Energy, Environment, and Sustainability
Series Editors: Avinash Kumar Agarwal · Ashok Pandey

Binod Parameswaran
Sunita Varjani
Sindhu Raveendran *Editors*

Green Bio-processes

Enzymes in Industrial Food Processing

Energy, Environment, and Sustainability

Series editors

Avinash Kumar Agarwal, Department of Mechanical Engineering, Indian Institute of Technology, Kanpur, Uttar Pradesh, India
Ashok Pandey, Distinguished Scientist, CSIR-Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India

This books series publishes cutting edge monographs and professional books focused on all aspects of energy and environmental sustainability, especially as it relates to energy concerns. The Series is published in partnership with the International Society for Energy, Environment, and Sustainability. The books in these series are editor or authored by top researchers and professional across the globe. The series aims at publishing state-of-the-art research and development in areas including, but not limited to:

- Renewable Energy
- Alternative Fuels
- Engines and Locomotives
- Combustion and Propulsion
- Fossil Fuels
- Carbon Capture
- Control and Automation for Energy
- Environmental Pollution
- Waste Management
- Transportation Sustainability

More information about this series at http://www.springer.com/series/15901

Binod Parameswaran · Sunita Varjani
Sindhu Raveendran
Editors

Green Bio-processes

Enzymes in Industrial Food Processing

Editors
Binod Parameswaran
Microbial Processes and Technology Division
CSIR-National Institute for Interdisciplinary Science and Technology (NIIST)
Thiruvananthapuram, Kerala, India

Sindhu Raveendran
Microbial Processes and Technology Division
CSIR-National Institute for Interdisciplinary Science and Technology (NIIST)
Thiruvananthapuram, Kerala, India

Sunita Varjani
Gujarat Pollution Control Board
Gandhinagar, Gujarat, India

ISSN 2522-8366 ISSN 2522-8374 (electronic)
Energy, Environment, and Sustainability
ISBN 978-981-13-3262-3 ISBN 978-981-13-3263-0 (eBook)
https://doi.org/10.1007/978-981-13-3263-0

Library of Congress Control Number: 2018962129

© Springer Nature Singapore Pte Ltd. 2019
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. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Energy demand has been rising remarkably due to increasing population and urbanization. Global economy and society are significantly dependent on the energy availability because it touches every facet of human life and its activities. Transportation and power generation are two major examples. Without the transportation by millions of personalized and mass transport vehicles and availability of 24×7 power, human civilization would not have reached contemporary living standards.

The International Society for Energy, Environment and Sustainability (ISEES) was founded at Indian Institute of Technology Kanpur (IIT Kanpur), India, in January 2014 with the aim of spreading knowledge/awareness and catalysing research activities in the fields of energy, environment, sustainability and combustion. The society's goal is to contribute to the development of clean, affordable and secure energy resources and a sustainable environment for the society and to spread knowledge in the above-mentioned areas and create awareness about the environmental challenges, which the world is facing today. The unique way adopted by the society was to break the conventional silos of specializations (engineering, science, environment, agriculture, biotechnology, materials, fuels, etc.) to tackle the problems related to energy, environment and sustainability in a holistic manner. This is quite evident by the participation of experts from all fields to resolve these issues. ISEES is involved in various activities such as conducting workshops, seminars and conferences in the domains of its interest. The society also recognizes the outstanding works done by the young scientists and engineers for their contributions in these fields by conferring them awards under various categories.

The second international conference on "Sustainable Energy and Environmental Challenges" (SEEC-2018) was organized under the auspices of ISEES from 31 December 2017 to 3 January 2018 at J N Tata Auditorium, Indian Institute of Science, Bangalore. This conference provided a platform for discussions between eminent scientists and engineers from various countries including India, USA, South Korea, Norway, Finland, Malaysia, Austria, Saudi Arabia and Australia. In this conference, eminent speakers from all over the world presented their views

related to different aspects of energy, combustion, emissions and alternative energy resources for sustainable development and a cleaner environment. The conference presented five high-voltage plenary talks from globally renowned experts on topical themes, namely “Is It Really the End of Combustion Engines and Petroleum?” by Prof. Gautam Kalghatgi, Saudi Aramco; “Energy Sustainability in India: Challenges and Opportunities” by Prof. Baldev Raj, NIAS, Bangalore; “Methanol Economy: An Option for Sustainable Energy and Environmental Challenges” by Dr. Vijay Kumar Saraswat, Hon. Member (S&T), NITI Aayog, Government of India; “Supercritical Carbon Dioxide Brayton Cycle for Power Generation” by Prof. Pradip Dutta, IISc Bangalore; and “Role of Nuclear Fusion for Environmental Sustainability of Energy in Future” by Prof. J. S. Rao, Altair Engineering.

