignocelluloses. In: Hofrichter M (ed) Industrial applications, vol 10. Springer, Berlin, pp 319–340. doi:10.1007/978-3-642-11458-8_15. ISBN 978-3-642-11457-1
Hatakka A, Lundell T, Tervilä-Wilo ALM, Brunow G (1991) Metabolism of non-phenolic β-O-4 lignin model compounds by the white-rot fungus *Phlebia radiata*. Appl Microbiol Biotechnol 36(2):270–277. doi:10.1007/BF00164433
Hofrichter M, Ullrich R, Pecyna M, Liers C, Lundell T (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87(3):871–897. doi:10.1007/s00253-010-2633-0
Holladay J, White J, Bozell J, Johnson D (2007) Top value-added chemicals from biomass—volume II—results of screening for potential candidates from biorefinery lignin Pacific Northwest National Laboratory. Richland, WA, U.S. Department of Energy, 87
Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol 2(12):602–619
Huang H-J, Ramaswamy S (2013) Overview of biomass conversion processes and separation and purification technologies in biorefineries. In: Ramaswamy S, Huang H-J, Ramarao BV (eds) Separation and purification technologies in biorefineries. Wiley, London, pp 3–36. ISBN 978-0-470-97796-5 (Hardcover)
Johnson TM, Pease EA, Li JKK, Tien M (1992) Production and characterization of recombinant lignin peroxidase isozyme H2 from *Phanerochaete chrysosporium* using recombinant baculovirus. Arch Biochem Biophys 296(2):660–666. doi:10.1016/0003-9861(92)90624-6
Jönsson A-S (2013) Microfiltration, ultrafiltration and diafiltration. In: Ramaswamy S, Huang H-J, Ramarao BV (eds) Separation and purification technologies in biorefineries. Wiley, London, pp 205–231. ISBN 978-0-470-97796-5 (Hardcover)

Jönsson AS, Wimmerstedt R (1985) The application of membrane technology in the pulp and paper industry. Desalination 53(1–3):181–196. doi:10.1016/0011-9164(85)85060-8
Kawai S, Asukai M, Ohya N, Okita K, Ito T, Ohashi H (1999) Degradation of a non-phenolic β-O-4 substructure and of polymeric lignin model compounds by laccase of *Coriolus versicolor* in the presence of 1-hydroxybenzotriazole. FEMS Microbiol Lett 170(1):51–57. doi:10.1111/j.1574-6968.1999.tb13354.x
Kettle AJ, Anderson RF, Hampton MB, Winterbourn CC (2007) Reactions of superoxide with myeloperoxidase. Biochemistry 46(16):4888–4897. doi:10.1021/bi602587k
Kinstre RB (1993) An overview of strategies for reducing the environmental impact of bleach-plant effluents. Tappi J 76(5):105–113
Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505
Landureau JC, Jolles P (1970) Lytic enzyme produced in vitro by insect cells: lysozymelor chitinase? Nature 225(5236):968–969. doi:10.1038/225968a0
Lange J-P (2007) Lignocellulose conversion: an introduction to chemistry, process and economics. Biofuels Bioprod Biorefin 1(1):39–48. doi:10.1002/9783527621118.ch2
Leiknes T (2009) Wastewater treatment by membrane bioreactors. In: Membrane operations. Wiley-VCH Verlag GmbH & Co. KGaA, pp 363–395. doi: 10.1002/9783527626779.ch16. ISBN 9783527626779
Lema J, López C, Eibes G, Taboada-Puig R, Moreira MT, Feijoo G (2010) Reactor engineering. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 245–290. doi:10.1007/978-3-642-12627-7_10. ISBN 978-3-642-12626-0
Liers C, Bobeth C, Pecyna M, Ullrich R, Hofrichter M (2010) DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl Microbiol Biotechnol 85(6):1869–1879. doi:10.1007/s00253-009-2173-7
Lin SY, Dence CW (1992) Methods in lignin chemistry. Springer, Berlin. doi:10.1007/978-3-642-74065-7. ISBN 9783642740671
Longoria A, Tinoco R, Torres E (2010) Enzyme technology of peroxidases: immobilization, chemical and genetic modification. In: Torres E, Ayala M (eds) Biocatalysis based on heme peroxidases. Springer, Berlin, pp 209–243. doi:10.1007/978-3-642-12627-7_9. ISBN 978-3-642-12626-0
López C, Moreira MT, Feijoo G, Lema JM (2007) Dynamic modeling of an enzymatic membrane reactor for the treatment of xenobiotic compounds. Biotechnol Bioeng 97(5):1128–1137. doi:10.1002/bit.21311
Lundell T, Schoemaker H, Hatakka A, Brunow G (1993a) New mechanism of the Ca–Cb cleavage in non-phenolic arylglycerol b-aryl ether lignin substructures catalyzed by lignin peroxidase. Holzforschung Int J Biol Chem Phys Technol Wood 47:219. doi:10.1515/hfsg.1993.47.3.219
Lundell T, Wever R, Floris R, Harvey P, Hatakka A, Brunow G, Schoemaker H (1993b) Lignin peroxidase L3 from *Phlebia radiata*. Pre-steady-state and steady-state studies with veratryl alcohol and a non-phenolic lignin model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol. Eur J Biochem 211(3):391–402
Lundell TK, Mäkelä MR, Hildén K (2010) Lignin-modifying enzymes in filamentous basidiomycetes—ecological, functional and phylogenetic review. J Basic Microbiol 50(1): 5–20. doi:10.1002/jobm.200900338
Mantzavinos D, Psillakis E (2004) Enhancement of biodegradability of industrial wastewaters by chemical oxidation pre-treatment. J Chem Technol Biotechnol 79(5):431–454. doi:10.1002/jctb.1020
Martínez ÁT (2007) High redox potential peroxidases. In: Polaina J, MacCabe A (eds) Industrial enzymes. Springer, Netherlands, pp 477–488. doi:10.1007/1-4020-5377-0_27. ISBN 9781402053771
Martínez MJ, Ruiz-Dueñas FJ, Guillén F, Martínez ÁT (1996) Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. Eur J Biochem 237(2): 424–432. doi:10.1111/j.1432-1033.1996.0424k.x

Martínez ÁT, Ruiz-Dueñas FJ, Martínez MJ, del Río JC, Gutiérrez A (2009) Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 20(3):348–357. doi:10.1016/j.copbio.2009.05.002

Melin T, Rautenbach R (2007) Membranverfahren Grundlagen der Modul- und Anlagenauslegung. Springer, Berlin. ISBN 978-3-540-34327-1

Méndez-Hernández JE, Eibes G, Arca-Ramos A, Lú-Chau TA, Feijoo G, Moreira MT, Lema JM (2015) Continuous removal of nonylphenol by versatile peroxidase in a two-stage membrane bioreactor. Appl Biochem Biotechnol 175(6):3038–3047. doi:10.1007/s12010-014-1474-1

Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by Bjerkandera species strain BOS55 in the absence of manganese. J Biol Chem 273(25):15412–15417. doi:10.1074/jbc.273.25.15412

Mika N, Zorn H, Rühl M (2013) Insect-derived enzymes: a treasure for industrial biotechnology and food biotechnology. In: Vilcinskas A (ed) Yellow biotechnology II. Springer, Berlin, pp 1–17. doi:10.1007/10_2013_204. ISBN 3642399010

Mohorčič M, Benčina M, Friedrich J, Jerala R (2009) Expression of soluble versatile peroxidase of Bjerkandera adusta in *Escherichia coli*. Bioresour Technol 100(2):851–858. doi:10.1016/j.biortech.2008.07.005

Moreira PR, Bouillenne F, Almeida-Vara E, Xavier Malcata F, Frère JM, Duarte JC (2006) Purification, kinetics and spectral characterisation of a new versatile peroxidase from a *Bjerkandera* sp. isolate. Enzyme Microb Technol 38(1–2):28–33. doi:10.1016/j.enzmictec.2004.12.035

Morrison M, Pope PB, Denman SE, McSweeney CS (2009) Plant biomass degradation by gut microbiomes: more of the same or something new? Curr Opin Biotechnol 20(3):358–363. doi:10.1016/j.copbio.2009.05.004

Mulder MHV (1996) Basic principles of membrane technology. Kluwer, The Netherlands. ISBN 0-7923-4247-X (HB)

Nakajima R, Yamazaki I (1987) The mechanism of oxyperoxidase formation from ferryl peroxidase and hydrogen peroxide. J Biol Chem 262(6):2576–2581

Nicell JA, Bewtra JK, Biswas N, Taylor E (1993) Reactor development for peroxidase catalyzed polymerization and precipitation of phenols from wastewater. Water Res 27(11):1629–1639. doi:10.1016/0043-1354(93)90127-4

Palmer JM, Harvey PJ, Schoemaker HE (1987) The role of peroxidases, radical cations and oxygen in the degradation of lignin [and discussion]. Philos Trans R Soc Lond A 321 (1561):495–505. doi:10.1098/rsta.1987.0027

Pauchet Y, Wilkinson P, Chauhan R, French-Constant RH (2010) Diversity of beetle genes encoding novel plant cell wall degrading enzymes. PLoS One 5(12):e15635. doi:10.1371/journal.pone.0015635

Pease EA, Aust SD, Tien M (1991) Heterologous expression of active manganese peroxidase from *Phanerochaete chrysosporium* using the baculovirus expression system. Biochem Biophys Res Commun 179(2):897–903. doi:10.1016/0006-291X(91)91903-P

Pérez-Boada M, Ruiz-Dueñas FJ, Pogni R, Basosi R, Choinowski T, Martínez MJ, Piontek K, Martínez ÁT (2005) Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. J Mol Biol 354(2):385–402. doi:10.1016/j.jmb.2005.09.047

Pfromm PH, Watkins EJ (1997) Fouling of electrodialysis membranes by organic macromolecules. IPST technical paper series number 676 12

Pogni R, Baratto MC, Giansanti S, Teutloff C, Verdin J, Valderrama B, Lendzian F, Lubitz W, Vazquez-Duhalt R, Basosi R (2005) Tryptophan-based radical in the catalytic mechanism of versatile peroxidase from *Bjerkandera adusta*. Biochemistry 44(11):4267–4274. doi:10.1021/bi047474l

Porter MC (1972) Concentration polarization with membrane ultrafiltration. Product R&D 11 (3):234–248. doi:10.1021/i360043a002

Rasmussen CB, Dunford HB, Welinder KG (1995) Rate enhancement of compound I formation of barley peroxidase by ferulic acid, caffeic acid and coniferyl alcohol. Biochemistry 34 (12):4022–4029. doi:10.1021/bi00012a021

Rodakiewicz-Nowak J, Jarosz-Wilkołazka A, Luterek J (2006) Catalytic activity of versatile peroxidase from *Bjerkandera fumosa* in aqueous solutions of water-miscible organic solvents. Appl Catal A 308:56–61. doi:10.1016/j.apcata.2006.04.009

Rodríguez Couto S, Toca Herrera JL (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24(5):500–513. doi:10.1016/j.biotechadv.2006.04.003

Rühl M, Kilaru S, Navarro-Gonzaléz M, Hoegger P, Kharazipour A, Kües U (2007) Production of laccase and other enzymes for the wood industry. In: Kües U (ed) Wood production, wood technology, and biotechnological impacts. Universitätsverlag Göttingen, Göttingen, pp 469–507. ISBN 0849369649

Rühl M, Fischer C, Kües U (2008) Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheatstraw-based substrate. Curr Trends Biotechnol Pharm 2(4):478–492. ISSN 0973-8916

Ruiz-Dueñas FJ, Martínez ÁT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2(2):164–177. doi:10.1111/j.1751-7915.2008.00078.x

Ruiz-Dueñas FJ, Morales M, García E, Miki Y, Martínez MJ, Martínez ÁT (2009a) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 60 (2):441–452. doi:10.1093/jxb/ern261

Ruiz-Dueñas FJ, Pogni R, Morales M, Giansanti S, Mate MJ, Romero A, Martínez MJ, Basosi R, Martínez ÁT (2009b) Protein radicals in fungal versatile peroxidase: catalytic tryptophan radical in both compound I and compound II and studies on W164Y, W164H, and W164S variants. J Biol Chem 284(12):7986–7994. doi:10.1074/jbc.M808069200

Saake B, Lehnen R (2012) Lignin. In: Ullmann's encyclopedia of industrial chemistry. Wiley-VCH Verlag GmbH & Co. KGaA. doi: 10.1002/14356007.a15_305.pub3

Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27(2):185–194. doi:10.1016/j.biotechadv.2008.11.001

Scharf ME, Karl ZJ, Sethi A, Boucias DG (2011) Multiple levels of synergistic collaboration in termite lignocellulose digestion. PLoS One 6(7):e21709. doi:10.1371/journal.pone.0021709

Schlipalius DI, Valmas N, Tuck AG, Jagadeesan R, Ma L, Kaur R, Goldinger A, Anderson C, Kuang J, Zuryn S, Mau YS, Cheng Q, Collins PJ, Nayak MK, Schirra HJ, Hilliard MA, Ebert PR (2012) A core metabolic enzyme mediates resistance to phosphine gas. Science 338 (6108):807–810. doi:10.1126/science.1224951

Schoemaker HE (1990) On the chemistry of lignin biodegradation. Recl Trav Chim Pays-Bas 109 (4):255–272. doi:10.1002/recl.19901090402

Schoemaker HE, Piontek K (1996) On the interaction of lignin peroxidase with lignin. Pure Appl Chem 68:2089–2096. doi:10.1351/pac199668112089

Schoemaker HE, Lundell TK, Floris R, Glumoff T, Winterhalter KH, Piontek K (1994a) Do carbohydrates play a role in the lignin peroxidase cycle? Redox catalysis in the endergonic region of the driving force. Bioorganic Med Chem 2(6):509–519. doi:10.1016/0968-0896(94)80021-9

Schoemaker HE, Lundell TK, Hatakka AI, Piontek K (1994b) The oxidation of veratryl alcohol, dimeric lignin models and lignin by lignin peroxidase: the redox cycle revisited. FEMS Microbiol Rev 13(2–3):321–331. doi:10.1111/j.1574-6976.1994.tb00052.x

Schüttmann I, Bouws H, Szweda RT, Suckow M, Czermak P, Zorn H (2014) Induction, characterization, and heterologous expression of a carotenoid degrading versatile peroxidase from *Pleurotus sapidus*. J Mol Catal B Enzym 103:79–84. doi:10.1016/j.molcatb.2013.08.007

Seelbach K, van Deurzen MPJ, van Rantwijk F, Sheldon RA, Kragl U (1997) Improvement of the total turnover number and space-time yield for chloroperoxidase catalyzed oxidation. Biotechnol Bioeng 55(2):283–288. doi:10.1002/(sici)1097-0290(19970720)55:2<283:aid-bit6>3.0.co;2-e

Singh D, Chen S (2008) The white-rot fungus *Phanerochaete chrysosporium*: conditions for the production of lignin-degrading enzymes. Appl Microbiol Biotechnol 81(3):399–417. doi:10.1007/s00253-008-1706-9

Sridhar S, Bhattacharya PK (1991) Limiting flux phenomena in ultrafiltration of kraft black liquor. J Membr Sci 57(2–3):187–206. doi:10.1016/S0376-7388(00)80678-X

Stöcker M (2008) Bio- und BTL-Kraftstoffe in der Bioraffinerie: katalytische Umwandlung Lignocellulose-reicher Biomasse mit porösen Stoffen. Angew Chem 120(48):9340–9351. doi:10.1002/ange.200801476

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83(1):1–11. doi:10.1016/S0960-8524(01)00212-7

Tien M (1987) Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. Crit Rev Microbiol 15(2):141–168. doi:10.3109/10408418709104456

Tien M, Kirk TK (1984) Lignin-degrading enzyme from Phanerochaete chrysosporium: Purification,characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. PNAS 81(8):2280–2284.doi:10.1073/pnas.81.8.2280

Towne V, Will M, Oswald B, Zhao Q (2004) Complexities in horseradish peroxidase-catalyzed oxidation of dihydroxyphenoxazine derivatives: appropriate ranges for pH values and hydrogen peroxide concentrations in quantitative analysis. Anal Biochem 334(2):290–296. doi:10.1016/j.ab.2004.07.037

Tsukihara T, Honda Y, Sakai R, Watanabe T, Watanabe T (2006) Exclusive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, *Pleurotus ostreatus*. J Biotechnol 126(4):431–439. doi:10.1016/j.jbiotec.2006.05.013

Valderrama B, Ayala M, Vazquez-Duhalt R (2002) Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes. Chem Biol 9(5):555–565. doi:10.1016/S1074-5521(02)00149-7

Van der Bruggen B (2009) Fundamentals of membrane solvent separation and pervaporation. In: Membrane operations. Wiley-VCH Verlag GmbH & Co. KGaA, pp 45–61. doi: 10.1002/9783527626779.ch3. ISBN 9783527626779

van Deurzen MPJ, Seelbach K, van Rantwijk F, Kragl U, Sheldon RA (1997) Chloroperoxidase: use of a hydrogen peroxide-stat for controlling reactions and improving enzyme performance. Biocatal Biotransform 15(1):1–16. doi:10.3109/10242429709003606

Viamajala S, Donohoe B, Decker S, Vinzant T, Selig M, Himmel M, Tucker M (2010) Heat and mass transport in processing of lignocellulosic biomass for fuels and chemicals. In: Singh OV, Harvey SP (eds) Sustainable biotechnology. Springer, Netherlands, pp 1–18. doi:10.1007/978-90-481-3295-9_1. ISBN 978-90-481-3294-2

Vlasits J, Jakopitsch C, Bernroitner M, Zamocky M, Furtmüller PG, Obinger C (2010) Mechanisms of catalase activity of heme peroxidases. Arch Biochem Biophys 500(1):74–81. doi:10.1016/j.abb.2010.04.018

Wang Y, Vazquez-Duhalt R, Pickard MA (2003) Manganese-lignin peroxidase hybrid from *Bjerkandera adusta* oxidizes polycyclic aromatic hydrocarbons more actively in the absence of manganese. Can J Microbiol 49(11):675–682. doi:10.1139/w03-091

Wariishi H, Gold MH (1989) Lignin peroxidase compound III: formation, inactivation, and conversion to the native enzyme. FEBS Lett 243(2):165–168. doi:10.1016/0014-5793(89)80122-X

Wariishi H, Gold MH (1990) Lignin peroxidase compound III. Mechanism of formation and decomposition. J Biol Chem 265(4):2070–2077

Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 157(2):174–209. doi:10.1007/s12010-008-8279-z

Yokota K, Yamazaki I (1965) The activity of the horseradish peroxidase compound 3. Biochem Biophys Res Commun 18:48–53. doi:10.1016/0006-291X(65)90880-6

Zorn H, Peters T, Nimtz M, Berger RG (2005) The secretome of *Pleurotus sapidus*. PROTEOMICS 5(18):4832–4838. doi:10.1002/pmic.200500015

Chapter 12
Fungal Aryl-Alcohol Oxidase in Lignocellulose Degradation and Bioconversion

Juan Carro, Ana Serrano, Patricia Ferreira and Angel T. Martínez

Abstract Bioconversion of lignocellulosic materials draws much interest as they are regarded as a renewable source of energy and platform chemicals. The enzyme aryl-alcohol oxidase (AAO) has been extensively studied, revealing its involvement in biodegradation of lignocellulose by several well-known white-rot fungi. Its physiological role is to supply hydrogen peroxide from the oxidation of aromatic substrates derived from fungal secondary metabolism or lignin degradation, which can: (i) be used by peroxidases to oxidise lignin; or (ii) give rise, through Fenton reaction, to hydroxyl radical that is able (itself) to depolymerise cellulose and oxidise lignin. Several features make this enzyme an appealing biocatalyst that has shown its potential for industrial applications: AAO has a broad range of substrates that it oxidises by stereoselective hydride transfer reaction mechanism, and reduces atmospheric molecular oxygen as a co-substrate producing hydrogen peroxide.