The conference included 27 technical sessions on topics related to energy and environmental sustainability including 5 plenary talks, 40 keynote talks and 18 invited talks from prominent scientists, in addition to 142 contributed talks, and 74 poster presentations by students and researchers. The technical sessions in the conference included Advances in IC Engines: SI Engines, Solar Energy: Storage, Fundamentals of Combustion, Environmental Protection and Sustainability, Environmental Biotechnology, Coal and Biomass Combustion/Gasification, Air Pollution and Control, Biomass to Fuels/Chemicals: Clean Fuels, Advances in IC Engines: CI Engines, Solar Energy: Performance, Biomass to Fuels/Chemicals: Production, Advances in IC Engines: Fuels, Energy Sustainability, Environmental Biotechnology, Atomization and Sprays, Combustion/Gas Turbines/Fluid Flow/Sprays, Biomass to Fuels/Chemicals, Advances in IC Engines: New Concepts, Energy Sustainability, Waste to Wealth, Conventional and Alternate Fuels, Solar Energy, Wastewater Remediation and Air Pollution. One of the highlights of the conference was the rapid-fire poster sessions in (i) Energy Engineering, (ii) Environment and Sustainability and (iii) Biotechnology, where more than 75 students participated with great enthusiasm and won many prizes in a fiercely competitive environment. More than 200 participants and speakers attended this four-day conference, which also hosted Dr. Vijay Kumar Saraswat, Hon. Member (S&T), NITI Aayog, Government of India, as the chief guest for the book release ceremony, where 16 ISEES books published by Springer under a special dedicated series “Energy, Environment, and Sustainability” were released. This is the first time that such significant and high-quality outcome has been achieved by any society in India. The conference concluded with a panel discussion on “Challenges, Opportunities & Directions for Future Transportation Systems”, where the panellists were Prof. Gautam Kalghatgi, Saudi Aramco; Dr. Ravi Prashanth, Caterpillar Inc.; Dr. Shankar Venugopal, Mahindra and Mahindra; Dr. Bharat Bhargava, DG, ONGC Energy Center; and Dr. Umamaheshwar, GE Transportation, Bangalore. The panel discussion was moderated by Prof. Ashok Pandey, Chairman, ISEES. This conference laid out the road map for technology development, opportunities and challenges in energy, environment and sustainability domains. All these topics are very relevant for the country and the world in the present context. We acknowledge the support received from various funding agencies and organizations for successful conduct of the second ISEES conference

SEEC-2018, where these books germinated. We would therefore like to acknowledge SERB, Government of India (special thanks to Dr. Rajeev Sharma, Secretary); ONGC Energy Center (special thanks to Dr. Bharat Bhargava); TAFE (special thanks to Sh. Anadrao Patil); Caterpillar (special thanks to Dr. Ravi Prashanth); Progress Rail, TSI, India (special thanks to Dr. Deepak Sharma); Tesscorn, India (special thanks to Sh. Satyanarayana); GAIL, VOLVO; and our publishing partner Springer (special thanks to Swati Mehershi).

The editors would like to express their sincere gratitude to a large number of authors from all over the world for submitting their high-quality work in a timely manner and revising it appropriately at short notice. We would to like express our special thanks to all the reviewers, who reviewed various chapters of this book and provided very valuable suggestions to the authors to improve their manuscript.

This book covers different aspects of the use of enzymes in food applications. With the advancement of technologies, novel enzymes with a wide range of application and specificity have been developed. Enzymes play a prominent role in the food industry. Enzymatic conversions have many advantages including less energy requirement and high specificity which make them attractive processors in the food industry. Various enzymes used in food processing such as proteases, asparaginases, xylanases, lipases, amylases, α-L-arabinofuranosidase, cellulases and tannase are discussed in this book. The enzymes for specific applications like fruit juice debittering, production of sweeteners, enzymes in the design of functional food and algal enzymes are discussed in this book. Detailed applications of enzymes used in food industries for various processes are included in this book.

Thiruvananthapuram, India — Binod Parameswaran
Thiruvananthapuram, India — Sindhu Raveendran
Gandhinagar, India — Sunita Varjani

Contents

1 Introduction to Green Bioprocesses: Industrial Enzymes for Food Applications 1
Parameswaran Binod, Emmanuel Papamichael, Sunita Varjani and Raveendran Sindhu

2 Production of Microbial Proteases for Food Industry 9
Irem Deniz

3 Aspartic Proteases in Food Industry 15
Indu C. Nair and K. Jayachandran

4 Influence of Proteases on Functional Properties of Food 31
Bindhumol Ismail, Hanif Mohammed and A. Jayakumaran Nair

5 Recent Development in the Uses of Asparaginase as Food Enzyme 55
Shahenvaz Alam, Kumar Pranaw, Rameshwar Tiwari and Sunil Kumar Khare

6 Applications of Asparaginase in Food Processing 83
G. Baskar, R. Aiswarya and S. Renganathan

7 Xylanases for Food Applications 99
Kedar Sharma, Abhijeet Thakur and Arun Goyal

8 Biotechnological Avenues for Fruit Juices Debittering 119
Lokesh Kumar Narnoliya and Jyoti Singh Jadaun

9 Enzymes in Sweeteners Production 151
Filipe Carvalho and Pedro Fernandes

10 Lipases: A Promising Tool for Food Industry 181
Sangeeta Negi

11 Amylases for Food Applications—Updated Information 199
Divya Balakrishnan, Swaroop S. Kumar and Shiburaj Sugathan

12 α-L-Arabinofuranosidase: A Potential Enzyme for the Food Industry 229
Abhijeet Thakur, Kedar Sharma and Arun Goyal

13 Agro-Industrial By-Products in the Synthesis of Food Grade Microbial Pigments: An Eco-Friendly Alternative 245
Bishwambhar Mishra, Sunita Varjani and G. Karthikeya Srinivasa Varma

14 Digestive Enzymes: Industrial Applications in Food Products 267
Luciana Porto de Souza Vandenberghe, Susan Grace Karp, Maria Giovana Binder Pagnoncelli, Cristine Rodrigues, Adriane Bianchi Pedroni Medeiros and Carlos Ricardo Soccol

15 Industrial Enzymes as Feed Supplements—Advantages to Nutrition and Global Environment 293
Sharrel Rebello, Divya Balakrishnan, A. N. Anoopkumar, Raveendran Sindhu, Parameswaran Binod, Ashok Pandey and Embalil Mathachan Aneesh

16 Intriguing Disposition of Marine Algae-Derived Enzymes in Food Biotechnology 305
Ravichandran Rathna, Ekambaram Nakkeeran, Sunita Varjani and Bethu Madhumitha

17 Role of Cellulases in Food, Feed, and Beverage Industries 323
V. Anoop Kumar, R. Suresh Chandra Kurup, C. Snishamol and G. Nagendra Prabhu

18 Production of a Transfructosylating Enzymatic Activity Associated to Fructooligosaccharides 345
Abril Flores-Maltos, Solange I. Mussatto, Juan C. Contreras-Esquivel, Raul Rodríguez, Jose A. Teixeira and Cristobal N. Aguilar

19 Tannase and Its Applications in Food Processing 357
Swaroop S. Kumar, Rakhy Sreekumar and Abdulhameed Sabu

20 Enzymes in the Design of Functional Foods or Their Constituents 383
Sarita G. Bhat and Venetia D'Rose

21 Biorefinery Approach for Red Seaweeds Biomass as Source for Enzymes Production: Food and Biofuels Industry 413
Arturo Siller-Sánchez, Héctor A. Ruiz, Cristóbal N. Aguilar and Rosa M. Rodríguez-Jasso

Editors and Contributors

About the Editors

Dr. Binod Parameswaran (Ph.D.) is Scientist in the Microbial Processes and Technology Division at the CSIR-National Institute for Interdisciplinary Science and Technology, Kerala, India. He obtained his Ph.D. in biotechnology from Kerala University, Trivandrum, in 2008 and then completed his postdoctoral studies in the area of lignocellulosic biofuels at the Korea Institute of Energy Research, Daejeon, South Korea. He has authored more than a hundred journal articles and 34 chapters. In 2001, he received the Young Scientist Award from the International Forum on Industrial Bioprocesses (IFIBiop), and in 2014, he received the Kerala State Young Scientist Award.

Dr. Sunita Varjani is Scientific Officer at Gujarat Pollution Control Board, Gandhinagar, Gujarat, India. She holds an M.Sc. in microbiology and Ph.D. in biotechnology. Her major areas of research are industrial and environmental microbiology/biotechnology. She has authored more than 60 publications, including research and review papers, books and chapters. She has won several awards and honours, including Young Scientist Award—2018 by International Society for Energy, Environment and Sustainability; Certificate of Appreciation—2017 from EPFL, Lausanne, Switzerland; Top Reviewer Award—2017 from *Bioresource Technology Journal*, Elsevier; Young Scientist Award at AFRO-ASIAN Congress on Microbes for Human and

Environmental Health, New Delhi (2014); and Best Paper Awards in national and international conferences in 2008, 2012 and 2013. She is Member of the editorial board of *Journal of Energy and Environmental Sustainability* and has served as Guest Editor of special issues of *Bioresource Technology Journal, Journal of Energy and Environmental Sustainability* and *Indian Journal of Experimental Biology*. She is the recipient of visiting scientist fellowship from EPFL, Lausanne, Switzerland (2017).