Abbreviations

AAO	Aryl-alcohol oxidase
DFF	2,5-Diformylfuran
FDCA	2,5-Furandicarboxylic acid
FFCA	5-Formylfurancarboxylic acid
GMC	Glucose-methanol-choline oxidase/dehydrogenase superfamily
HMF	5-Hydroxymethylfurfural
HMFCA	5-Hydroxymethylfurancarboxylic acid
LiP	Lignin peroxidase
MnP	Manganese peroxidase

J. Carro · A. Serrano · A.T. Martínez (✉)
Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain
e-mail: ATMartinez@cib.csic.es

P. Ferreira
Department of Biochemistry and BIFI, University of Zaragoza, Pedro Cerbuna 12, 50009 Zaragoza, Spain

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_12

PEF Poly(ethylene furandicarbolylate)
UPO Unspecific peroxygenase
VP Versatile peroxidase

12.1 Introduction to AAO

Aryl-alcohol oxidase (AAO) is a fungal enzyme secreted by different fungi, including basidiomycetes involved in the degradation of lignocellulose. It belongs to the glucose-methanol-choline oxidase/dehydrogenase (GMC) protein superfamily (Cavener 1992) that includes oxidoreductases, although there are exceptions such as hydroxynitrile lyase, with different substrate specificities. They all share a common fold in two subunits (the cofactor-binding and substrate-binding ones) and the presence of FAD as a cofactor.

AAO activity was reported during the 1960s in cultures of the lignicolous fungus *Trametes versicolor* by Farmer et al. (1960), although this enzyme was not further characterised. It was during the late 1980s and early 1990s that AAO was isolated from several Agaricales species, such as *Pleurotus sajor-caju* (Bourbonnais and Paice 1988), *Pleurotus eryngii* (Guillén et al. 1990) and *Pleurotus ostreatus* (Sannia et al. 1991), and identified as the source of the H_2O_2 (Guillén et al. 1994) and aromatic aldehydes found in cultures of the above fungi (Guillén and Evans 1994; Gutiérrez et al. 1994). During subsequent years, its involvement in the ligninolytic process was demonstrated. AAO is responsible, along with other GMC oxidoreductases and copper-radical oxidases, for the H_2O_2 production that, as described below, is required by: (i) the activity of ligninolytic peroxidases; and (ii) the formation of oxygen radicals that exert oxidative and depolymerising activities on polysaccharides and also on lignin.

Some AAOs have been exhaustively characterized, being those of *P. eryngii* (Ferreira et al. 2005; Guillén et al. 1992) and *Bjerkandera adusta* (de Jong et al. 1994; Romero et al. 2009) two remarkable examples. Their substrate specificity showed to be broad, probably due to its extracellular and ligninolytic nature, since it proved to use secondary fungal metabolites, *p*-methoxybenzyl alcohol and other benzylic alcohols, as substrates. In fact, it is currently known that AAO can oxidise both phenolic and non-phenolic aryl-alcohols, together with other polyunsaturated (aliphatic) primary alcohols, to their corresponding aldehydes, as well as aromatic secondary alcohols, albeit with much lower efficiency (Table 12.1). Moreover, the presence of low amounts of acids attributed to its activity in some fungal cultures (and in vitro reactions) paved the way for the demonstration of its activity on aryl aldehydes (Ferreira et al. 2010).

This plethora of AAO substrates (Table 12.1), among which several reduced species derived from lignin decay products are noticeable, makes it a promising biocatalyst. The lignocellulosic materials are the main renewable resource on Earth

Table 12.1 Comparison of the catalytic efficiencies of *P. eryngii* and *B. adusta* AAO oxidising representative alcohol and aldehyde substrates

		k_{cat}/K_m (s^{-1} mM^{-1})	
		P. eryngii	*B. adusta*
Benzyl alcohol		47 ± 9	18 ± 1
p-Fluorobenzyl alcohol		59 ± 6	22 ± 2
m-Fluorobenzyl alcohol		13 ± 1	47 ± 2
p-Chlorobenzyl alcohol		398 ± 32	361 ± 15
m-Chlorobenzyl alcohol		203 ± 4	1050 ± 30
p-Methoxybenzyl alcohol		5230 ± 620	646 ± 45
m-Methoxybenzyl alcohol		65 ± 24	349 ± 8
Veratryl alcohol		210 ± 5	22 ± 1
Isovanillyl alcohol		152 ± 5	51 ± 1
Vanillyl alcohol		0	31 ± 1
3-Chloro-*p*-methoxybenzyl alcohol		4090 ± 200	1480 ± 110
2-4-Hexadien-1-ol		1270 ± 60	186 ± 7
Cinnamyl alcohol		78 ± 11	305 ± 11
Coniferyl alcohol		0	5 ± 0.2
m-Chlorobenzaldehyde		0.64 ± 0.05	1 ± 0.09
3-Chloro-*p*-methoxy-benzaldehyde		0.085 ± 0.006	0.050 ± 0.002
m-Fluorobenzaldehyde		0.40 ± 0.02	0.05 ± 0.005
p-Nitrobenzaldehyde		0.31 ± 0.006	0.010 ± 0.001

(continued)

Table 12.1 (continued)

		k_{cat}/K_m (s^{-1} mM^{-1})	
		P. eryngii	*B. adusta*
5-Hydroxymethyl-2-furfural		0.21 ± 0.02	n.d.
2,5-Diformylfuran		0.150 ± 0.008	n.d.

n.d. not determined

because they are widespread and abundant (forests cover 27 % of world's area) and, thus, they are cheap and can be easily stored. As a consequence, the conversion of these materials into biofuels in biorefineries is of great interest. However, not only biofuels, that is heat and power, are important, but other by-products obtained during the biorefinery processes are also being carefully examined aiming at using them for the production of valorised chemicals (Bozell and Petersen 2010). AAO is a candidate for the enzymatic delignification of plant biomass (in synergy with other oxidoreductases) and for the production of aromatic aldehydes and acids that can originate from this renewable resource.

In this chapter, AAO involvement in lignocellulose decay owing to its capacity to produce H_2O_2, as well as its potential applications in different industrial processes, are discussed.

12.2 AAO and Lignocellulose Decay

Lignocellulosic biomass (in woody and nonwoody vascular plants) accounts for the 60 % of the total carbon fixed by land photosynthesis, and is constituted of three main polymers: cellulose, hemicellulose and lignin (Higuchi 1997). Both cellulose and lignin are the two most abundant polymers on Earth. Lignin is located in the middle lamella, where it attains its highest concentration, and the secondary wall of vascular plants, together with the above polysaccharides. Its main functions are conferring rigidity to the plants, waterproofing vessels and providing protection against desiccation, pathogens and irradiation (Gellerstedt and Henriksson 2008). Since lignin is very recalcitrant, it constitutes the main sink of carbon in land ecosystems and, as a consequence, its degradation is a key step to complete the carbon cycle. In fact, before it started to be biodegraded and mineralised, at the end of the Carboniferous period (~300 million year ago), the carbon that the plants fixed accumulated and therefore gave rise to the coal deposits we currently use as source of fossil fuels (Floudas et al. 2012).

The only organisms capable of degrading wood are some saprotrophic agaricomycetes, feeding on the simple sugars produced when cellulose and

hemicelluloses are hydrolysed. However, to accomplish this task, fungi must overcome a very recalcitrant barrier: lignin. Lignin is an amorphous polymer including a variety of bonds established among the phenylpropanoid units that form its structure. Although these fungi do not use lignin as a nutrient, they have to remove or modify lignin to gain access to carbohydrates. The development of an extracellular system capable of altering the structure of lignin was, thus, a great achievement in evolutionary and ecological terms, since the polymer is mineralised and the carbon "sequestered" in it may go back to the atmosphere as CO_2. It is generally accepted that the first lignin-degrading organism must have been a basidiomycete (Floudas et al. 2012), which developed some strategies to act on the recalcitrant lignin polymer oxidising its subunits, causing bond breakages and releasing the carbohydrates embedded within its matrix.

The role of H_2O_2 in lignocellulose decay was studied as it proved to be produced simultaneously with the ligninolytic system. It was seen that the addition of catalase, an enzyme that degrades H_2O_2, to cultures of the white-rot fungus *Phanerochaete chrysosporium* diminished its lignin-degrading ability (Faison and Kirk 1983). Two families of oxidoreductases include oxidases being able to produce this strong oxidant: copper-radical oxidases and GMC oxidoreductases.

The copper-radical oxidases are proteins that have one copper ion and a protein radical involved in catalysis, such as extracellular glyoxal oxidase (Kersten and Kirk 1987) and the related enzymes recently identified from sequenced genomes (Kersten and Cullen 2014), among others. GMC oxidoreductases are a superfamily of enzymes that share common structural patterns, including the FAD cofactor and a histidine catalytic base, although they differ in their substrate specificities. Extracellular GMC enzymes involved in the lignocellulose-degradation process are cellobiose dehydrogenase (Ayers et al. 1978; Bao et al. 1993), pyranose 2-oxidase (Daniel et al. 1994), aryl-alcohol oxidase (Guillén et al. 1990) and methanol oxidase (Nishida and Eriksson 1987). The latter enzyme lacks a signal peptide to transport the protein to the extracellular space, but its presence out of the hyphae has been revealed, and it is supposed to be secreted by alternative secretion pathways not known yet (Daniel et al. 2007). Glucose oxidase (Eriksson et al. 1986) is an intracellular enzyme, so its role in lignocellulose degradation is controversial, since a transport system for the H_2O_2 formed would be required in order that it could carry out functions in the extracellular medium.

AAO activity has been reported in different fungi including *T. versicolor* (Farmer et al. 1960), *Fusarium solani* (Iwahara et al. 1980), *Rigidoporus microporus* (Waldner et al. 1988), *Pleurotus* species (Bourbonnais and Paice 1988; Guillén et al. 1992; Sannia et al. 1991), *B. adusta* (Kimura et al. 1990; Muheim et al. 1990b; Romero et al. 2009, 2010) and *Botrytis cinerea* (Goetghebeur et al. 1993); and the corresponding genes have been identified in many basidiomycete genomes (Ferreira et al. 2015; Floudas et al. 2012; Hernández-Ortega et al. 2012a), as described below. Further evidence of the simultaneous expression of AAO along with enzymes typically expressed under ligninolytic condition such as lignin peroxidase (LiP) in *B. adusta* (Muheim et al. 1990a) and manganese-oxidising peroxidases in *Pleurotus* cultures (Camarero et al. 1996), supported the involvement of this oxidase in lignin

degradation. Moreover, the enzyme was located in the hyphal sheath (which is formed by secreted fungal polysaccharide) during lignocellulose degradation (Barrasa et al. 1998), as it had been previously reported for ligninolytic peroxidases and laccases (Gallagher et al. 1989; Green et al. 1992).

The activity of AAO is supported by both non-phenolic and phenolic aryl alcohols that can derive from fungal metabolism (de Jong et al. 1994; Gutiérrez et al. 1994) and from lignin degradation (Kirk and Farrell 1987; Shimada and Higuchi 1991) and are substrates for this enzyme in cooperation with related dehydrogenases. It was seen that H_2O_2 is produced when adding aromatic alcohols, as well as the corresponding aldehydes and acids, to the mycelium of *P. eryngii* (Guillén et al. 1994). Thus, it was postulated that the redox cycling of these compounds results in a continuous supply of H_2O_2. The product of the reaction with AAO (aldehyde or acid) needs to be reduced in order to be re-used by the enzyme. Therefore, it is hypothesised that the oxidised species is transported to the intracellular space, where aryl-alcohol or aryl-aldehyde dehydrogenases, both NADPH-dependent enzymes, convert it into alcohol again (de Jong et al. 1994; Guillén and Evans 1994). Supply of reduced NADPH must be supported by the high carbon availability for a fungus under ligninolytic conditions (Fig. 12.1).

P. eryngii AAO's preferential substrate is *p*-methoxybenzyl alcohol (Table 12.1), which is a secondary metabolite detected in fungal cultures grown

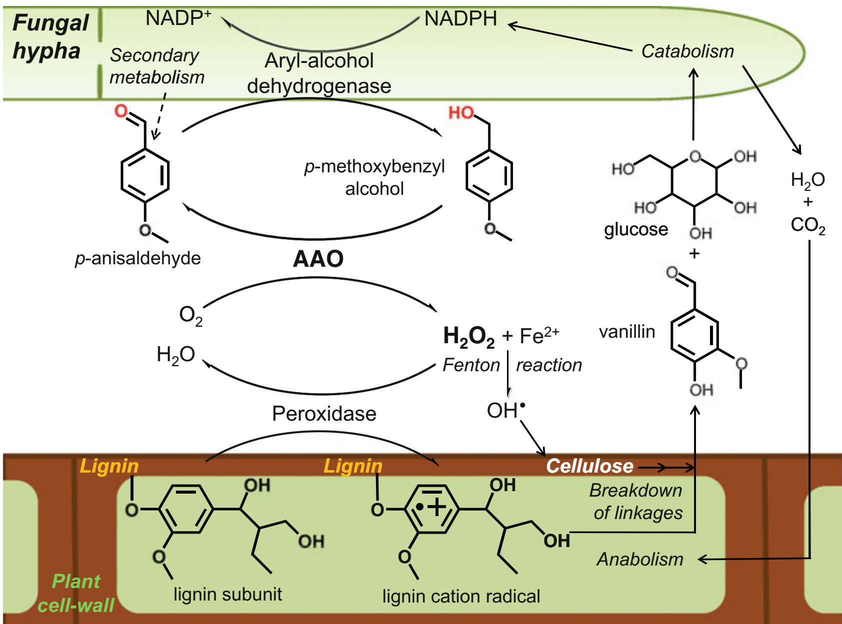


Fig. 12.1 Scheme of the natural role of AAO in decay of plant cell-wall producing H_2O_2 for: (i) activation of lignin-degrading peroxidases; and (ii) formation of cellulose-depolymerising hydroxyl radical (also causing lignin oxidation)

both in glucose and lignin media (Gutiérrez et al. 1994). It is known that aromatic alcohols, aldehydes and acids are derived from the shikimic acid pathway of fungal secondary metabolism (Turner and Aldridge 1983). It was seen that the oxidised product, *p*-methoxybenzaldehyde (also known as *p*-anisaldehyde), was much more abundant in the *Pleurotus* cultures than its alcoholic counterpart (Gutiérrez et al. 1994). High levels of related compounds, such as 3-chloro-*p*-anisaldehyde, were also found to be abundant in *B. adusta* cultures (de Jong et al. 1992) and agree with catalytic efficiencies towards the corresponding alcohol for *B. adusta* AAO, for which seems to be the preferential substrate (Table 12.1). These chlorinated derivatives are also minor extracellular metabolites in *P. eryngii* and *P. ostreatus* (Gutiérrez et al. 1994; Okamoto et al. 2002).

These findings further supported the hypothesis postulating that AAO was acting in the extracellular space involved in the said redox-cycling process for H_2O_2 production.

Based on macroscopic and chemical composition features, the wood-degrading processes were split into two different types involving different decay mechanisms. Some fungi leave a whitish residue, and thus were named white-rot fungi, while others produce a brown residue and were called brown-rot fungi (Martínez et al. 2005; Schwarze et al. 2000; Zabel and Morrell 1992). Owing to genomic and enzymatic studies, it is now thought that AAO could act as an auxiliary enzyme in both processes due to its H_2O_2-producing activity and presence in the sequenced genomes of Agaricomycotina responsible for the two types of wood-decay processes.

12.2.1 White-Rot Decay

White-rot fungi developed an enzymatic machinery to degrade lignin and, hence, be able to use cellulose and hemicellulose as a source of carbon and energy. The main enzymes involved are metalloproteins including LiP, manganese peroxidase (MnP) and versatile peroxidase (VP), as well as laccases (Ruiz-Dueñas and Martínez 2009). Ligninolytic peroxidases are heme proteins that use H_2O_2 as the electron-accepting substrate to oxidise the lignin units. In contrast, laccases have copper as a cofactor, use O_2 as electron acceptor, and are thought to often act through small intermediate compounds, redox mediators, which in turn oxidise lignin. Since most enzymes cannot penetrate the intricate and compact structure of sound wood, small chemical oxidisers, activated oxygen species (as hydroxyl radical), metal cations (as Mn^{3+} produced by MnP and VP) and aromatic radicals (formed by different oxidoreductases) are probably responsible for the first stages of lignin decay (Evans et al. 1994). In white-rot decay there is a need of a continuous H_2O_2 flow in the extracellular environment in order that peroxidases be able to act on lignin. Moreover, hydroxyl radical can be produced through Fenton reaction between Fe^{2+} and H_2O_2, and participate in the oxidative modification of cellulose

(as described below for brown-rot decay) and also of lignin (Fig. 12.1) (Bes et al. 1983; Forney et al. 1982; Gómez-Toribio et al. 2009).