Dr. Sindhu Raveendran is Scientist at the Centre for Biofuels, Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, India. She has done her Ph.D. in biotechnology from the School of Biosciences, Mahatma Gandhi University, Kottayam, India. Her research interests include producing bioethanol from lignocellulosic biomass and working with microbial enzymes, biopolymers and metagenomics. She has authored more than 151 research publications, which include 79 journal articles and 26 book chapters, and has been the recipient of many prestigious awards and fellowships, including the Marie Curie Fellowship, Visiting Fellowship at EPFL, Switzerland.

Contributors

Cristobal N. Aguilar Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico

Cristóbal N. Aguilar Biorefinery Group, Food Research Department, Faculty of Chemical Sciences, Autonomous University of Coahuila, Saltillo, Coahuila, Mexico

R. Aiswarya Department of Biotechnology, St. Joseph's College of Engineering, Chennai, India

Shahenvaz Alam Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, Hauz Khas, New Delhi, India

Embalil Mathachan Aneesh Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Kerala, India

V. Anoop Kumar Centre for Research on Aquatic Resources, S. D. College, University of Kerala, Alleppey, India

A. N. Anoopkumar Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Kerala, India

Divya Balakrishnan Division of Microbiology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India

G. Baskar Department of Biotechnology, St. Joseph's College of Engineering, Chennai, India

Sarita G. Bhat Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India

Parameswaran Binod Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum, India

Filipe Carvalho Department of Bioengineering and IBB, Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

Juan C. Contreras-Esquivel Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico

Venetia D'Rose Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India

Irem Deniz Bioengineering Department, Engineering Faculty, Manisa Celal Bayar University, Muradiye-Manisa, Turkey

Pedro Fernandes Department of Bioengineering and IBB, Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal; DREAMS and Faculty of Engineering, Universidade Lusófona de Humanidades e Tecnologias, Lisbon, Portugal

Abril Flores-Maltos Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico

Arun Goyal Carbohydrate Enzyme Biotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

Bindhumol Ismail Department of Biotechnology, University of Kerala, Thiruvananthapuram, India

Jyoti Singh Jadaun Botany Department, Dayanand Girl's P.G. College, Kanpur, Uttar Pradesh, India

K. Jayachandran School of Biosciences, Mahatma Gandhi University, Athirampuzha, Kottayam, Kerala, India

Susan Grace Karp Federal University of Parana, Curitiba-PR, Brazil

G. Karthikeya Srinivasa Varma Department of Biotechnology, Sreenidhi Institute of Science and Technology, Hyderabad, Telangana, India

Sunil Kumar Khare Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, Hauz Khas, New Delhi, India

Swaroop S. Kumar Department of Biotechnology and Microbiology, Kannur University, Kannur, Kerala, India

Bethu Madhumitha Research Laboratory, Department of Biotechnology, Sri Venkateswara College of Engineering (Autonomous), Sriperumbudur, Tamil Nadu, India

Adriane Bianchi Pedroni Medeiros Federal University of Parana, Curitiba-PR, Brazil

Bishwambhar Mishra Department of Biotechnology, Centurion University of Technology and Management, Bhubaneswar, Odisha, India

Hanif Mohammed Department of Biotechnology, University of Kerala, Thiruvananthapuram, India

Solange I. Mussatto Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark

G. Nagendra Prabhu Centre for Research on Aquatic Resources, S. D. College, University of Kerala, Alleppey, India

A. Jayakumaran Nair Department of Biotechnology, University of Kerala, Thiruvananthapuram, India

Indu C. Nair Department of Biotechnology, SAS SNDP Yogam College, Konni, Pathanamthitta, Kerala, India

Ekambaram Nakkeeran Research Laboratory, Department of Biotechnology, Sri Venkateswara College of Engineering (Autonomous), Sriperumbudur, Tamil Nadu, India

Lokesh Kumar Narnoliya Department of Biotechnology (DBT), Center of Innovative and Applied Bioprocessing (CIAB), Mohali, Punjab, India

Sangeeta Negi Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

Maria Giovana Binder Pagnoncelli Federal Technological University of Parana, Dois Vizinhos-PR, Brazil

Ashok Pandey CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, India

Emmanuel Papamichael Department of Chemistry, University of Ioannina, Ioannina, Greece

Kumar Pranaw Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, Hauz Khas, New Delhi, India

Ravichandran Rathna Research Laboratory, Department of Biotechnology, Sri Venkateswara College of Engineering (Autonomous), Sriperumbudur, Tamil Nadu, India

Sharrel Rebello Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Kerala, India

S. Renganathan Centre for Biotechnology, A. C. Technology, Anna University, Chennai, India

Cristine Rodrigues Federal University of Parana, Curitiba-PR, Brazil

Raul Rodríguez Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico

Rosa M. Rodríguez-Jasso Biorefinery Group, Food Research Department, Faculty of Chemical Sciences, Autonomous University of Coahuila, Saltillo, Coahuila, Mexico; Cluster of Bioalcoholes, Mexican Centre for Innovation in Bioenergy (Cemie-Bio), Mexico, Mexico

Héctor A. Ruiz Biorefinery Group, Food Research Department, Faculty of Chemical Sciences, Autonomous University of Coahuila, Saltillo, Coahuila, Mexico; Cluster of Bioalcoholes, Mexican Centre for Innovation in Bioenergy (Cemie-Bio), Mexico, Mexico

Abdulhameed Sabu Department of Biotechnology and Microbiology, Kannur University, Kannur, Kerala, India

Kedar Sharma Carbohydrate Enzyme Biotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

Arturo Siller-Sánchez Biorefinery Group, Food Research Department, Faculty of Chemical Sciences, Autonomous University of Coahuila, Saltillo, Coahuila, Mexico

Raveendran Sindhu Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum, India

C. Snishamol Department of Microbiology, S. D. College, University of Kerala, Alleppey, India

Carlos Ricardo Soccol Federal University of Parana, Curitiba-PR, Brazil

Luciana Porto de Souza Vandenberghe Federal University of Parana, Curitiba-PR, Brazil

Rakhy Sreekumar Department of Biotechnology and Microbiology, Kannur University, Kannur, Kerala, India

Shiburaj Sugathan Division of Microbiology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India

R. Suresh Chandra Kurup P. G. Department of Zoology, M. G. College, University of Kerala, Trivandrum, India

Jose A. Teixeira Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

Abhijeet Thakur Carbohydrate Enzyme Biotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

Rameshwar Tiwari Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, Hauz Khas, New Delhi, India

Divya Ullas Cashew Exporters Promotion Council of India (CEPCI) Laboratory and Research Institute, Kollam, India

Sunita Varjani Gujarat Pollution Control Board, Gandhinagar, Gujarat, India

Chapter 1
Introduction to Green Bioprocesses: Industrial Enzymes for Food Applications

Parameswaran Binod, Emmanuel Papamichael, Sunita Varjani and Raveendran Sindhu

Abstract The use of enzymes in food preparations is an age-old process. With the advancement of technologies, novel enzymes with a wide range of application and specificity have been developed. Enzymes play a prominent role in the food industry. Enzymatic conversions have many advantages including less energy requirement and high specificity which make them attractive processors in the food industry. Various enzymes used in food processing such as proteases, asparaginases, xylanases, lipases, amylases, α-L-arabinofuranosidase, cellulases, tannase, etc. are discussed in this book. The enzymes for specific applications like fruit juice debittering, production of sweeteners, enzymes in the design of functional food, and algal enzymes are discussed in this book. Detailed applications of enzymes used in food industries for various processes are included in this book.

Keywords Food enzymes · Proteases · Asparaginases · Xylanases
Lipases · Amylases · α-L-arabinofuranosidase · Cellulases · Tannase

Enzymes play an important role in the food industry in both traditional and novel products. The ancient processes of brewing and cheese-making rely on enzymes. Among the various enzymes used in food applications, proteases have a long history. Proteases have become more and more attractive in the food industry regarding its specific properties, such as high production yield, specificity for a certain substrate, and high activity as well as being environmentally friendly. Proteases have also activity in a wide range of temperature (20–80 °C) and pH values (3–13), which increases the fields of application. Chymosin and papain are

P. Binod (✉) · R. Sindhu
Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum 695019, India
e-mail: binodkannur@niist.res.in

E. Papamichael
Department of Chemistry, University of Ioannina, Ioannina 45110, Greece

S. Varjani
Gujarat Pollution Control Board, Sector-10A, Gandhinagar 382010, Gujarat, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_1

the well-known proteases and recently, novel enzymes and production techniques are studied due to the increase in application areas. Proteases are available in a broad diversity of microorganisms, plants and animals. However, microbial protease productions offer numerous benefits in terms of technical and economic properties such as higher yields in less time and less cost with a higher overall productivity.