White-rot fungi can be classified into two groups according to their gross degradation patterns. Some of them degrade lignin and cellulose simultaneously as it is the case for *P. chrysosporium*, one of the most studied lignin-degrading organisms. Instead, others, such as *Ceriporiopsis subvermispora*, degrade lignin before cellulose (Otjen and Blanchette 1986). The main differences between the sequenced genomes of these two fungi appear to be related to (i) the peroxidase repertoire, and (ii) the genes involved in lipid metabolism (Fernández-Fueyo et al. 2012). The latter is related to the fact that free radicals from unsaturated lipids are supposed to also play a role in lignin attack (Bao et al. 1994). Selective white-rot fungi are the most interesting for industrial (biotechnological) applications in which carbohydrates are the raw material, since they release cellulose from the lignin matrix without significantly consuming it.

The analysis of genomes of white-rot fungi in which AAO appeared to be produced, along with peroxidases, further confirmed its involvement in the white-rot process as an auxiliary enzyme producing H_2O_2. For instance, Floudas et al. (2012) analysed 24 basidiomycete genomes to search for enzymes involved in the degradation of lignin. On the one hand, all white-rot genomes studied possessed AAO genes with the only exception of *Auricularia delicata*. On the other hand, AAO appears to be the most common H_2O_2-producing GMC, since methanol oxidase genes are not as abundant, glucose oxidase genes are absent, and pyranose 2-oxidase genes are only found in two of the genomes studied (Table 12.2).

Table 12.2 Inventory of peroxidase and GMC genes in eleven white-rot Agaricomycotina genomes

		AD	PST	FM	DS	TV	SH	BA	PB	PC	GS	CS
Peroxidases		21	21	33	21	39	11	34	16	18	14	25
GMCs	AAO	0	4	2	9	3	14	11	3	3	9	4
	CDH	1	1	1	1	1	1	1	1	1	1	1
	MOX	3	3	2	4	4	7	5	6	3	4	1
	P_2O	2	0	0	0	2	0	1	1	1	0	0

From Floudas et al. (2012) and Ruiz-Dueñas et al. (2013) and Ferreira et al. (2015)

AAO, aryl-alcohol oxidase; CDH, cellobiose dehydrogenase; MOX, methanol oxidase and P2O, pyranose 2-oxidase

AD, *Auricularia delicata*; PST, *Punctularia strigosozonata*; FM, *Fomitiporia mediterranea*; DS, *Dichomitus squalens*; TV, *Trametes versicolor*; SH, *Stereum hirsutum*; BA, *Bjerkandera adusta*; PB, *Phlebia brevispora*; PC, *Phanerochaete chrysosporium*; GS, *Ganoderma* sp. in the *Ganoderma lucidum* complex and CS, *Ceriporiopsis subvermispora*

12.2.2 Brown-Rot Decay

The brown-rot decay is based on a non-enzymatic attack on wood that leads to lignin modification, instead of degradation/mineralisation and cellulose depolymerisation. On having no longer the machinery for degrading lignocellulose (Table 12.3), brown-rot fungi are the only organisms known to be able to nearly completely remove all polysaccharides from wood without causing substantial lignin degradation (Arantes et al. 2012). Nevertheless, it has been demonstrated that they do alter lignin in order to gain access to the carbohydrates leaving it partially modified but still polymeric and with a recognisable chemical structure (Martínez et al. 2011). Genomic studies have proved that these fungi stem from the white-rot lineage, and that they have lost most of the energy-consuming apparatus their white-rot ancestors used to have, which means that peroxidases are absent or residual in their genomes (only one generic peroxidase gene in some species) (Ruiz-Dueñas et al. 2013). They have originated independently in several lineages and have representatives in five basidiomycete orders (Floudas et al. 2012).

The initial oxidative step alters the plant cell-wall structure in order to make it more accessible to enzymes involved in subsequent decay (Goodell et al. 1997). Reactive oxygen species, such as hydroxyl radical, generated by Fenton chemistry, play a key role in this oxidative process (Fig. 12.1) (Baldrian and Valaskova 2008; Halliwell 1965). Fenton chemistry is based on reactions among H_2O_2 and Fe^{2+} ions giving rise to hydroxyl free radical. This is a radical-chain mechanism where Fe^{2+} is generated from Fe^{3+} in a cycle that involves fungal enzymes. The hydroxyl radical is the most powerful (non-specific) oxidant in biological systems but it has an extremely short half-life (10^{-9} s) and, as a consequence, Fenton reactions need occur immediately adjacent to the site of oxidative action within the plant cell wall because of spatial diffusion limitations (Arantes et al. 2012).

Since one of the most remarkable chemical alterations that brown-rot decay cause to lignin is demethoxylation (Niemenmaa et al. 2008; Yelle et al. 2008), which ultimately gives rise to methanol, methanol oxidase is thought to be the most important H_2O_2-producing enzyme in brown-rot decay. Nevertheless, genomic

Table 12.3 Inventory of peroxidase and GMC genes (see Table 12.2 for enzyme abbreviations) in six brown-rot Agaricomycotina genomes

		CP	GT	FP	WC	DSP	RP
Peroxidases		0	0	1	1	0	1
GMCs	AAO	0	2	1	0	0	2
	CDH	1	1	0	0	0	0
	MOX	2	1	4	4	1	4
	P_2O	0	0	0	0	0	0

From Floudas et al. (2012) and Ferreira et al. (2015)
CP, *Coniophora puteana*; GT, *Gloeophyllum trabeum*; FP, *Fomitopsis pinicola*; WC, *Wolfiporia cocos*; DSP, *Dacryopinax* sp. and RP, *Rhodonia placenta*

studies have shown that brown-rot fungi have AAO too (Martinez et al. 2009), suggesting that the latter oxidase might play a role in Fenton chemistry. Floudas et al. (2012) analysed seven brown-rot fungal genomes and found AAO genes in several of them, although they were not as abundant as those of methanol oxidases (Table 12.3).

12.3 Paper Pulp Industry: Biopulping and Biobleaching

One of the main steps of pulp and paper industry is the treatment of wood chips to separate cellulosic fibres (the actual raw material used) from the lignin forming the middle lamella, a process called pulping. The concern about environment and energy wasting has stimulated studies on the use of microorganisms to accomplish this task. The resulting biopulping process is thought to be energetically and environmentally more favourable than chemical and/or mechanical treatments (Blanchette et al. 1992; Rasmussen et al. 2010).

The biopulping process starts with the colonisation of wood xylem and parenchyma by the fungi. After hyphae have grown on the substrate, the fungus will start producing its ligninolytic enzymatic system in order to degrade the middle lamellae and separate fibres (Breen and Singleton 1999). Since the loss of cellulose is undesirable, the organisms of choice are white-rot fungi showing preference for lignin degradation rather than cellulose degradation, whose task is to make cellulosic fibres accessible for the papermaking process (Scott and Swaney 1998). Several selective ligninolytic fungi, such as *C. subvermispora* and *P. eryngii*, together with the model white-rot fungus *P. chrysosporium*, and brown-rot fungi such as *Postia placenta*, among others, have been investigated for biopulping of wood and annual plants (Akhtar et al. 1997; Camarero et al. 1998; Ferraz et al. 2008; Giles et al. 2014; Masarin et al. 2009; Vicentim et al. 2009). AAO is supposed to act as an auxiliary enzyme providing H_2O_2 in these processes, as it is the case of natural lignin degradation.

Pulp bleaching, which is the removal of chromophores in order to obtain white paper pulp, is another process in which AAO has shown to contribute. In a study in which two flax pulps were treated with fungal enzymes—laccases, peroxidases and feruroyl esterases—the ability of AAO from *Pleurotus pulmonarius* CBS 507.85, which is a natural hyperproducer of this enzyme, to aid in this process was tested (Sigoillot et al. 2005). The results showed that the presence of AAO along with laccase improved the bleaching process probably due to the ability of AAO to prevent the repolymerisation of the phenoxy radicals released by laccases by using them as electron acceptors (as an alternative for O_2). In a similar way, AAO can be combined with ligninolytic peroxidases, in the presence of a substrate enabling it to release the H_2O_2 required by the former enzymes.

12.4 Bioconversions

12.4.1 Flavour Synthesis

White-rot fungi are among the most versatile flavour and aroma producers in nature (Fraatz and Zorn 2011; Lapadatescu et al. 2000). These compounds are mainly of aromatic nature and synthesised through biotransformations by plant, enzymatic or microbial processes (Serra et al. 2005). Since there exists demand for naturally produced compounds, the biotechnological production of flavours and aromas attracts much attention (Krings and Berger 1998) due to the great economic importance this industry has.

One of the most important flavours is vanillin, naturally produced by orchids of the *Vanilla* genus, but this source only represents the 1 % of the commercial vanilla flavour. As a consequence, several methods of obtaining vanillin have been developed (Priefert et al. 2001) that use bioconversion of lignin and phenyl-propanoids, such as eugenol (Overhage et al. 2003). The ability of *B. adusta* AAO to oxidise vanillyl alcohol reported by Romero et al. (2009), could be exploited in the biotechnological production of this flavour. It has been suggested that AAO may be used to avoid formation of vanillyl alcohol as a by-product diminishing the yield of vanillin formation (Fig. 12.2a) by the fungi of the genus *Pycnoporus* (Lomascolo et al. 2011).

Fig. 12.2 Catalytic efficiencies of *P. eryngii* and *B. adusta* AAOs in bioconversion of some flavours and aromas. **a** Oxidation of vanillyl alcohol into vanillin. **b** Oxidation of benzyl alcohol into benzaldehyde. **c** Oxidation of *p*-methoxybenzyl alcohol into *p*-methoxybenzaldehyde

Another very remarkable commercial aromatic compound is benzaldehyde. Some research has been carried out using the fungus *B. adusta* for the production of this chemical from L-phenylalanine (Lapadatescu et al. 2000; Lapadatescu and Bonnarme 1999). In this reaction of L-phenylalanine for flavour production, aryl-alcohols are the main products obtained, as reported for *B. adusta* (Lapadatescu et al. 2000) and *P. chrysosporium* (Jensen et al. 1994). Consequently, the application of AAO to such biotransformations could result in the obtention of higher levels of aromatic aldehydes, since their alcohol counterparts are substrates of the enzyme (Fig. 12.2b). Finally, using AAO plus an unspecific peroxygenase (UPO) from the fungus *Agrocybe aegerita* (Ullrich et al. 2004), an oxidoreductase cascade can be used for toluene conversion into benzaldehyde, with the second enzyme using the peroxide generated by AAO.

Another flavour that AAO produces as a consequence of its auxiliary role in lignin degradation is *p*-anisaldehyde (4-methoxybenzaldehyde) (Fig. 12.2c). It was shown that the corresponding alcohol is the physiological and preferential substrate of the *P. eryngii* enzyme (Ferreira et al. 2005; Guillén et al. 1992) and that AAO and mycelium-associated aromatic dehydrogenases establish a concerted anisaldehyde redox cycle to produce H_2O_2 continuously as described above (Fig. 12.1). Therefore, AAO can be used for biotransformations aiming at the production of vanillin, benzaldehyde, anisaldehyde and other aromas.

12.4.2 Deracemization of Chiral Secondary Alcohols

Many of the drugs and potential drug candidates possess chiral centres and most of them need to be commercialised as enantiomers rather than racemates given that enantiomers often carry out different activities within biological systems (Carey et al. 2006). Therefore, chiral intermediates for pharmaceuticals are synthesised through enantioselective asymmetric reactions (Patel 2013). As an alternative, deracemization of chiral mixtures is used by the pharmaceutical industry to obtain pure enantiomers. Among chiral compounds, some secondary alcohols are used as chiral intermediates and analytical reagents, and the development of synthesis procedures for the production of enantiomerically enriched (enantioenriched) alcohols has gained importance in the pharmaceutical industry.

Biological systems are generally chiral and, as a consequence, many enzymes are regio- and enantioselective. These properties are regarded to be a consequence of the active sites' architecture and the enzyme's mechanism. Therefore, many microorganisms and enzymes offer attractive alternatives for easy production (asymmetrical synthesis or deracemization) of enantiomeric compounds of interest in the fine chemicals and pharmaceutical sectors (de Albuquerque et al. 2015; Matsuda et al. 2009). The AAO catalytic mechanism consists in a hydride abstraction from the benzylic position of the alcohol by the oxidised flavin, in a reaction aided by an active-site histidine acting as a catalytic base to form the alkoxide intermediate (Hernández-Ortega et al. 2012a). Due to active site

architecture, and the simultaneous nature of the hydride and proton abstractions, hydride transfer by AAO is stereoselective (only takes place from the pro-*R* position) as shown using the two α-monodeuterated enantiomers of *p*-methoxybenzyl alcohol (Hernández-Ortega et al. 2012b). Taking advantage of this information, Hernández-Ortega et al. (2012b) assayed the transformation of racemic secondary alcohols using *P. eryngii* AAO. They saw that the enzyme was able to oxidise the racemic 1-(*p*-methoxyphenyl)-ethanol, although it showed an apparent efficiency orders of magnitude smaller than the one for *p*-methoxybenzyl alcohol. In this reaction, AAO enantioselectivity towards the (*S*) isomer was shown using chiral HPLC, which allows for the isolation of (*R*) isomer from chiral mixtures (Fig. 12.3). Moreover, the AAO enantioselectivity towards another secondary alcohol 1-(*p*-fluorophenyl)-ethanol was estimated as a *S*/*R* ratio of 21, in reactions using the individual enantiomers.

Therefore, it is plausible that AAO could be used for the isolation of isomers from racemates taking advantage of its kinetic behaviour. However, AAO activity on secondary alcohols is low, due to some hindrances among such substrates and the residues forming the active site (Fernández et al. 2009). A mutated variant (F501A), in which the side chain of a bulky aromatic residue was removed to make room in the cavity, was created with the purpose of facilitating oxidation of secondary alcohols. The removal of this side chain resulted in a stereoselectivity *S*/*R* ratio on 1-(*p*-fluorophenyl)-ethanol three-fold higher than that of the wild-type enzyme. Hence, improved variants by directed evolution, as it has been done with galactose oxidase for these purposes (Escalettes and Turner 2008), or further site-directed mutagenesis would result in better deracemization reactions.

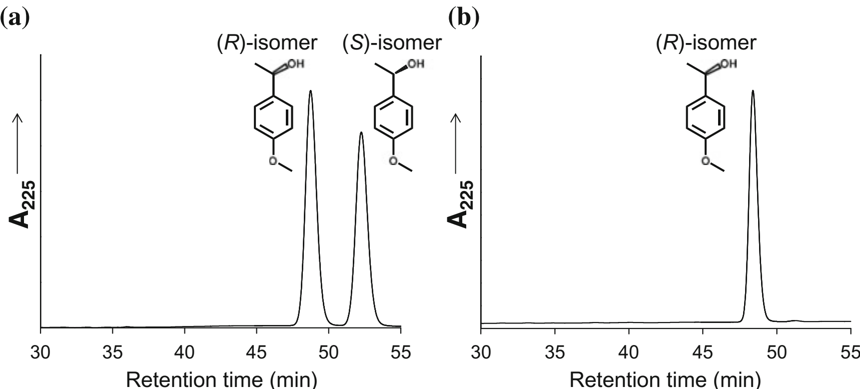


Fig. 12.3 Chiral HPLC chromatograms showing the deracemization of 1-(*p*-methoxyphenyl)-ethanol by *P. eryngii* AAO. **a** Chromatogram of the untreated racemate. **b** Chromatogram after 24-h reaction with AAO, where only the peak of the (*R*) isomer is detected (the peak of the *p*-methoxyacetophenone, formed from oxidation of the (*S*) isomer, eluted in a different region of the chromatogram)

12.4.3 Oxidation of Furfurals

Another remarkable class of chemicals derived from lignocellulosics, which are substrates of AAO, is furfurals. They are formed as by-products of the pretreatment of lignocellulosics in the paper pulp and the bioethanol industries. In spite of the fact that they imply the loss of raw material in such processes, they are now regarded as valorised by-products in biorefinery processes. Moreover, 5-hydroxymethylfurfural (HMF hereinafter) is commercially produced from the dehydration of hexoses, mainly fructose, (Karinen et al. 2011) but direct production from glucose is also possible (Zhao et al. 2007).

HMF is regarded as a chemical building block due to the presence of several functionalities within the molecule (furan ring and hydroxyl and carbonyl groups) (Rosatella et al. 2011), which may undergo chemical reactions that give rise to a bunch of compounds: phenolic resins, bioplastics, Schiff bases, polyurethane foams of epoxy resins, as well as disubstituted furan derivatives (Moreau et al. 2004). The obtention of 2,5-furandicarboxylic acid (FDCA hereinafter) from HMF (Fig. 12.4) is of great interest since FDCA can polymerise to give rise to polyesters that may substitute for those produced from fossil fuels. Such polymers are called poly (ethylene furandicarbolylate) polyesters (PEFs) and show good mechanical and barrier properties, are biodegradable and come from renewable resources (Papageorgiou et al. 2014).

Several processes involving enzymes and organisms have been reported aiming at the obtention of FDCA from HMF (Carro et al. 2015; Dijkman et al. 2014, 2015; Dijkman and Fraaije 2014; Hanke 2012; Koopman et al. 2010; van Deurzen et al. 1997). Regarding the use of AAO with this purpose, Hanke (2012) screened for available AAOs capable of oxidising HMF with variable results. With the same purpose, Carro et al. (2015) analysed the ability of *P. eryngii* AAO to oxidise HMF.