Aspartic proteases play a role in the degradation of proteinaceous materials. Aspartic proteases comprise a small group of enzymes which mainly include cathepsin, rennin, and pepsin. The enzymes carry two aspartate residues in its active site and act in association with a bound water molecule in acidic pH. They are highly specific on dipeptide with hydrophobic residues and beta methylene group. Their applications are well established in food processing in the manufacturing of both traditional and novel products and the applications are now extended to new areas like beverage clarification. They are extensively used in cheese manufacturing, in the preservation of wine and also to effect clarification of beverages. Detailed knowledge regarding the mechanism of action, influencing factors and the structure of the enzyme is sure to bring further meaningful utilization of the enzyme in food industry.

Another important enzyme used in the food industry is Bromelain, a cysteine protease. It is isolating from pineapple stem, fruit or other parts of pineapple plants and it has a wide range of uses ranging from industrial to pharmaceutical applications. Due to these varieties of applications, the demand for bromelain is on the rise. The price of bromelain depends upon the purity of the bromelain which based upon the end application. For most of the industrial applications, conventional production methods such as extraction, concentration and drying are being employed. However, high-end applications in pharmaceutical industry, much more pure bromelain is essential and is achieved through various chromatographic methods such as gen filtration or affinity chromatography.

Asparaginases (EC 3.5.1.1) another important enzyme used for the conversion of asparagine into aspartic acid and ammonia. It is one of the most utilized clinical enzymes used in the treatment of different types of cancers. However, there has been a renewed interest in other application of this enzyme especially for minimizing the acrylamide content in baked/fried starchy food products. Acrylamide is generated as a by-product of Maillard reactions between asparagine and reducing sugars. The reactions usually occur at temperature above 100 °C and account for colour and flavour developments in fried/baked starchy foods. In the year 1994, Acrylamide was first time classified in Group B2, i.e. as probably carcinogenic to humans by the International agency for research on cancer. Significant contents of acrylamide have been detected in range of food products including roasted potatoes, root vegetables, chips, crisps, toasts, cakes, biscuits, cereals and coffee. Extensive efforts have been made to reduce the formation of acrylamide during baking/toasting or frying by incorporating asparaginase enzyme as pretreatment.

Enzyme engineering offers an effective pathway in the design of application according to the specificity and the structural changes. The rational design of enzymes helps in evaluating the purpose of enzymes in food processing industries

and bulk chemicals. The commercial asparaginase for the mitigation of acrylamide in food isolated from fungal species was regarded to be safe with high specificity and minimum activity towards glutamine. The enzyme asparaginase has been declared as safe and favourable additive by various food committee experts. Presently, asparaginase is from various bacterial and fungal sources. Free and immobilized asparaginases are used for the mitigation of acrylamide level in food during the blanching process. The immobilized asparaginase is repeatedly reused without any loss in the activity of the enzyme. The main drawback on using asparaginase lies in the commercialization of the product at various countries due to the issues associated at industrial level. Incorporation of asparaginase in food industries needs extensive research on the enzymatic effect and pre/post-processing conditions. The purification of enzyme needs an extensive attention as they influence the mitigation of acrylamide. The adverse effect using asparaginase on sensory properties of food and commercialization of the enzymatic approach towards the mitigation process at various sectors of food industries is the next future scope in the food processing industries.

The growing world population with rapidly rising demand for the development of new food products, improvement in food quality, and ease of food production process is of prime concern. This concern makes it a necessity for the use of various enzymes such as glycoside hydrolases, lipases, proteases, transglutaminases, etc., in the processing of food and food ingredients. Crops and fruits used in the food and brewing industry contain considerable xylan a content. Xylan is a branched heteropolysaccharide and its main chain is composed of xylose subunits linked by β-(1 → 4) glycosidic bonds and includes substitutions in the side chain. Xylanase hydrolyses the xylan polysaccharides randomly. In the last decade, xylanase has received appreciable attention owing to its applications in various food processing industries such as cereal food processing for the improvement of gluten agglomeration, baking industry for the improved texture of bread and cookies, clarification of fruit juices, production of xylooligosaccharide or arabinoxylooligosaccharides as prebiotic food supplements.

Nowadays, majority of the population follows the tight working schedule so it became difficult for them to maintain their balanced diet. Therefore, to obtain proper healthy nutrition, food supplements need to be included in their diet. Fruits are the best choices as a food supplement to provide nutritional constituents. The long-term storage of fruits is the major problem, therefore, the use of packed fruit juices is increased day by day. People are using these packed materials more frequently because these can be stored for a long time, availability of juice of all fruits in all seasons and easy to carry anywhere, without any problem of leakage and spoiling. So, this is the blooming phase of all fruit based industries throughout the world. The demand and market of citrus fruit juice are worldwide and it is quite high due to their significant nutritional value. There is huge hurdle in citrus juice market, which is related to its bitterness. There are two main components, naringin and limonin, responsible for 'immediate' and 'delayed' bitterness, respectively. During processing, this bitterness is increased with time due to the conversion of limonoate A-ring lactone (non-bitter) into limonin (bitter) and this conversion is

facilitated under acidic pH of juice. Bitterness can be reduced up to acceptance range of the consumer by the use of certain enzymes like naringinase and limonoate dehydrogenases but production of these enzymes in such a large amount, which would be sufficient for treatment of juice at industrial scale, is a big issue of concern. Current research activities are focusing on this target to develop technology for achieving the highly efficient enzyme sources either from native or recombinant sources by using synthetic biology and modern biotechnological approaches. Other aspect to enhance this technology is the immobilization of such enzymes for their reuses, which will minimize the total cost of production.

The eco-friendly and highly specific nature of enzymes has made these biocatalysts widely used in the production of sweeteners. Traditionally, their application is mostly associated with the production of starch-derived high-calorie sugars, and at a minor scale, to the production of invert sugar syrup. Such pattern still stands, albeit with significant developments towards improved biocatalysts for those particular roles. These have involved several approaches such as enzyme screening/ modification through genetic or chemical approaches, and enhanced enzyme formulations. Additionally, in recent years, the public perception on the impact of diet in public health has established the need for alternative, low-calorie sweeteners. These abridge a diversity of compounds, from high-intensity sweeteners to oligosaccharides with low sweetening power but with a prebiotic role.

Lipases (triacylglycerol acylhydrolases EC: 3.1.1.3) are universal enzymes, present in all living creatures, i.e. plants, animals, fungi and bacteria. Their basic function is to catalyse the hydrolysis of lipid into free fatty acid and glycerol at the interface of aqueous and organic solvent, which broaden its applications in enormous industries. Lipases catalyse a wide range of industrially important reactions: transesterifications, esterifications, interesterifications, etc., and also show enantio-selectivity due which they are considered as indispensable tools in food, pharmaceuticals, biofuel, diagnostics, chiral chemistry, drug, detergent, oleochemicals, cosmetics, leather and biosensor industry.

The opportunity for discovery of new industrial applications from microorganisms is as large as the variety of environments they confront. The majority of existing biotechnological applications are of microbial origin and enzymes are the most important among them. Microbial enzymes surpass those from animals and plant sources since their ease of production and genetic manipulation, diverse catalytic activities, etc. The role of enzymes in many processes has been known for a long time in which the enzymes from microorganisms had been used for baking, brewing, alcohol production, cheese-making, etc. Starch represents one of the most ubiquitous and an important renewable biological resource and forms a major source of food to a large population. Starch hydrolysis forms the basis of many industrial processes and acid hydrolysis was significant during the earlier days. However, this was almost completely replaced by enzymatic hydrolysis nowadays since the availability and abundance of starch producing microorganisms, corrosion free reaction and specificity of the reaction. One of the major applications of these enzymes is in food industry and starch hydrolysis yields diverse range of products such as glucose, maltose and fructose syrups, cyclodextrins, fat mimetics

substances, etc. They also find application as brewing and baking agents. Enzymatic liquefaction and saccharification of starch requiring higher temperatures demand thermostable amylases.

Cellulose, hemicellulose, pectin and lignin are the major components of the plant cell wall. Hemicellulose is the second most abundant carbohydrates polymer present on earth. Hemicelluloses are branched, hetero-polysaccharides formed by β-(1 → 4)-linked backbones of hexoses like glucose, mannose or pentose like xylose, arabinose such as xylans, mannans, arabinan and glucans. Xylan contains the backbone of 1, 4-linked-β-D-xylopyranose with various side chain substitutions like arabinose, acetic acid, glucuronic acid, ferulic acid and *p*-coumaric acid. L-Arabinose side chain is found in hemicelluloses like arabinan, arabinoxylan, oat spelt xylan and arabinogalactan. The side chain substitution depends on the source of the xylans and it makes the xylan structure complex and hindersits enzymatic hydrolysis. α-L-Arabinofuranosidase has potential application in agro-industrial processes because of its functioning synergistically effect with other hemicellulases. α-L-arabinofuranosidase hydrolyses arabinose side chain present at α-1,2-, α-1,3- and α-1,5-positions on arabinoxylan, thus potentiate other xylanolytic enzymes to act efficiently on the backbone. Therefore, α-L-arabinofuranosidases are used in various industries such as in bread industry as a natural improver of bread quality, wine industry for improvement of flavour, for clarification of fruit juices in juice industry, as supplement for feedstock for enhancing digestion, in production of medicinal compounds and production and modification of the side chains in oligosaccharide.