Fig. 12.4 Oxidative pathways from HMF (**1**) to FDCA (**5**). FFCA (**4**) formation can take place through two alternative intermediate compounds: DFF (**2**) or HMFCA (**3**)

In this way, it proved to be catalytically active towards HMF and to be able to catalyse two subsequent oxidations in its molecule giving rise to 5-formylfurancarboxylic acid (FFCA, hereinafter; 84 %) and FDCA (6 %) in 24 h. When they tested the ability to oxidise the intermediates between HMF and FDCA, AAO proved to be catalytically active on diformylfuran (DFF), producing 86 % of conversion into FFCA and a 14 % into FDCA. However, it did not react either with 5-hydroxymethylfurancarboxylic acid (HMFCA) or with FFCA. NMR studies of the compounds involved in this chemical pathway demonstrated that carbonyl groups of both DFF and FFCA were hydrated in aqueous medium giving rise to diols in 53 and 8 % of abundance, respectively. These *gem*-diols resulting from the hydration of aldehydes had been reported to be substrates of the *P. eryngii* AAO (Ferreira et al. 2010). However, HMF underwent no hydration, which agrees with AAO being active towards DFF (formed by oxidation of the HMF hydroxyl group), but not towards HMFCA, which would be formed by a prior oxidation of the HMF carbonyl group. Such results suggest that the oxidative pathway for the oxidation of HMF to FFCA catalysed by this AAO proceeded via DFF. Although this enzyme was not able to oxidise FFCA, this constraint was overcome through the application of a sequential enzymatic cascade together with UPO (Ullrich et al. 2004). This enzyme used the H_2O_2 produced by AAO to introduce a hydroxyl group in the formyl group of FFCA, thus producing FDCA, attaining 91 % of conversion after 120 h (Fig. 12.5).

These findings paved the way to implement AAO together with the peroxygenase to obtain a valorised by-product originated from lignocellulosic biomass. Furthermore, they widened the spectrum of its aromatic substrates, which is now thought to range from benzylic carbocycles to heterocycles, as HMF and DFF.

Fig. 12.5 Sequential enzymatic cascade for the production of FDCA (**5**) from HMF (**1**) using *P. eryngii* AAO and *A. aegerita* UPO *via* DFF (**2**) and its hydrated *gem*-diol counterpart (**2′**) (abundance of DFF and its *gem*-diol at equilibrium shown as percentage), and FFCA (**4**). AAO catalytic efficiencies are indicated

12.5 Conclusions

The finite character of fossil fuels makes it necessary to find new resources, preferentially renewable ones, which allow for the substitution of the classical resources. In this way, lignocelluloses are a remarkable material due to their ubiquitous and renewable character. Furthermore, the concern about the environment impels us to search for new catalytic procedures that take advantage from natural processes, instead of the chemical environmentally polluting and energy-wasting ones. Hence, the use of organisms or enzymes to carry out catalytic industrial processes is gaining importance.

AAO is a very promiscuous enzyme that catalyses the oxidation of a great deal of polyunsaturated alcohols (and hydrated aldehydes). Its applicability on some biotechnology-based industrial processes has been reviewed here and it has showed to be promising as a biocatalyst in several conversions. AAO, because of its involvement in the lignocellulose decay process, has potential to be applied to lignocellulose biorefineries. AAO has so far shown its potential applications in paper pulp manufacture and bioconversion of lignocellulose-derived compounds (such as HMF), synthesis of flavours, and deracemization of chiral alcohols (Fig. 12.6). In spite of the fact that the use of enzymes (among which AAO) for these purposes is not yet sufficiently developed, much progress has been done the recent years in this field as one can infer from the huge amount of publications available on enzymatic biocatalysis.

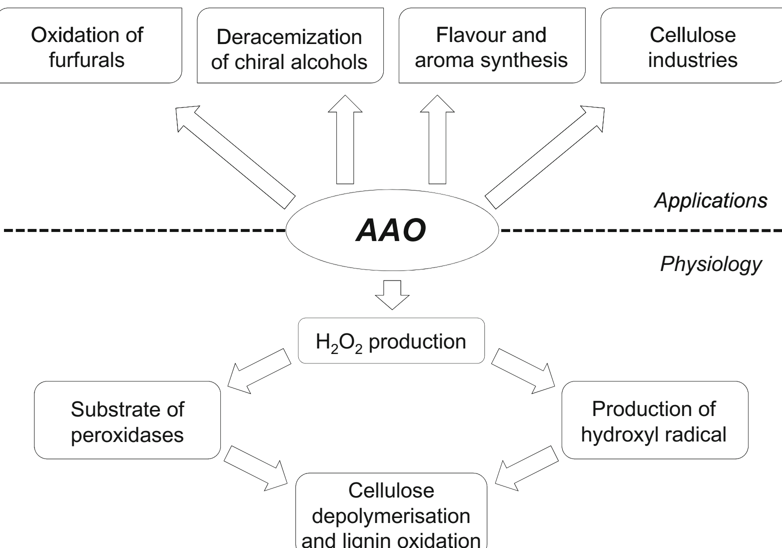


Fig. 12.6 Scheme depicting the physiological role of AAO in lignocellulose degradation and its potential applications in the cellulose and other chemical sectors

Acknowledgments This work was supported by the HIPOP (BIO2011-26694) and the NOESIS (BIO2014-56388-R) projects of the Spanish Ministry of Economy and Competitiveness, and the INDOX (KBBE-2013-7-613549) and EnzOx2 (H2020-BBI-PPP-2015-RIA-720297) European projects. J.C. acknowledges a FPU fellowship of the Spanish Ministry of Education, Culture and Sport.

References

Akhtar M, Blanchette RA, Kirk TK (1997) Fungal delignification and biomechanical pulping of wood. In: Scheper T (ed) Advances in biochemical engineering/biotechnology. Springer, Berlin, pp 160–195

Arantes V, Jellison J, Goodell B (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol 94:323–338

Ayers AR, Ayers SB, Eriksson K-E (1978) Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*. Eur J Biochem 90:171–181

Baldrian P, Valaskova V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501–521

Bao WJ, Usha SN, Renganathan V (1993) Purification and characterization of cellobiose dehydrogenase, a novel extracellular hemoflavoenzyme from the white-rot fungus *Phanerochaete chrysosporium*. Arch Biochem Biophys 300:705–713

Bao WL, Fukushima Y, Jensen KA, Moen MA, Hammel KE (1994) Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett 354:297–300

Barrasa JM, Gutiérrez A, Escaso V, Guillén F, Martínez MJ, Martínez AT (1998) Electron and fluorescence microscopy of extracellular glucan and aryl-alcohol oxidase during wheat-straw degradation by *Pleurotus eryngii*. Appl Environ Microbiol 64:325–332

Bes B, Ranjeva R, Boudet AM (1983) Evidence for the involvement of activated oxygen in fungal degradation of lignocellulose. Biochimie 65:283–289

Blanchette RA, Burnes TA, Eerdmans MM, Akhtar M (1992) Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. Holzforschung 46:109–115

Bourbonnais R, Paice MG (1988) Veratryl alcohol oxidases from the lignin degrading basidiomycete *Pleurotus sajor-caju*. Biochem J 255:445–450

Bozell JJ, Petersen GR (2010) Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chem 12:539–554

Breen A, Singleton FL (1999) Fungi in lignocellulose breakdown and biopulping. Curr Opin Biotechnol 10:252–258

Camarero S, Barrasa JM, Pelayo M, Martínez AT (1998) Evaluation of *Pleurotus* species for wheat-straw biopulping. J Pulp Pap Sci 24:197–203

Camarero S, Böckle B, Martínez MJ, Martínez AT (1996) Manganese-mediated lignin degradation by *Pleurotus pulmonarius*. Appl Environ Microbiol 62:1070–1072

Carey JS, Laffan D, Thomson C, Williams MT (2006) Analysis of the reactions used for the preparation of drug candidate molecules. Org Biomol Chem 4:2337–2347

Carro J, Ferreira P, Rodríguez L, Prieto A, Serrano A, Balcells B, Ardá A, Jiménez-Barbero J, Gutiérrez A, Ullrich R, Hofrichter M, Martínez AT (2015) 5-Hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J 282 (16):3218–3229

Cavener DR (1992) GMC oxidoreductases. A newly defined family of homologous proteins with diverse catalytic activities. J Mol Biol 223:811–814

Daniel G, Volc J, Filonova L, Plihal O, Kubátová E, Halada P (2007) Characteristics of *Gloeophyllum trabeum* alcohol oxidase, an extracellular source of H_2O_2 in brown rot decay of wood. Appl Environ Microbiol 73:6241–6253

Daniel G, Volc J, Kubátová E (1994) Pyranose oxidase, a major source of H_2O_2 during wood degradation by *Phanerochaete chrysosporium, Trametes versicolor*, and *Oudemansiella mucida*. Appl Environ Microbiol 60:2524–2532

de Albuquerque NCP, de Gaitani CM, de Oliveira ARM (2015) A new and fast DLLME-CE method for the enantioselective analysis of zopiclone and its active metabolite after fungal biotransformation. J Pharm Biomed Anal 109:192–201

de Jong E, Cazemier AE, Field JA, de Bont JAM (1994) Physiological role of chlorinated aryl alcohols biosynthesized de novo by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl Environ Microbiol 60:271–277

de Jong E, Field JA, Dings JAFM, Wijnberg JBPA, de Bont JAM (1992) De novo biosynthesis of chlorinated aromatics by the white-rot fungus *Bjerkandera* sp. BOS55. Formation of 3-chloro-anisaldehyde from glucose. FEBS Lett 305:220–224

Dijkman WP, Fraaije MW (2014) Discovery and characterization of a 5-hydroxymethylfurfural oxidase from *Methylovorus* sp strain MP688. Appl Environ Microbiol 80:1082–1090

Dijkman WP, Groothuis DE, Fraaije MW (2014) Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid. Angew Chem 126:6633–6636

Dijkman WP, Binda C, Fraaije MW, Mattevi A (2015) Structure-based enzyme tailoring of 5-hydroxymethylfurfural oxidase. ACS Catal 5:1833–1839

Eriksson K-E, Pettersson B, Volc J, Musílek V (1986) Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 23:257–262

Escalettes F, Turner NJ (2008) Directed evolution of galactose oxidase: generation of enantioselective secondary alcohol oxidases. ChemBioChem 9:857–860

Evans CS, Dutton MV, Guillén F, Veness RG (1994) Enzymes and small molecular mass agents involved with lignocellulose degradation. FEMS Microbiol Rev 13:235–240

Faison BD, Kirk TK (1983) Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. Appl Environ Microbiol 46:1140–1145

Farmer VC, Henderson MEK, Russell JD (1960) Aromatic-alcohol-oxidase activity in the growth medium of *Polystictus versicolor*. Biochem J 74:257–262

Fernández IS, Ruiz-Dueñas FJ, Santillana E, Ferreira P, Martínez MJ, Martínez AT, Romero A (2009) Novel structural features in the GMC family of oxidoreductases revealed by the crystal structure of fungal aryl-alcohol oxidase. Acta Crystallogr D Biol Crystallogr 65:1196–1205

Fernández-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P, Larrondo L, James TY, Seelenfreund D, Lobos S, Polanco R, Tello M, Honda Y, Watanabe T, Watanabe T, Ryu JS, Kubicek CP, Schmoll M, Gaskell J, Hammel KE, St. John FJ, Vanden Wymelenberg A, Sabat G, Bondurant SS, Syed K, Yadav J, Doddapaneni H, Subramanian V, Lavín JL, Oguiza JA, Perez G, Pisabarro AG, Ramírez L, Santoyo F, Master E, Coutinho PM, Henrissat B, Lombard V, Magnuson JK, Kües U, Hori C, Igarashi K, Samejima M, Held BW, Barry K, LaButti K, Lapidus A, Lindquist E, Lucas S, Riley R, Salamov A, Hoffmeister D, Schwenk D, Hadar Y, Yarden O, de Vries RP, Wiebenga A, Stenlid J, Eastwood DC, Grigoriev IV, Berka R, Blanchette RA, Kersten P, Martínez AT, Vicuña R, Cullen D (2012) Comparative genomics of *Ceriporiopisis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Natl Acad Sci USA 109:5458–5463

Ferraz A, Guerra A, Mendonça R, Masarin F, Vicentim MP, Aguiar A, Pavan PC (2008) Technological advances and mechanistic basis for fungal biopulping. Enzyme Microb Technol 43:178–185

Ferreira P, Carro J, Serrano A, Martínez AT (2015) A survey of genes encoding H_2O_2-producing GMC oxidoreductases in 10 Polyporales genomes. Mycologia 107:1105–1119

Ferreira P, Hernández-Ortega A, Herguedas B, Rencoret J, Gutiérrez A, Martínez MJ, Jiménez-Barbero J, Medina M, Martínez AT (2010) Kinetic and chemical characterization of aldehyde oxidation by fungal aryl-alcohol oxidase. Biochem J 425:585–593

Ferreira P, Medina M, Guillén F, Martínez MJ, van Berkel WJH, Martínez AT (2005) Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochem J 389:731–738

Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martínez AT, Otillar R, Spatafora JW, Yadav JS, Aerts A, Benoit I, Boyd A, Carlson A, Copeland A, Coutinho PM, de Vries RP, Ferreira P, Findley K, Foster B, Gaskell J, Glotzer D, Górecki P, Heitman J, Hesse C, Hori C, Igarashi K, Jurgens JA, Kallen N, Kersten P, Kohler A, Kües U, Kumar TKA, Kuo A, LaButti K, Larrondo LF, Lindquist E, Ling A, Lombard V, Lucas S, Lundell T, Martin R, McLaughlin DJ, Morgenstern I, Morin E, Murat C, Nolan M, Ohm RA, Patyshakuliyeva A, Rokas A, Ruiz-Dueñas FJ, Sabat G, Salamov A, Samejima M, Schmutz J, Slot JC, St. John F, Stenlid J, Sun H, Sun S, Syed K, Tsang A, Wiebenga A, Young D, Pisabarro A, Eastwood DC, Martin F, Cullen D, Grigoriev IV, Hibbett DS (2012) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336:1715–1719

Forney LJ, Reddy CA, Tien M, Aust SD (1982) The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. J Biol Chem 257:11455–11462

Fraatz MA, Zorn H (2011) Fungal flavours. In: Hofrichter M (ed) The mycota. X industrial applications. Springer, Berlin, pp 249–268

Gallagher IM, Fraser MA, Evans CS, Atkey PT (1989) Ultrastructural localization of lignocellulose-degrading enzymes. In: Lewis NG, Paice MG (eds) ACS symposium “plant cell-wall polymers: biogenesis and biodegradation”, vol 399, American Chemical Society, pp 426–442

Gellerstedt G, Henriksson G (2008) Lignins: major sources, structure and properties. In: Belgacem M, Gandini A (eds) Monomers, polymers and composites from renewable resources. Elsevier, Amsterdam, pp 201–224

Giles RL, Galloway ER, Zackeru JC, Naithani V, Parrow MW (2014) Two stage fungal biopulping solubilizes lignocellulosic carbohydrates without supplemental enzymatic hydrolysis. Int Biodeterior Biodegradation 86:265–271

Goetghebeur M, Brun S, Galzy P, Nicolas M (1993) Benzyl alcohol oxidase and laccase synthesis in *Botrytis cinerea*. Biosci Biotechnol Biochem 57:1380–1381

Gómez-Toribio V, García-Martín AB, Martínez MJ, Martínez AT, Guillén F (2009) Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. Appl Environ Microbiol 75:3944–3953

Goodell B, Jellison J, Liu J, Daniel G, Paszczynski A, Fekete F, Krishnamurthy S, Jun L, Xu G (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. J Biotechnol 53:133–162

Green F III, Clausen CA, Larsen MJ, Highley TL (1992) Immuno-scanning electron microscopic localization of extracellular wood-degrading enzymes within the fibrillar sheath of the brown-rot fungus *Postia placenta*. Can J Microbiol 38:898–904

Guillén F, Evans CS (1994) Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. Appl Environ Microbiol 60:2811–2817

Guillén F, Martínez AT, Martínez MJ (1990) Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. Appl Microbiol Biotechnol 32:465–469

Guillén F, Martínez AT, Martínez MJ (1992) Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. Eur J Biochem 209:603–611

Guillén F, Martínez AT, Martínez MJ, Evans CS (1994) Hydrogen peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase. Appl Microbiol Biotechnol 41:465–470

Gutiérrez A, Caramelo L, Prieto A, Martínez MJ, Martínez AT (1994) Anisaldehyde production and aryl-alcohol oxidase and dehydrogenase activities in ligninolytic fungi from the genus *Pleurotus*. Appl Environ Microbiol 60:1783–1788

Halliwell G (1965) Catalytic decomposition of cellulose under biological conditions. Biochem J 95:35–40

Hanke PD (2012) Enzymatic oxidation of hydroxymethylfurfural. Patent (USA) 8,183,020 B2

Hernández-Ortega A, Ferreira P, Martínez AT (2012a) Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. Appl Microbiol Biotechnol 93:1395–1410

Hernández-Ortega A, Ferreira P, Merino P, Medina M, Guallar V, Martínez AT (2012b) Stereoselective hydride transfer by aryl-alcohol oxidase, a member of the GMC superfamily. ChemBioChem 13:427–435

Higuchi T (1997) Biochemistry and molecular biology of wood. Springer, London

Iwahara S, Nishihira T, Jomori T, Kuwahara M, Higuchi T (1980) Enzymic oxidation of α, β-unsaturated alcohols in the side chains of lignin-related aromatic compounds. J Ferment Technol 58:183–188

Jensen KA Jr, Evans KMC, Kirk TK, Hammel KE (1994) Biosynthetic pathway for veratryl alcohol in the ligninolytic fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 60:709–714

Karinen R, Vilonen K, Niemelä M (2011) Biorefining: heterogeneously catalyzed reactions of carbohydrates for the production of furfural and hydroxymethylfurfural. Chemsuschem 4:1002–1016

Kersten P, Cullen D (2014) Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. Fungal Genet Biol 72:124–130

Kersten PJ, Kirk TK (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J Bacteriol 169:2195–2201

Kimura Y, Asada Y, Kuwahara M (1990) Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl Microbiol Biotechnol 32:436–442

Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ (2010) Efficient whole-cell biotransformation of 5-(hydroxymethyl)furfural into FDCA, 2,5-furandicarboxylic acid. Bioresour Technol 101:6291–6296

Krings U, Berger RG (1998) Biotechnological production of flavours and fragrances. Appl Microbiol Biotechnol 49:1–8

Lapadatescu C, Bonnarme P (1999) Production of aryl metabolites in solid-state fermentations of the white-rot fungus *Bjerkandera adusta*. Biotechnol Lett 21:763–769

Lapadatescu C, Giniès C, Le Quéré J-L, Bonnarme P (2000) Novel scheme for biosynthesis of aryl metabolites from L-phenylalanine in the fungus *Bjerkandera adusta*. Appl Environ Microbiol 66:1517–1522

Lomascolo A, Uzan-Boukhris E, Herpoel-Gimbert I, Sigoillot JC, Lesage-Meessen L (2011) Peculiarities of *Pycnoporus* species for applications in biotechnology. Appl Microbiol Biotechnol 92:1129–1149

Martínez AT, Rencoret J, Nieto L, Jiménez-Barbero J, Gutiérrez A, del Río JC (2011) Selective lignin and polysaccharide removal in natural fungal decay of wood as evidenced by in situ structural analyses. Environ Microbiol 13:96–107

Martínez AT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, del Río JC (2005) Biodegradation of lignocellulosics: microbiological, chemical and enzymatic aspects of fungal attack to lignin. Int Microbiol 8:195–204

Martinez D, Challacombe J, Morgenstern I, Hibbett DS, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Dueñas FJ, Martínez AT, Kersten P, Hammel KE, Vanden Wymelenberg A, Gaskell J, Lindquist E, Sabat G, Bondurant SS, Larrondo LF, Canessa P, Vicuña R, Yadav J, Doddapaneni H, Subramanian V, Pisabarro AG, Lavín JL, Oguiza JA, Master E, Henrissat B, Coutinho PM, Harris P, Magnuson JK, Baker SE, Bruno K, Kenealy W, Hoegger PJ, Kues U,

Ramaiya P, Lucas S, Salamov A, Shapiro H, Tu H, Chee CL, Misra M, Xie G, Teter S, Yaver D, James T, Mokrejs M, Pospisek M, Grigoriev IV, Brettin T, Rokhsar D, Berka R, Cullen D (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA 106:1954–1959

Masarin F, Pavan PC, Vicentim MP, Souza-Cruz PB, Loguercio-Leite C, Ferraz A (2009) Laboratory and mill scale evaluation of biopulping of *Eucalyptus grandis* Hill ex Maiden with *Phanerochaete chrysosporium* RP-78 under non-aseptic conditions. Holzforschung 63:259–263

Matsuda T, Yamanaka R, Nakamura K (2009) Recent progress in biocatalysis for asymmetric oxidation and reduction. Tetrahedron-Asymmetry 20:513–557

Moreau C, Belgacem MN, Gandini A (2004) Recent catalytic advances in the chemistry of substituted furans from carbohydrates and in the ensuing polymers. Top Catal 27:11–30

Muheim A, Leisola MSA, Schoemaker HE (1990a) Aryl-alcohol oxidase and lignin peroxidase from the white-rot fungus *Bjerkandera adusta*. J Biotechnol 13:159–167

Muheim A, Waldner R, Leisola MSA, Fiechter A (1990b) An extracellular aryl-alcohol oxidase from the white-rot fungus *Bjerkandera adusta*. Enzyme Microb Technol 12:204–209

Niemenmaa O, Uusi-Rauva A, Hatakka A (2008) Demethoxylation of [O14CH3]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria* (*Postia*) *placenta*. Biodegradation 19:555–565

Nishida A, Eriksson K-E (1987) Formation, purification, and partial characterization of methanol oxidase, a H_2O_2-producing enzyme in *Phanerochaete chrysosporium*. Biotechnol Appl Biochem 9:325–338

Okamoto K, Narayama S, Katsuo A, Shigematsu I, Yanase H (2002) Biosynthesis of *p*-anisaldehyde by the white-rot basidiomycete *Pleurotus ostreatus*. J Biosci Bioeng 93:207–210

Otjen L, Blanchette RA (1986) A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. Can J Bot 64:905–911

Overhage J, Steinbüchel A, Priefert H (2003) Highly efficient biotransformation of eugenol to ferulic acid and further conversion to vanillin in recombinant strains of Escherichia coli. Appl Environ Microbiol 69:6569–6576

Papageorgiou GZ, Tsanaktsis V, Bikiaris DN (2014) Synthesis of poly(ethylene furandicarboxylate) polyester using monomers derived from renewable resources: thermal behavior comparison with PET and PEN. Phys Chem Chem Phys 16:7946–7958

Patel RN (2013) Biocatalytic synthesis of chiral alcohols and amino acids for development of pharmaceuticals. Biomolecules 3:741–777

Priefert H, Rabenhorst J, Steinbüchel A (2001) Biotechnological production of vanillin. Appl Microbiol Biotechnol 56:296–314

Rasmussen ML, Shrestha P, Khanal SK, Pometto AL, van Leeuwen J (2010) Sequential saccharification of corn fiber and ethanol production by the brown rot fungus *Gloeophyllum trabeum*. Bioresour Technol 101:3526–3533

Romero E, Ferreira P, Martínez AT, Martínez MJ (2009) New oxidase from *Bjerkandera* arthroconidial anamorph that oxidizes both phenolic and nonphenolic benzyl alcohols. Biochim Biophys Acta 1794:689–697

Romero E, Martínez AT, Martínez MJ (2010) Manganese peroxidase and aryl-alcohol oxidase: a ligninolytic tandem in *Bjerkandera* arthroconidial anamorph. In: Proc OxiZymes in Leipzig, 14–16 June

Rosatella AA, Simeonov SP, Frade RFM, Afonso CAM (2011) 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological properties, synthesis and synthetic applications. Green Chem 13:754–793

Ruiz-Dueñas FJ, Lundell T, Floudas D, Nagy LG, Barrasa JM, Hibbett DS, Martínez AT (2013) Lignin-degrading peroxidases in Polyporales: an evolutionary survey based on ten sequenced genomes. Mycologia 105:1428–1444

Ruiz-Dueñas FJ, Martínez AT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2:164–177

Sannia G, Limongi P, Cocca E, Buonocore F, Nitti G, Giardina P (1991) Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim Biophys Acta 1073:114–119

Schwarze FWMR, Baum S, Fink S (2000) Dual modes of degradation by *Fistulina hepatica* in xylem cell walls of *Quercus robur*. Mycol Res 104:846–852

Scott GM, Swaney R (1998) New technologies for papermaking: biopulping economics. Tappi J 81:153–157

Serra S, Fuganti C, Brenna E (2005) Biocatalytic preparation of natural flavours and fragrances. Trends Biotechnol 23:193–198

Shimada M, Higuchi T (1991) Microbial, enzymatic and biomimetic degradation of lignin. In: Hon DNS, Shiraishi N (eds) Wood and cellulosic chemistry. Marcel Dekker, New York, pp 557–619

Sigoillot C, Camarero S, Vidal T, Record E, Asther M, Pérez-Boada M, Martínez MJ, Sigoillot J-C, Asther M, Colom J, Martínez AT (2005) Comparison of different fungal enzymes for bleaching high-quality paper pulps. J Biotechnol 115:333–343

Turner WB, Aldridge DC (1983) Fungal metabolites II. Academic Press, London

Ullrich R, Nuske J, Scheibner K, Spantzel J, Hofrichter M (2004) Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. Appl Environ Microbiol 70:4575–4581

van Deurzen MPJ, van Rantwijk F, Sheldon RA (1997) Chloroperoxidase-catalyzed oxidation of 5-hydroxymethylfurfural. J Carbohydr Chem 16:299–309

Vicentim MP, Faria RD, Ferraz A (2009) High-yield kraft pulping of *Eucalyptus grandis* Hill ex Maiden biotreated by *Ceriporiopsis subvermispora* under two different culture conditions. Holzforschung 63:408–413

Waldner R, Leisola MSA, Fiechter A (1988) Comparison of ligninolytic activities of selected white-rot fungi. Appl Microbiol Biotechnol 29:400–407

Yelle DJ, Ralph J, Lu F, Hammel KE (2008) Evidence for cleavage of lignin by a brown rot basidiomycete. Environ Microbiol 10:1844–1849

Zabel R, Morrell J (1992) Wood microbiology: decay and its prevention. Academic Press, London

Zhao H, Holladay JE, Brown H, Zhang ZC (2007) Metal chlorides in ionic liquid solvents convert sugars to 5-hydroxymethylfurfural. Science 316:1597–1600

Chapter 13
Monosaccharide Oxygenase

Avnish Kumar, Monika Asthana, Hirawati Deval and Sarika Amdekar

Abstract Oxygenases are enzymes that catalyze the insertion of oxygen atom into an organic substrate. With the intention to perform this kind of reactions, those enzymes want to prompt molecular oxygen to conquer its spin-forbidden response with the organic substrate. In most cases, oxygenases make use of organic cofactors to transfer electron to molecular oxygen for its activation. Due to chemo-, regio-, and/or enantio-selective activity both mono- or dioxygenases are attractive biocatalysts. Oxygenases having many organic substrates, of these monosaccharide reactive oxygenases are focused in this chapter. Most abundant protein on earth known as Rubisco monooxygenase and 5-carbon monosaccharide, Sialic acid monooxygenase is well studied but any dioxygenase capable to incorporate O_2 atom in monosachharide is still unknown.

13.1 Introduction of Oxygenases and Monosaccharides

Biochemical reactions in which electrons are exchanged starting with one molecule then onto the next are catalyzed by a wide assortment of enzymes, alluded to as oxidoreductases or redox catalysts (EC 1.x.x.x) (Dixon and Webb 1979). Taking into account the kind of reactions they catalyze, oxidoreductases have been separated into 22 diverse EC-subclasses. A more broad order was proposed by Xu (2005), in which these proteins were separated into four subgroups: (i) oxidases,

A. Kumar · M. Asthana (✉)
Department of Biotechnology, School of Life Sciences,
Dr. Bhim Rao Ambedkar University, Agra, U.P. 282004, India
e-mail: mailtomonikasaxena@gmail.com

H. Deval
National Institute of Virology (NIV), Indian Council of Medical Research,
Ministry of Health and Family Welfare, Govt. of India, Gorakhpur Unit,
B.R.D. Medical College Campus, Gorakhpur 273013, India

S. Amdekar
Department of Microbiology, Barkatullah University, Bhopal, M.P 462026, India

© Springer International Publishing Switzerland 2016

V.K. Gupta (ed.), *Microbial Enzymes in Bioconversions of Biomass*,
Biofuel and Biorefinery Technologies 3, DOI 10.1007/978-3-319-43679-1_13

Table 13.1 Dioxygenases and their cofactors

Enzyme	Type of reaction	Cofactors
Metapyrocatechase	Ring cleavage	Fe^{2+}
Pyrocatechase	Ring cleavage	Fe^{3+}
Prostaglandin cyclooxygenase	Formation of a cyclic peroxide	Heme, Tryptophane, glutathione
Lipoxygenase	Formation of an open peroxide	Fe^{2+}
Cysteamine dioxygenase	Oxygenation of sulfur	Fe^{2+}, sulfides
Tryptophane 2,3-dioxygenase	Ring cleavage	Heme
4-Hydroxyphenyl-pyruvate dioxygenase	Hydroxylation, oxidative decarboxylation	Fe^{2+}, ascorbic acid, required α-ketoglutarate

Monosaccharide dioxgenases are not found to be explained so far in our search study

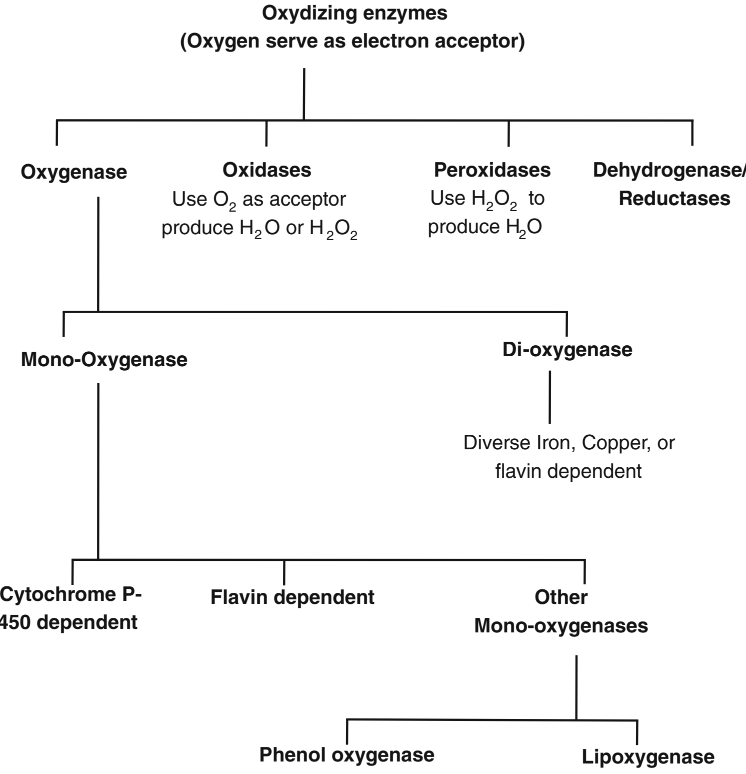


Fig. 13.1 Classification of oxidative enzymes

(ii) peroxidases, (iii) oxygenases/hydroxylases and (iv) dehydrogenases/reductases (Table 13.1; Fig. 13.1).

13.1.1 Oxygenases

Catalyze the incorporation of oxygen atom into the substrate. They are classified into two groups: monooxygenase and dioxygenases. Dioxygenases consolidate both oxygen atoms into the substrate, while monooxygenases incorporate a single oxygen atom as a hydroxyl group into the substrate and the second oxygen atom is reduced to water. So as to do this sort of reactions, these catalysts need to actuate molecular oxygen to overcome its spin-forbidden reaction with the organic substrate. Mostly, monooxygenases use (in)organic cofactors to exchange electrons to molecular oxygen for its activation.

13.1.2 Monosaccharides

Monosaccharides are a class of carbohydrates that cannot be separated to less complex sugars by hydrolysis and that constitute the building blocks of oligosaccharides and polysaccharides. Monosaccharide comprise of no less than three carbon molecules, one of which is connected to an oxygen atom to shape an aldehyde group (CHO) or a ketone, and the others of which are each appended to a hydroxyl group (OH), e.g., glucose, fructose, mannose, glucosamine, mannosamine, gluconic acid, sialic acid, uronic acid, *N*-acetyl glucosamine, *N*-acetyl muramic acid, and so forth. Monosaccharide can be occurred as chains or rings. Six-carbon sugars (hexoses) and five-carbon sugars (pentoses) are the most frequently encountered monosaccharide in nature.

13.2 Monooxygenase Reactions

Oxidoreductases are getting more attention these days for selective oxygenation of aromatic compounds and a wide range of reactions have been documented using these enzymes from various microbial sources. Monooxygenases are an emerging class of biotechnologically relevant enzymes due to their chemo-, regio-, and/or enantio-selective, making those appealing biocatalysts. Monooxygenases incorporate one atom from O_2 into a substrate that accepts oxygen and using another substrate that furnishes the two H atoms to reduce the other oxygen to water. Monooxygenases responds with O_2 and supplement one atom of oxygen into the substrate and second oxygen atom forms water with a reduced co-substrate.

$$S + O_2 + RH_2 \rightarrow SO + H_2O + R$$

Here, RH_2 is reduced co-substrate and R is oxidized co-substrate.

If monooxygenase split hydrogen from the substrate itself then it is known as internal monooxygenase otherwise it is called external monooxygenase.

In monooxygenation reactions, monooxygenases require electrons rather than O_2. Reactions in which organic compounds are oxygenated or hydroxylated are of great value for organic synthesis. In any case, specific oxyfunctionalization of organic substrates can be a specific issue in organic synthesis. These reactions are frequently completed with solid oxidizing operators and they additionally happen with little chemo-, regio-, and enantio selectivity (Loughlin 2000). These issues are taken away by managing the use of oxygenases. Under optimum conditions of pH scale and temperature, aqueous solvent, etc. these enzymes catalyze a specific oxyfunctionalization of organic substrates. For instance, styrene monooxygenase was demonstrated enantio specifically catalyze the epoxidation of styrene subordinates, yielding the comparing (S)-epoxides with >99 % e.e. (Panke et al. 1998; van Hellemond et al. 2007).

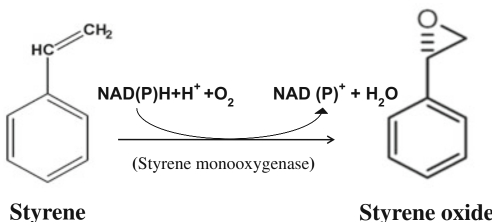
Chemoselectivity refers to the selective reactivity of one functional group in the presence of others; often this process in convoluted and protecting groups are on the molecular connectivity alone.

Regio selectivity is the preference of one direction of chemical bond making or breaking of over all other possible directions, e.g., selectively generation of one constitutional isomer rather than the other.

Enantio selectivity is the production of a compound by a method that selectively preferred the formation of a specific enantiomer or diastereomer.

All styrene monooxygenases (Fig. 13.2) perform enantio-selective epoxidations of styrene and chemically analogous compounds, which makes them interesting for biotechnological applications (Montersino et al. 2011).

In the reaction, monooxygenases (EC 1.13.x.x and EC1.14.x.x) need to enact molecular oxygen, as no reaction will proceed without actuation due to the spin state of O_2. This actuation endless supply of electrons to sub-atomic oxygen, after which oxygenation of the natural substrate can happen. The formation of reactive oxygen intermediate could be identified according to the cofactor present in the



Styrene + $FADH_2$ + O_2 $\longleftrightarrow$ (S)-2-phenyloxirane + FAD + H_2O

Fig. 13.2 Styrene oxygenase reaction

monooxygenase. Sometimes, no cofactor at all is available. internal monooxygenases get these electrons from the substrate itself, though outside monooxygenases are subject to outer electron contributors, e.g., coenzyme NAD(P)H (van Berkel et al. 2006a). Torres et al. (2010) has reviewed a brief discourses of the distinctive groups of monooxygenases, in light of the types of cofactor they require for catalysis are as take after. Monooxygenases is presented, based on the type of cofactor that these enzymes require. This includes not only the cytochrome P450 and flavin-dependent monooxygenases, but also enzymes that utilize pterin, metal ions (copper or iron) or no cofactor at all. As most of these monooxygenases require nicotinamide coenzymes as electron donors.

Heme-dependent Monooxygenases Additionally alluded to as cytochrome P450 monooxygenases or CYPs (EC 1.14.13.x, EC 1.14.14.x and EC 1.14.15.x) can be found in numerous life shapes: eukaryotes (warm blooded animals, plants and organisms) and microbes express a wide assortment of these catalysts (Nelson et al. 1996). CYPs are most inexhaustible in eukaryotes. They are β-sort heme-subordinate outer monooxygenases and they owe their name to the exceptional light assimilation at 450 nm of the lessened CO-bound heme-complex. This high retention is utilized to assess the centralization of the monooxygenase (Omura and Sato 1964).

Rather than CYPs, qualities encoding **Flavin-dependent monooxygenases** have all the earmarks of being moderately bottomless in prokaryotic genomes. Likewise eukaryotes have been appeared to contain large portions of these qualities (e.g., the genome of *Arabidopsis thaliana* contains 29 quality homologs of purported 'flavin-containing monooxygenases') (Schlaich 2007). The flavins used by these catalysts are either FMN or FAD and are either bound firmly to the chemical (prosthetic gathering) or capacity as a substrate (coenzyme) (van Berkel et al. 2006b). Flavin-subordinate monooxygenases can catalyze different responses, for instance epoxidations, Baeyer–Villiger oxidations (Fig. 13.3) and halogenations. Keeping in mind the end goal to perform these responses, flavin-subordinate monooxygenases create a receptive transitional by shaping a covalent bond between atomic oxygen and the C4a of the flavin (Ghisla and Massey 1989). Depending upon the protonation state, this intermediate is proposed to lead to either

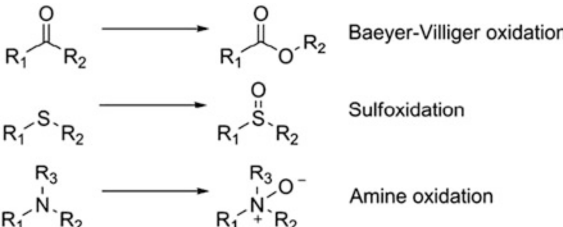


Fig. 13.3 Asymmetric chemical synthetic routes that catalyze selective oxygen insertion are usually mediated by heavy metals with complex ligands and/or explosive peroxidic oxidizing agents. A way to circumvent the use of these hazardous materials is by applying the flavin-dependent Type I Baeyer–Villiger monooxygenases. These enzymes are able to catalyze a broad range of oxidative reactions such as Baeyer–Villiger and heteroatom oxidations

nucleophilic or electrophilic oxygenations. Although this intermediate is well stablized within the enzyme. (Entsch and van Berkel 1995), it will rot to the oxidized flavin without a suitable natural substrate or halide, yielding hydrogen peroxide (H_2O_2) as an item (Massey 1994).

Copper-dependent Monooxygenases (EC 1.14.17.x) constitute a comparatively tiny family of enzymes that need copper ions for hydroxylation of their substrates. Enzymes belonging to this family have primarily been found in organism organisms. The best-studied member of this family is dopamine-monooxygenase (DβM), a protein that hydroxylates the carbon of Dopastat at the expense of one equivalent of molecular element and 2 equivalents of ascorbate, thereby yielding norepinephrine as the main product (Levin et al. 1960). In *Drosophila melanogaster*, the reaction of Dopastat is administered by aminoalkanoic acid monooxygenase (TβM) (Monastirioti et al. 1996). Besides DβM or TβM, these organisms contain a peptidylglycine–hydroxylating monooxygenase (PHM) and a monooxygenase X (MOX) (Xin et al. 2004). The latter monooxygenase was identified for the 1st time throughout a hunt for genes whose expression is altered in aging human fibroblasts (Chambers et al. 1998). Furthermore, copper-dependent monooxygenases have been identified that do not need ascorbate as molecule. An example of these monooxygenases is that the membrane-associated methane series monooxygenase (pMMO) from *Methylococcus capsulatus*. The monooxygenase utilizes two ascorbate molecules to scale back each copper cofactor from Cu^{2+} to Cu^{+}. Thereafter, the enzyme binds the organic substrate and the reduced copper particle reacts with molecular element to create a copper-peroxide (Aboelella et al. 2004; Prigge et al. 2004). This reactive intermediate then oxygenates the substrate, yielding water and the hydroxylated substrate.

Non-heme Iron-dependent Monooxygenases Utilize two iron atoms as chemical compound for his or her aerophilic activity (Fig. 13.4). These di-iron-dependent enzymes are conjointly stated as microorganism multicomponent monooxygenases

Fig. 13.4 Proposed catalytic cycle of non-heme iron-dependent monooxygenases (Valentine et al. 1999)

(BMMs), catalyze hydroxylation and epoxidation reactions and consist of 3 components; a monooxygenase, a reductase and a tiny restrictive macromolecule (Wallar and Lipscomb 1996; Leahy et al. 2003). The best-characterized member of this family is the soluble methane monooxygenase (sMMO) from *M. capsulatus* (Murrell et al. 2000).

Other members of this family include alkene monooxygenase (Gallagher et al. 1997), phenol hydroxylases (Whited and Gibson 1991), membrane-bound alkane hydroxylases (van Beilen and Funhoff 2007) and four-component alkene/aromatic monooxygenases (e.g., toluene 4-monooxygenase).

Pterin-dependent Monooxygenases (EC 1.14.16.x) is a family of monooxygenases known to hydroxylate the amino acids essential amino acid, tyrosine and tryptophane at their aromatic ring. Although some of these enzymes are found in bacterium, they are primarily from organism origin. These monooxygenases bind one iron atom in their situation with two histidines and a salt and utilize tetrahydrobiopterin (BH_4) as a molecule. Upon hydroxylation of the substrate, this coenzyme is regenerate to 4-hydroxybiopterin that, after dehydration, forms quinonoid dihydrobiopterin. In vivo, a NAD(P)H-dependent dihydropteridine reductase recycles its latter product to BH4 (Fitzpatrick 1999). One of the best-studied members of this monooxygenase-family is phenylalanine 4-monooxygenase (PheH, EC 1.14.16.1). However, during chemical change no accumulation of the ferrous protein has been determined. Therefore, the ferrous protein is thought-about to be the resting type in absence of substrates. In this state, the enzyme is projected to bind molecular element in its active site. It has been suggested that O_2 is activated by each the ferrous-ion and therefore the sure reduced tetrahydrobiopterin, after that the side-product 4-hydroxybiopterin is fashioned additionally to the reactive FeIVO-intermediate. This intermediate then hydroxylates the substrate, yielding the product and ferrous protein. However, it is important to notice that the existence of the reactive intermediates has not been confirmed, however (Fitzpatrick 2003).

Cofactor-independent Monooxygenases These are in majority of monooxygenases. It requires an organic or bronze chemical compound for their chemical change activity. However, several monooxygenases have been found that do not need any cofactors. In 1993, tetracenomycin F1 monooxygenase (TcmH) from *Streptomyces glauscens* was refined and characterized. This enzyme was shown to be an internal cofactor-independent monooxygenase, as it required solely molecular element for its activity and used the substrate as chemical agent. Furthermore, inhibition studies suggested that sulfhydryl teams and essential amino acid residues were essential for its aerophilic activity (Shen and Hutchinson 1993). The finding of new members of this monooxygenase-family showed that a lot of those enzymes are primarily gift in streptomycetes. For example, ActVA-orf6 from *Streptomyces coelicolor*A3(2) is a monooxygenase that is thought to turn the formation of dihydrokalafungin, an actinorhodin precursor. An exception to these findings could be a recently known quinolmonooxygenase (YgiN) from *E. coli* (Adams and Jia 2005). In streptomycetes, the cofactor-independent enzymes appear to be concerned

in the synthesis of polyketide antibiotics, such as the antitumor antibiotic tetracenomycin C. Another role was suggested for a theoretic monooxygenase from eubacteria T.B. that features a similar crystal structure as ActVA-orf6. This protein is steered to neutralize reactive element and chemical element species, which are generated by host cells that are attacked by *M. tuberculosis* (Lemieux et al. 2005).

Other cofactor-dependent monooxygenases—In 2005, a methyltransferase homolog was found to hydroxylate the C-10 carbon atom of 15-demethoxy–rhodomycin. This aclacinomycin 10-hydroxylase from *Streptomyces purpurascens* was found to insert an element atom from molecular element into the substrate. Surprisingly, this enzyme needed *S*-adenosyl-L-methionine as a cofactor (Jansson et al. 2003, 2005). It is proposed that the charge of the chemical compound facilitates the delocalization of electrons upon chemical change of the substrate. Thereafter, molecular oxygen is activated by the substrate, yielding a hydroxyperoxide intermediate. Thus far, this is the sole monooxygenase that has been identified that does not belong to at least one of the antecedently mentioned monooxygenase-families.

13.3 Dioxygenase Reactions

Dioxygenases introduce both gas atom of associate gas molecule into a substrate. In most instances, the two oxygen atoms react with one substrate molecule (intramolecular dioxygenase)

$$S + O_2 \rightarrow SO_2$$

Some dioxygenase, however, incorporate one atom into every of the oxygen molecule into completely different molecules of the same substrate.

$$S_2 + O_2 \rightarrow 2SO$$

Or into two completely different substrate (intermolecular dioxygenase)

$$S + S' + O_2 \rightarrow SO + S'O$$

Dioxygenases include two major classes: haem-dependent iron sulfur dioxygenases and Rieske iron–sulfur non-haem dioxygenases the majority of that is NADH dependent (Wackett and Hershberger 2001; Burton 2003). The reactions catalyzed by these enzymes include aromatic ring cleavage and hydroperoxidation or *cis*-dihydroxylation (Fig. 13.5). Rieske non-heme iron-dependent oxygenases are important enzymes that catalyze a wide variety of reactions in the biodegradation of xenobiotics and the biosynthesis of bioactive natural products.

Fig. 13.5 Toluene Dioxygenase reaction

In result of an expansion in the accessibility of catalysts that can perform particular oxygenations; an increasing enthusiasm is demonstrated by the enzyme industries for oxidative biocatalysts.

Rieske oxygenases were first identified as enzymes involved in the degradation of aromatic compounds by *Pseudomonas putida* (Fig. 13.6) (Axcell and Geary 1975; Gibson et al. 1968). These enzymes were ultimately characterized as the three component systems naphthalene dioxygenase and toluene dioxygenase (Yeh et al. 1977; Ensley and Gibson 1983; Ensley et al. 1982). Nowadays, such enzymes are best known as catalysts of the first step in the oxidative degradation of aromatic compounds via regio—and stereospecific *cis*-dihydroxylation to produce dihydrodiols (Jerina et al. 1971). As regards their substrate specificity some compounds such as arenes (toluene, naphthalene) and carboxylates (benzoate, phthalate) are substrate of this type of enzymes.

Nitropropane Dioxygenase FMN Dependent Reaction

2-Nitropropane dioxygenase from *Hansenula mrakii* is a flavin-dependent enzyme that catalyzes the oxidation of anionic nitroalkanes into the corresponding carbonyl compounds and nitrite, with oxygen as the electron acceptor (Fig. 13.6). Although nitroalkanes are anticipated to be toxic and carcinogenic, they are used widely in chemical industry for a quick and effective way of synthesizing common reagents. Consequently, the biochemical and biophysical analysis of 2-nitropropane dioxyganase has a potential for bioremediation purposes.

Fig. 13.6 Nitropropane Dioxygenase reaction

Nearly all dioxygenases contains iron or copper, which in most instances is a right away constituent of the enzyme protein. Iron will also be component or ferroprotoporphyrin IX (Heme). The metallic turns on oxygens connected to the enzyme, a manner that is associated with polarization of the complex. Inside the case of the iron containing enzyme this will be formulated as below:

$$E{-}Fe^{2+} + O_2 \Leftrightarrow E{-}Fe^{2+} - O{-}O^{-}$$

The enzyme oxygen complex adds to the substrate with the elimination of a proton and the $E{-}Fe^{2+}$ complex is regenerated.

13.4 Monosaccharides Monooxygenase

Monooxygenases are much more common than dioxygenases. The oxygenases if utilize monosaccharides to produce reactive oxygen-intermediate are termed here as monosacharride oxygenases.

Ribulose 1,5-Bisphosphate (RuBP) Carboxylase/Oxygenase (RubisCO)
Rubisco, the most abundant enzyme in the biosphere (Ellis 1979), fixes CO_2 into organic carbon that supports nearly all life on Earth. In today's atmosphere, O_2 competes with CO_2 at Rubisco's catalytic site, producing the toxic compound phosphoglycolate (Somerville and Ogren 1979). Phosphoglycolate must be metabolized at the expense of energy and loss of fixed carbon and nitrogen (Bauwe et al. 2010). To overcome Rubisco's limitations, many photosynthetic organisms have evolved carbon-concentrating mechanisms (CCMs) (Sage et al. 2012; Giordano et al. 2005). CCMs increase the CO_2 concentration around Rubisco, decreasing O_2 competition and enhancing carbon fixation. Ribulose is a ketopentose (monosaccharide) that comprise 5 carbon atoms, and together with a ketone functional group. Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) is one of the precise enzymes of the reductive pentose phosphate

pathway, or Calvin–Bassham–Benson cycle on this pathway, RubisCO capabilities to catalyze the real CO_2 assimilatory step to convert RuBP and CO_2 into two molecules of 3-phosphoglyceric acid via a six-carbon carboxylated intermediate. The unique activity of oxygenase relative to carboxylase became extraordinarily small and the most beneficial pH changed into about 9, at which pH the carboxylase activity was nil. Kinetics and immunological studies confirmed that the large subunit moiety share the catalytic web site for both carboxylase and oxygenase response (Nishimura and Akazawa 1974; Takabe and Akazawa 1975). As a consequence RuBP carboxylase is unique in its bifunctional nature and additionally due to the fact, in contrast to most different oxygenase it lacks characteristics oxygenase prosthetic groups, i.e., flavin, Cu, and Fe (Chollet et al. 1975); this activity of the enzyme may be characterized as an internal monooxygenase.

Under conditions of high O_2 and low CO_2, however, rubisco has oxygenase activity, initiating the phenomenon called photorespiration (Fig. 13.7). Rubisco-carboxylase-oxygenase: within the oxygenase reaction, O_2 reacts with rubisco's 2nd substrate, RuBP, to shape 3 PG and 2 phosphoglycolate.

The charge of carboxylase reaction is four times that of the oxygenase reaction below everyday atmospheric situations at 25 °C; the stromal awareness of carbon dioxide is then 10 μM and that of oxygen is 250 μM. The oxygenase response, just like the carboxylase reaction, requires that lysine 201 be in the carbamate shape. Due to the fact this carbamate forms only inside the presence of carbon dioxide, this assets might save you rubisco from catalyzing the oxygenase reaction exclusively whilst carbon dioxide is absent.

Fig. 13.7 The likely mechanism of oxygenase reactions catalyzed by way of RuBP carboxylase–oxygenase reactions

CO_2 is likewise the sole carbon source for the predominant life-forms on this planet, and its efficient incorporation into organic matter is directly related to the productivity of important ecosystems, including agriculturally significant plants. For maximum organisms, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) catalyzes the number one step of CO_2 fixation (Gutteridge and Gatenby 1995; Hartman and Harpel 1994; Tabita 1995); in spite of the reality that it's miles the most considerable protein located on earth (Ellis 1979), RubisCO's catalytic performance is seriously limited by way of the ability to catalyze a competing O_2 fixation response. This ends in inefficient CO_2 fixation and occasional productiveness. Right now, the molecular foundation for CO_2/O_2 discrimination is not completely understood; however, the relative capacity of this enzyme to choose either carboxylation or oxygenation is not immutable, but, varies for different source of RubisCO (Jordan and Ogren 1981; Read and Tabita 1994).

The pyrenoid is a spherical structure in the chloroplast stroma, discovered more than 130 years ago (Schmitz 1882; Vaucher 1803; Brown 1967). Pyrenoids have been found in nearly all of the major oceanic eukaryotic primary producers and mediate ~28–44 % of global carbon fixation (Field et al. 1998; Behrenfeld et al. 2001; Rousseaux and Gregg 2013; Mann 1996; Thierstein and Young 2004; Meyer and Griffiths 2013). A pyrenoid typically consists of a matrix surrounded by a starch sheath and traversed by membrane tubules continuous with the photosynthetic thylakoid membranes (Engel et al. 2015). This matrix is thought to consist primarily of tightly packed Rubisco and its chaperone, Rubisco activase (McKay and Gibbs 1991). In higher plants and non-pyrenoid-containing photosynthetic eukaryotes, Rubisco is instead soluble throughout the chloroplast stroma. The molecular mechanism by which Rubisco aggregates to form the pyrenoid matrix remains enigmatic.

Two mechanisms for Rubisco accumulation in the pyrenoid have been proposed: (i) Rubisco holoenzymes could bind each other directly through hydrophobic residues (Meyer et al. 2012), or (ii) a linker protein may link Rubisco holoenzymes together (Engel et al. 2015; Meyer et al. 2012). The second model is based on analogy to the well-characterized prokaryotic carbon-concentrating organelle, the β-carboxysome, where Rubisco aggregation is mediated by a linker protein consisting of repeats of a domain resembling the Rubisco small subunit (Long et al. 2007).

The reaction, which occurs in C4 plants and bacteria, uses the high energy phosphate of PEP to drive the incorporation of a carboxyl group in order to form oxaloacetate. In C4 plants, PEP carboxylase is located in the mesophyll cells on the external surfaces of the plant. Incorporation of CO_2 in this manner helps to shuttle CO_2 to the bundle sheath cells, where the Calvin cycle enzymes are concentrated. It also helps to avoid the wasteful photorespiration cycle due to the oxygenase activity of the CO_2 fixing enzyme, rubisco.

By catalyzing carbon dioxide fixation during photosynthesis, this enzyme is responsible for virtually all of the reduced carbon found in living organisms. Rubisco couples the inorganic and organic carbon pools on earth and is the most significant route for linking these pools together by the synthesis of carbohydrate. It

Photosynthesis :

$$CO_2 + H_2O + \text{ribulose-1,5-}P_2 \longrightarrow 2(\text{3-phospho-D-glycerate}) \quad \text{-----(Eq.1)}$$

Photorespiration :

$$O_2 + \text{ribulose-1,5-}P_2 \longrightarrow \text{3-phospho-D-glycerate} + \text{2- phosphoglycolate} \quad \text{-----(Eq.2)}$$

Fig. 13.8 Equation of rubisco carboxylase/oxygenase catalytic mechanism during photosynthesis and photorespiration

is, however, a poor catalyst, having both a low affinity for carbon dioxide and a small turnover number. Autotrophic organisms must devote a major part of their synthetic capacity to produce sufficient enzyme to sustain life.

Rubisco catalyzes the first reaction in the pathways of both photosynthesis and photorespiration, which are, respectively, its carboxylase and oxygenase activities (Fig. 13.8).

Photosynthesis and photorespiration are interlocking metabolic cycles, with Rubisco determining the relative rates of carbon flow through the two pathways (Fig. 13.9). In the dark, plants are carrying out mitochondrial respiration by the oxidation of substrates to CO_2 and the conversion of O_2 to H_2O. On top of that, there is another process in plants that, like mitochondrial respiration, consumes O_2 and produces CO_2 and, like photosynthesis, is driven by light. This process is called photorespiration and is a costly side reaction of photosynthesis. Rubisco requires both CO_2 and Mg as obligatory cofactors, resulting in carbamylation of an active site lysine residue by CO_2 and coordination of Mg^{2+}.

In the Oxygenase Reaction (Eq. 2) of Photorespiration However, the enediol intermediate reacts with molecular oxygen to form a hydroperoxy derivative that breaks down to 2-phosphoglycolate and 3-phosphoglycerate. Photorespiration consumes ribulose bisphosphate, which results in no net gain of carbon, and also requires the consumption of energy to recycle the lost carbon. Partitioning of ribulose bisphosphate between the two reactions of photosynthesis and photorespiration can vary significantly between different photosynthetic organisms, which has stimulated interest in improving the carboxylation efficiency of crop plants.

Rubisco activity is modulated in plants by an inhibitor and an activator. The inhibitor 2′-carboxy arabinitol 1-phosphate (2CAIP) accumulates in some plants during darkness and binds to the active site of Rubisco. 2CAIP is degraded by a specific phosphatase, which presumably allows Rubisco to function during photosynthesis in the light. Rubisco can be severely inhibited by a range of sugar bisphosphates, including substrate analogs. The enzyme Rubisco activase has the ability to relieve the inhibition caused by sugar bisphosphates, possibly by interacting with Rubisco and altering the affinity of the enzyme for bisphosphates.

Fig. 13.9 The reactions catalyzed by Rubisco. The first intermediate of catalysis is the C2, C3 *cis*-enediol form of ribulose bisphosphate (ribulose 1,5-P2) after abstraction of the C3 proton. The enediol can partition a number of ways, the majority into the products of carboxylation (upper reactions) or oxygenation (lower reactions). However, a number of misprotonated isomers of ribulose 1,5-P2, for example, xylulose-bisphosphate, have been detected with the wild-type enzyme that are produced in quantity by mutations of specific amino acid residues involved in proton transfer. Phosphate elimination of the carbanion forms of intermediates are also produced by some mutants. R,—CHOH—CH20P02 v 3P D-glycerate, 3-phospho glycerate; 2P glycolate, 2-phosphoglycolate. Five discrete partial reactions have been described for the carboxylation reaction (Eq. 1) of ribulose bisphosphate. The initial formation of a C2, C3-enediol intermediate of the ribulose bisphosphate substrate is followed by its carboxylation, where the enediol reacts with CO_2 at the C2 position. The resulting six-carbon intermediate is hydrolytically cleaved to two molecules of 3-phosphoglycerate, resulting in an overall gain of carbon during photosynthesis

Regulation of Its Enzymatic Activity

RuBisCO is usually only active throughout the day as ribulose 1,5-bisphosphate is not regenerated within the dark. This can be as a result of the regulation of many alternative enzymes within the Calvin cycle. Additionally, the activity of RuBisCO is coordinated thereupon of the opposite enzymes of the Calvin cycle in several ways.

Regulation by Ions

Upon illumination of the chloroplasts, the pH of the stroma rises from 7.0 to 8.0 as a result of the proton (hydrogen particle, H^+) gradient created across the thylakoid membrane. Simultaneously, magnesium ions (Mg^{2+}) transported outside the thylakoids to raise the concentration level of magnesium within the stroma of the chloroplasts. RuBisCO includes a high optimum hydrogen ion concentration (can be >9.0, counting on the metal particle concentration) and, thus, becomes "activated" by the addition of CO_2 and magnesium to the active sites as delineated above.

Regulation by RuBisCO Activase

In plants and a few alga, another enzyme, RuBisCO activase, is needed to permit the speedy formation of the crucial carbamate within the site of RuBisCO. RuBisCO Activase is needed as a result of the ribulose 1,5-bisphosphate (RuBP) substrate binds more powerfully to the active sites lacking the carbamate and markedly slows down the "activation" method. During Day time light, RuBisCO activase promotes the discharge of the repressive, or—in some views—storage RuBP from the catalytic sites. Activase is also needed in some plants (e.g., tobacco and lots of beans) as a result of, darkly, RuBisCO is suppressed (or protected against hydrolysis) by a competitive inhibitor synthesized by these plants, a substrate analog 2-carboxy-D-arabitinol 1-phosphate (CA1P) (Andralojc et al. 1994). CA1P binds tightly to the site of carbamylated RuBisCO and inhibits catalytic activity. Within the light, RuBisCO Activase conjointly promotes the release of CA1P from the catalytic sites. Once the CA1P is released from RuBisCO, it is speedily converted to a non-inhibitory type by a light-activated CA1P-phosphatase. Finally, once each many hundred reactions, the traditional reactions with CO_2 or oxygen are not completed, and other repressive substrate analogs square measure shaped within the site. Once again, RuBisCO Activase will promote the discharge of those analogs from the chemical action sites and maintain the catalyst in an exceedingly catalytically active type. The properties of Activase limit the chemical action potential of plants at high temperatures (Crafts-Brandner and Salvucci 2000). CA1P has conjointly been shown to stay RuBisCO in an exceedingly conformation that is protected against chemical action (Khan et al. 1999). At high temperatures, RuBisCO Activase aggregates and might now not activate RuBisCO. This contributes to the attenuated carboxylating capability discovered throughout heat stress (Salvucci et al. 2001).

Regulation by ATP/ADP and Stromal Reduction/Oxidation State Through the Activase

The removal of the inhibitory RuBP, CA1P, and the other inhibitory substrate analogs by activase requires the consumption of ATP. This reaction is inhibited by

the presence of ADP, and, thus, activase activity depends on the ratio of these compounds in the chloroplast stroma. Furthermore, in most plants, the sensitivity of activase to the ratio of ATP/ADP is modified by the stromal reduction/oxidation (redox) state through another small regulatory protein, thioredoxin. In this manner, the activity of activase and the activation state of RuBisCO can be modulated in response to light intensity and, thus, the rate of formation of the ribulose 1,5-bisphosphate substrate (Zhang et al. 2002).

Regulation by Phosphate

In cyanobacteria, inorganic phosphate (Pi) participates in the coordinated regulation of photosynthesis. Pi binds to the RuBisCO active site and to another site on the large chain where it can influence transitions between activated and less active conformations of the enzyme. Activation of bacterial RuBisCO might be particularly sensitive to Pi levels, which can act in the same way as RuBisCO activase in higher plants (Marcus and Gurevitz 2000).

Regulation by Carbon Dioxide

Since carbon dioxide and oxygen compete at the active site of RuBisCO, carbon fixation by RuBisCO can be enhanced by increasing the carbon dioxide level in the compartment containing RuBisCO (chloroplast stroma). Several times during the evolution of plants, mechanisms have evolved for increasing the level of carbon dioxide in the stroma (see C4 carbon fixation). The use of oxygen as a substrate appears to be a puzzling process, since it seems to throw away captured energy. However, it may be a mechanism for preventing overload during periods of high light flux. This weakness in the enzyme is the cause of photorespiration, such that healthy leaves in bright light may have zero net carbon fixations when the ratio of O_2 to CO_2 reaches a threshold at which oxygen is fixed instead of carbon. This phenomenon is primarily temperature-dependent. High temperature decreases the concentration of CO_2 dissolved in the moisture in the leaf tissues. This phenomenon is also related to water stress. Since plant leaves are evaporatively cooled, limited water causes high leaf temperatures. C4 plants use the enzyme PEP carboxylase initially, which has a higher affinity for CO_2. The process first makes a 4-carbon intermediate compound, which is shuttled into a site of C3 photosynthesis then de-carboxylated, releasing CO_2 to boost the concentration of CO_2, hence the name C4 plants.

Crassulacean acid metabolism (CAM) plants keep their stomata (on the underside of the leaf) closed during the day, which conserves water but prevents the light-independent reactions (i.e. the Calvin Cycle) from taking place, since these reactions require CO_2 to pass by gas exchange through these openings. Evaporation through the upper side of a leaf is prevented by a layer of wax.

Sialic Acid Monooxygenase

Sialic acids are a family of nine-carbon acidic monosaccharides that occur obviously at the cease of sugar chains attached to the surfaces of cells and soluble proteins. Sialic acids are sugars usually discovered on the outer terminal role of glycan chains that cover the surface of all vertebrate cells inside the human body, the very best concentration of sialic acid (as *N*-acetylneuraminic acid) takes place

within the brain in which it participates as an necessary a part of ganglioside structure in synaptogenesis and neural transmission.

The maximum generally expressed sialic acid is *N*-acetylneuraminic acid (Neu5Ac) that is the precursor for *N*-glycolylneuraminic acid (Neu5Gc) synthesis—a conversion mediated by means of motion of the CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) enzyme.

Various reactions involving the sialic acids has been given in figure 13.10.

Cells from higher animals and various microorganisms produce sialic acid in a protracted pathway beginning from glucose.

The CMP-N-acetylneuraminate monooxygenase (CMAH) enzyme converts Neu5Ac to the Neu5Gc "non-human" form of this sugar, (Fig. 13.10) (Irie et al. 1998). However, the Varki group has proposed that the functional loss of CMP-N-acetylneuraminate monooxygenase (CMAH) during evolution of humans from the great apes leads to several possible implications for its role in human development, the most notable of which being less constrained brain growth (Varki 1992). This premise is supported by evidence that the mutation that abrogated the activity of CMAH transcripts found in present-day humans occurred after the Homo-Pan divergence (Chou et al. 1998) but prior to brain expansion during human evolution (Chou et al. 2002). Unlike most primate brains that stop growing relatively soon after birth, human brains continue to grow postnatally at a rate similar to the whole body, presumably because they were freed from the constraints imposed by Neu5Gc in other mammals that limit brain expansion after birth (Chou et al. 2002).

In the human body, the highest concentration of sialic acid (as *N*-acetylneuraminic acid) occurs in the brain where it participates as an integral part of ganglioside structure in synaptogenesis and neural transmission. Human milk also contains a high concentration of sialic acid attached to the terminal end of free oligosaccharides, but its metabolic fate and biological role are currently unknown.

Sialic acids are components of the carbohydrate chains of glycoconjugates and are involved in cell–cell recognition (Rutishauser et al. 1988; Kelm et al. 1996) and cell-pathogen interactions (Smit et al. 1984; Suzuki et al. 1987). Sialic acid is a generic designation used for *N*-acylneuraminic acids and their derivatives (Varki 1992). *N*-Acetylneuraminic acid (NeuAc)1 and *N*-glycolylneuraminic acid (NeuGc) are two of the most abundant derivatives.

N-Glycolylneuraminic acid (NeuGc) is abundantly expressed in most mammals, but it is not detectable in humans. The expression of NeuGc is controlled by cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) hydroxylase activity (Irie et al. 1998).

Monosaccharide monooxygenases are noticeably specialized enzymes that have evolved to catalyze the insertion of single oxygen atom from molecular oxygen (O_2) into a monosacchride. These enzymes activate molecular oxygen through generation of a peroxy-intermediate that is capable to carry out the monooxygenation reaction. In most cases, monooxygenases utilize bound cofactor(s) which include flavin, or metal ions to facilitate the activation of molecular oxygen.

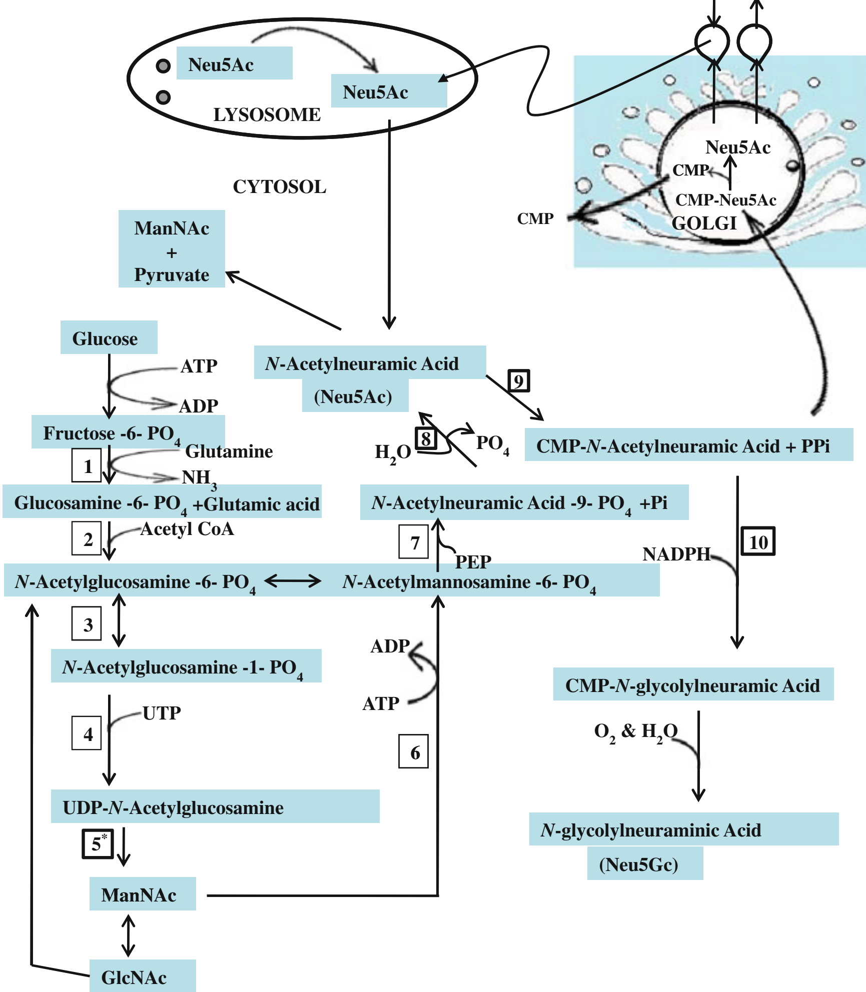


Fig. 13.10 Schema displaying the metabolism of sialic acids modified from Varki et al. (1999): *1* Glycosamine-6-phoshate synthase; *2* Glucosamine-6-phosphate *N*-acetyltransferase; *3* *N*-acetylglucosamine-!6-phosphate mutase; *4* UDP-*N*-acetylglucosaminepyrophos-phorylase; *5* *key enzyme: UDP-*N*-acetylglucosamine-2-epimerase; activity is initially low in young rats; *6* *N*-acetylmannosamine kinase; *7* *N*-acetylneuraminate-9-phosphate synthase; *8* *N*-acetylneuraminate-9-phosphatephatase; *9* CMP-*N*-acetylneuraminatesynthetase; *10* monooxygenase

Examples were described in which monooxygenases utilize the substrate as a "cofactor" to generate the active peroxy-intermediate. By formation of a peroxy-intermediate, these enzymes are capable of catalyze an extensive range of oxidative reactions. The oxidative power of monooxygenases enables to catalyze

wide range of oxidation reactions; e.g., hydroxylations, epoxidations, heteroatom-dealkylations, dehalogenations, dehydrogenations and oxidation of heteroatoms. Monooxygenases containing other (or no) cofactors are more limited of their reactivity scope. Besides the formation of the peroxy-intermediate, cofactor-dependent monooxygenases have some common characteristic; the electrons which can be required for the reduction of cofactor and thus for the activation of molecular oxygen are (in)directly provided by nicotinamide coenzymes NADH or NADPH. Therefore, cost-effective regeneration of these coenzymes is imperative for successful industrial applications of monooxygenases. Of the various possible approaches which are described in this chapter. The enzymatic regeneration of nicotinamide coenzymes is the most advanced and successful so far. Unfortunately, this confined substrate acceptance limits the applicability of these monooxygenases as biocatalyst.

13.5 Future Recommendations

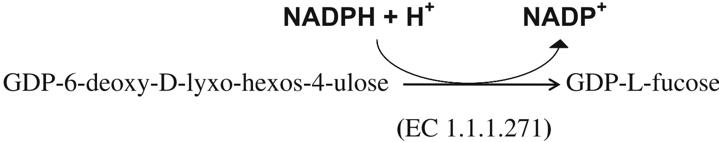
A few unidentified monosaccharide monooxygenases and dioxygenses can be existed in nature. With the identification of new members of cofactor-independent monooxygenase-family, various studies had been accomplished in order to understand how these monooxygenases perform their oxidative reactions in the absence/presence of cofactors. This is indicated by the proposed catalytic mechanism of these enzymes; in contrast to other monooxygenases, molecular oxygen is activated by the substrate, after which the oxygenation reaction occurs. This suggests that the nature of the substrate is important for the catalytic activity of these monooxygenases. Internal cofactor-independent monooxygenases are interesting enzymes for biotechnological applications as they do not require expensive cofactors/coenzymes.

Appendix 1: Some Reactions of Oxidoreductases

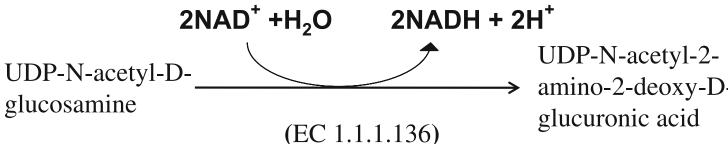
1. Higher plants incorporate large amounts of D-GluA residues into cell wall glucuronoarabinoxylans. D-GluA forms irreversibly from UDP-D-Glc by UDP-D-glucose dehydrogenase (EC 1.1.1.22)

$$\text{UDP-}D\text{-glucose} \xrightarrow[\text{(EC1.1.1.22)}]{\mathbf{2NAD^+} \;\curvearrowright\; \mathbf{2NADH + H^+}} \text{UDP-}D\text{-glucuronic acid}$$

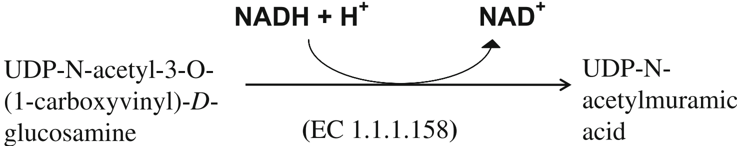
2. GDP-L-fucose formation requires subsequent activities of GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47) and a bifunctional GDP-L-fucose synthase (EC 1.1.1.271).



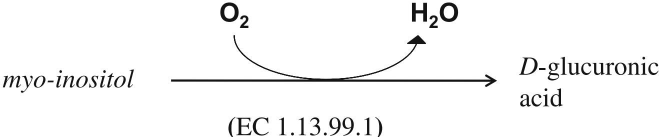
3. *N*-acetyl-D-glucosamine can be further converted to UDP-*N*-acetyl-D-glucuronic acid (UDP-*N*-acetylglucosamine 6-dehydrogenase, EC 1.1.1.136).



4. The peptidoglycan building stone, UDP-*N*-MurAc, forms from UDP-*N*-acetyl-D-glucosamine and phosphoenolpyruvic acid via the intermediate UDP-*N*-acetyl-3-*O*-(1-carboxyvinyl)-D-glucosamine (UDP-*N*-acetyl-D-glucosamine 1-carboxyvinyl transferase, EC 2.5.1.7), which is reduced to UDP-*N*-MurAc by UDP-*N*-acetylmuramate dehydrogenase (EC 1.1.1.158)



5. Biogenesis of UDP-D-GlcA can also occur via an alternative pathway from myo-inositol, which involves the action of myo-inositol oxygenase (EC 1.13.99.1)



Appendix 2

Table 13.2 List of oxedoreductases as per their EC number—the enzymes oxidizes a substrate by transferring the oxygen from molecular oxygen O_2 (as in air) to it are called as oxygenases (EC 1.13 or EC 1.14.)

EC 1.1	Acting on the CH–OH group of donors
EC 1.2	Acting on the aldehyde or oxo group of donors
EC 1.3	Acting on the CH–CH group of donors
EC 1.4	Acting on the $CH–NH_2$ group of donors
EC 1.5	Acting on the CH–NH group of donors
EC 1.6	Acting on NADH or NADPH
EC 1.7	Acting on other nitrogenous compounds as donors
EC 1.8	Acting on a sulfur group of donors
EC 1.9	Acting on a heme group of donors
EC 1.10	Acting on diphenols and related substances as donors
EC 1.11	Acting on a peroxide as acceptor
EC 1.12	Acting on hydrogen as donor
EC 1.13	Acting on single donors with incorporation of molecular oxygen (oxygenases)
EC 1.14	Acting on paired donors, with incorporation or reduction of molecular oxygen
EC 1.15	Acting on superoxide radicals as acceptor
EC 1.16	Oxidizing metal ions
EC 1.17	Acting on CH or CH_2 groups
EC 1.18	Acting on iron-sulfur proteins as donors
EC 1.19	Acting on reduced flavodoxin as donor
EC 1.20	Acting on phosphorus or arsenic in donors
EC 1.21	Acting on the reaction X–H + Y–H = X–Y
EC 1.22	Acting on halogen in donors
EC 1.23	Reducing C–O–C group as acceptor
EC 1.97	Other oxidoreductases

References

Aboelella NW, Kryatov SV, Gherman BF, Bernnessel WW, Young VG Jr, Sarangi R, Rybak-Akimova EV, Hogdson KO, Hedman B, Solomon EI, Cramer CJ, Tolman WB (2004) Dioxygen activation at a single copper site: structure, bonding and mechanism of formation of 1:1 Cu-O_2 adducts. J Am Chem Soc 126:16896–16911

Adams MA, Jia Z (2005) Structural and biochemical evidence for an enzymatic quinone redox cycle in *Escherichia coli*—identification of a novel quinol monooxygenase. J Biol Chem 280:8358–8363

Andralojc PJ, Dawson GW, Parry MA, Keys AJ (1994). Incorporation of carbon from photosynthetic products into 2-carboxyarabinitol-1-phosphate and 2-carboxyarabinitol. Biochem J 304(3):781–786. PMC 1137402. PMID 7818481

Axcell BC, Geary PJ (1975) Purification and some properties of a soluble benzene-oxidizing system from a strain of Pseudomonas. Biochem J 146:173–183

Bauwe H, Hagemann M, Fernie AR (2010) Photorespiration: players, partners and origin. Trends Plant Sci 15(6):330–336

Behrenfeld MJ et al (2001) Biospheric primary production during an ENSO transition. Science 291 (5513):2594–2597. Abstract/FREE Full Text

Brown R (1967) Pyrenoid: its structure distribution and function. J Phycol 3(Suppl 1):5–7

Burton SG (2003) Oxidising enzymes as biocatalysts. Trends Biotechnol 21:543–549

Chambers KJ, Tonkin LA, Chang E, Shelton DN, Linskens MH, Funk WD (1998) Identification and cloning of a sequence homologue of dopamine betahydroxylase. Gene 218:111–120

Chollet R, Anderson LL, Hovsepian LC (1975) The absence of tightly bound copper, iron, and flavin nucleotide in crystalline ribulose 1,5-bisphosphate carboxylase–oxygenase from tobacco. Biochem Biophy Res Commun 64:97–107

Chou HH, Takematsu H, Diaz S, Iber J, Nickerson E, Wright KL, Muchmore EA, Nelson DL, Warren ST, Varki A (1998) A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc Natl Acad Sci USA 95:11751–11756

Chou HH, Hayakawa T, Diaz S, Krings M, Indriati E, Leakey M, Paabo S, Satta Y, Takahata N, Varki A (2002) Inactivation of CMP-*N*-acetylneuraminic acid hydroxylase occurred prior to brain expansion during human evolution. Proc Natl Acad Sci USA 99:11736–11741

Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc Natl Acad Sci USA 97(24):13430–13435. Bibcode:2000PNAS…9713430C. doi:10.1073/pnas.230451497. PMC 27241. PMID 11069297

Dixon M, Webb EC (1979) Enzymes, 3rd edn. pp 218–220, Academic Press: New York

Ellis RJ (1979) The most abundant protein in the world. Trends Biochem Sci 4(11):241–244

Engel BD et al (2015) Native architecture of the Chlamydomonas chloroplast revealed by in situ cryo-electron tomography. eLife 4:e04889

Ensley BD, Gibson DT (1983) Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. J Bacteriol 155:505–511

Ensley BD, Gibson DT, Laborde AL (1982) Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816. J Bacteriol 149:948–954

Entsch B, van Berkel WJH (1995) Flavoprotein structure and mechanism. 1. Structure and mechanism of p-hydroxybenzoate hydroxylase. FASEB J 9:476–483

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281(5374):237–240

Fitzpatrick PF (1999) Tetrahydropterin-dependent amino acid hydroxylases. Ann Rev Biochem 68:355–381

Fitzpatrick PF (2003) Mechanism of aromatic amino acid hydroxylation. Biochemistry 42:14084–14091

Gallagher SC, Cammark R, Dalton H (1997) Alkene monooxygenase from *Nocardia corallina* B-276 is a member of the class of dinuclear iron proteins capable of stereospecific epoxygenation reactions. Eur J Biochem 247:635–641

Ghisla S, Massey V (1989) Mechanisms of flavoprotein catalyzed reactions. Eur J Biochem 181:1–17

Gibson DT, Koch JR, Kallio RE (1968) Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. Biochemistry 7:2653–2662

Giordano M, Beardall J, Raven JA (2005) CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annu Rev Plant Biol 56:99–131

Gutteridge S, Gatenby AA (1995) RubisCO synthesis, meeting, mechanism, and regulation. Plant Mobile 7:809–819

Hartman FC, Harpel MR (1994) Shape, function, law and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. Annu Rev Biochem 63:197–234

Irie A, Koyama S, Kozutsumi Y, Kawasaki T, Suzuki A (1998) The molecular basis for the absence of *N*-glycolylneuraminic acid in humans. J Biol Chem 273:15866–15871

Jansson A, Niemi J, Lindqvist Y, Mantsala P, Schneider G (2003) Crystal structure of aclacinomycin-10-hydroxylase, a S-adenosyl-L-methionine-dependent methyltransferase homolog involved in anthracycline biosynthesis in *Streptomyces purpurascens*. J Mol Biol 334:269–280

Jansson A, Koskiniemi H, Erola A, Wang J, Mäntsälä P, Schneider G, Niemi J (2005) Aclacinomycin 10-hydroxylase is a novel substrate-assisted hydroxylase requiring S-adenosyl-L-methionine as cofactor. J Biol Chem 280:3636–3644

Jerina DM, Daly JW, Jeffrey AM, Gibson DT (1971) *cis*-1,2-dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch Biochem Biophys 142:394–396

Jordan DB, Ogren WL (1981) Species variant inside the specificity of ribulosebisphosphate carboxylase/oxygenase. Nature 291:513–515

Kelm S, Schauer R, Crocker PR (1996) Glycoconj J 13:913–926

Khan S, Andralojc PJ, Lea PJ, Parry MA (1999). 2′-carboxy-D-arabitinol 1-phosphate protects ribulose 1,5-bisphosphate carboxylase/oxygenase against proteolytic breakdown. Eur J Biochem 266(3):840–847. doi:10.1046/j.1432-1327.1999.00913.x. PMID 10583377

Leahy JG, Batchelor PJ, Morcomb SM (2003) Evolution of the soluble diiron monooxygenases. FEMS Microbiol Rev 27:449–479

Lemieux MJ, Ference C, Cherney MM, Wang M, Garen C, James MNG (2005) The crystal structure of Rv0793, a hypothetical monooxygenase from *M. tuberculosis*. J Struct Funct Genom 6:245–257

Levin EY, Levenberg B, Kaufman S (1960) Enzymatic conversion of 3,4-dihydroxypheylethylamine to norepinephrine. J Biol Chem 235:2080–2086

Long BM, Badger MR, Whitney SM, Price GD (2007) Analysis of carboxysomes from Synechococcus PCC7942 reveals multiple Rubisco complexes with carboxysomal proteins CcmM and CcaA. J Biol Chem 282(40):29323–29335

Loughlin WA (2000) Biotransformations in organic synthesis. Bioresour Technol 74:49–62

Mann GD (1996) Chloroplast morphology, movements and inheritance in diatoms. In: Chaudhary BR, Agrawal SB (Eds) Cytology, genetics and molecular biology of algae. SPB Academic Publishing, Amsterdam, pp 249–274. Google Scholar

Marcus Y, Gurevitz M (2000) Activation of cyanobacterial RuBP-carboxylase/oxygenase is facilitated by inorganic phosphate via two independent mechanisms. Eur J Biochem 267 (19):5995–6003. doi:10.1046/j.1432-1327.2000.01674.x. PMID 10998060

Massey V (1994) Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem 269:22459–22462

McKay RML, Gibbs SP (1991) Composition and function of pyrenoids: cytochemical and immunocytochemical approaches. Can J Bot 69(5):1040–1052

Meyer M, Griffiths H (2013) Origins and diversity of eukaryotic CO_2-concentrating mechanisms: lessons for the future. J Exp Bot 64(3):769–786

Meyer MT et al (2012) Rubisco small-subunit α-helices control pyrenoid formation in Chlamydomonas. Proc Natl Acad Sci USA 109(47):19474–19479

Monastirioti M, Linn CEJ, White K (1996) Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci 16:3900–3911

Montersino S, Tischler D, Gassner GT, van Berkel WJH (2011) Catalytic and structural features of flavoprotein hydroxylases and epoxidases. Adv Synth Catal 353:2301–2319

Murrell JC, Gilbert B, McDonald IR (2000) Molecular biology and regulation of methane monooxygenase. Arch Microbiol 173:325–332

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1–42

Nishimura M, Akazawa T (1974) Research on spinach leaf ribulosebisphosphate carboxylase. Carboxylase and oxygenase response examined through immunochemical techniques. Biochemistry 13(11):2277–2281

Omura T, Sato R (1964) Carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemoprotein nature. J Biol Chem 239:2370–2378
Panke S, Witholt B, Schmid A, Wubbolts MG (1998) Towards a biocatalyst for (S)-styrene oxide production: characterization of the styrene degradation pathway of Pseudomonas sp. strain. Appl Environ Microbiol 64:2032–2043
Prigge ST, Eipper BA, Mains RE, Amzel LM (2004) Dioxygen binds end-on to mononuclear copper in a precatalytic enzyme complex. Science 304:864–867
Read BA, Tabita FR (1994) High substrate specificity element ribulosebisphosphate carboxylase/oxygenase from eukaryotic marine algae and properties of recombinant cyanobacterial Rubisco containing "algal" residue adjustments. Arch Biochem Biophys 312:210–218
Rousseaux CS, Gregg WW (2013) Interannual variation in phytoplankton primary production at a global scale. Remote Sens 6(1):1–19. CrossRefGoogle Scholar
Rutishauser U, Acheson A, Hall AK, Mann DM, Sunshine J (1988) Science 240:53–57
Sage RF, Sage TL, Kocacinar F (2012) Photorespiration and the evolution of C4 photosynthesis. Annu Rev Plant Biol 63:19–47
Salvucci ME, Osteryoung KW, Crafts-Brandner SJ, Vierling E (2001) Exceptional sensitivity of Rubisco activase to thermal denaturation in vitro and in vivo. Plant Physiol 127(3):1053–1064. doi:10.1104/pp.010357
Schlaich NL (2007) Flavin-containing monooxygenases in plants: looking beyond detox. Trends Plant Sci 12:412–418
Schmitz F (1882) Die Chromatophoren der Algen: Vergleichende Untersuchungen über Bau und Entwicklung der Chlorophyllkörper und der analogen Farbstoffkörper der Algen. M. Cohen & Sohn, Bonn
Shen B, Hutchinson CR (1993) Tetracenomycin F1-monooxygenase—oxidation of a naphthacenone to a naphthacenequinone in the biosynthesis of tetracenomycin-C in *Streptomyces glaucescens*. Biochemistry 32:6656–6663
Smit H, Gaastra W, Kamerling JP, Vliegenthart JF, de Graaf FK (1984) Infect Immun 46:578–584
Somerville CR, Ogren WL (1979) A phosphoglycolate phosphatase-deficient mutant of Arabidopsis. Nature 280(5725):833–836
Suzuki Y, Nagao Y, Kato H, Suzuki T, Matsumoto M, Murayama J (1987) Biochim Biophys Acta 903:417–424
Tabita FR (1995) The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in red micro organism. In: Blankenship RE, Madigan MT, Bauer CE (eds) Anoxygenic photosynthetic micro organism. Kluwer, Dordrecht, pp 885–914
Takabe T, Akazawa T (1975) Biochemistry 14:46–50
Thierstein HR, Young JR (eds) (2004) Coccolithophores: from molecular processes to global impact. Springer, Heidelberg
Torres PDE, Winkler M, Glieder A, Fraaije MW (2010) Monooxygenases as biocatalysts: classification, mechanistic aspects and biotechnological applications. J Biotechnol 146:9–24
Valentine AM, Stahl SS, Lippard SJ (1999) Mechanistic studies of the reaction of reduced methane monooxygenase hydroxylase with dioxygen and substrates. J Am Chem Soc 121:3876–3887
van Beilen JB, Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21
van Berkel WJH, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Biotechnol 124:670–689
van Hellemond EW, Janssen DB, Fraaije MW (2007) Discovery of a novel styrene monooxygenase originating from the metagenome. Appl Environ Microbiol 73:5832–5839
Varki A (1992) Diversity in the sialic acids. Glycobiology 2:25–40
Varki A, Cummings R, Kesko J, Freeze H, Hart G, Marth J (1999) Sialic acids. In: Varki A, Cummings R, Kesko J et al. (eds) The Essentials of Glycobiology. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 195–209

Vaucher J-P (1803) Histoire des Conferves D'eau Douce: Contenant Leurs Différents Modes De Reproduction, Et La Description De Leurs Principales Espèces, Suivie De L'histoire Des Trémelles Et Des Ulves D'eau Douce. JJ Paschoud, Geneva

Wackett LP, Hershberger CD (2001) Biocatalysis and biodegradation: microbial transformation of organic compounds, st edn. ASM Press, Washington

Wallar BJ, Lipscomb JD (1996) Dioxygen activation by enzymes containing binuclear non-heme iron clusters. Chem Rev 96:2625–2657

Whited GM, Gibson DT (1991) Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to p-cresol in *Pseudomonas mendocina* KR1. J Bacteriol 173:3010–3016

Xin X, Mains RE, Eipper BA (2004) Monooxygenase X, a member of the copper-dependent monooxygenase family localized to the endoplasmic reticulum. J Biol Chem 279:48159–48167

Xu F (2005) Applications of oxidoreductases: recent progress. Ind Biotechnol 1:38–50

Yeh W-K, Gibson DT, Liu T-N (1977) Toluene dioxygenase: a multicomponent enzyme system. Biochem Biophys Res Commun 78:401–410

Zhang N, Kallis RP, Ewy RG, Portis AR (2002) Light modulation of Rubisco in Arabidopsis requires a capacity for redox regulation of the larger Rubisco activase isoform. Proc Natl Acad Sci USA 99(5):3330–3334. Bibcode:2002PNAS…99.3330Z. doi:10.1073/pnas.042529999. PMC 122518. PMID 11854454