Digestion is a very complex process involving many different enzymes expressed by the human cells and by the microbial community in the digestive tract. Digestive problems such as lactose intolerance and poor digestion of vegetable oligosaccharides affect a great part of the human population, causing discomforts due to their fermentation by gas-producing microorganisms. Although the treatment may involve the supplementation with digestive enzymes, such as lactase (beta-galactosidase) and alpha-galactosidase, respectively, the industrial processing of food products is another alternative. Gluten intolerant and celiac individuals could potentially be benefited by the administration of peptidases or the consumption of peptidase-treated food, however, this is not yet considered a treatment option that substitutes the complete avoidance of gluten. Enzymes have been applied in food processing for various purposes including the removal of undesired components. Besides the galactosidases that remove specific saccharides, another example is the use of L-asparaginase to avoid the formation of acrylamide, a possible carcinogen.

The relevance of enzyme supplementation in animal feeds has been well established and exploited to generate a high income generating sector of industrial enzymes. Apart from aiding the better nutritional uptake and utility of the food constituents from the animal feed, the economic benefits gained by the production of better meat yield from livestock have prompted the acceptability of these enzymes in animal feeds.

Marine algae bestow with surfeit of bioactive molecules that play an important role in the field of food and pharmaceuticals for its idiosyncratic properties and prodigious applications. The bioactive compounds have a wide application in food and nutraceuticals as preservatives, additives, functional supplements and so on. Marine algae are specially recognized for its unprecedented enzymes that are comparatively unique from terrestrial derived enzymes.

The activities of microbial enzymes have been observed and utilized for many centuries, but it has been only in relatively recent times that the use of microbial enzymes has been commercialized. Cellulases are a group of enzymes consisting of three major components, endoglucanase, exoglucanase and ß-D glucosidase of which endoglucanase acts on carboxymethyl cellulose causing random scission of cellulose chains yielding glucose and cello-oligosaccharides, exoglucanase acts on micro-crystalline cellulose (avicel) liberating cellobiose as the primary product and ß-glucosidase works on cellobiose to release glucose. All these enzymes act synergistically to release glucose as the end product. Cellulases have a wide range of applications in Industrial Biotechnology. It is the second most used industrial enzyme after protease. In most of the cases, they are used in combination with other enzymes like pectinase, hemicellulase, ligninase, etc. Some of the most important applications of cellulase are in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes. However, the most promising applications are in the food, feed and beverage industries.

Biotransformation of sucrose to fructooligosaccharides (FOS) was investigated using the catalytic action of fructosyltransferase (FFase) originated from solid-state fermentation of agro-industrial wastes (sugarcane bagasse, sotol bagasse, *Agave* fibres and polyurethane) using four fungal strains (*Aspergillus niger* GH1, *A. niger* PSH, *Penicillium citrinum* and *P. purpurogenum*), which have demonstrated ability to produce great diversity of metabolites of industrial interest. Microorganisms and supports were selected based on transfructosylating activity and FOS production. *Agave* fibres were the best support material since permitted the highest amounts of FOS and FFase, with a FOS productivity of 10.88 g/L * h and yield of 2.70 g/g based on total substrate. Moreover, the At/Ah ratio of FFase was higher for cells cultivated on *Agave* fibres than those values obtained for the other wastes. Such results showed that *Agave* fibres can be successfully used as support of *A. niger* PSH strain for FOS and FTase production.

Tannases represent a group of enzymes finding its applications in food, brewing and pharmaceutical industries. They have a wide range of distribution and are reported form animals, plants and microbial sources. However, tannase from microbial source is preferred over other sources for industrial uses. They act upon hydrolysable tannins by cleaving the ester and depside bonds so as to release glucose and gallic acid. Gallic acid production is one of the most important commercial applications of tannase. Apart from that, they are extensively used in food industry, especially in instant tea production, where it enhances the extractability and cold water solubility of key compounds. Another important application of tannase is the removal of haze formation and unflavored phenolic compounds from

beer and wine. Quality of fruit juices also can be improved by tannase enzyme. Haze formation and bitterness of the fruit juices can be minimized by the application of these enzymes. Tannins are considered as anti-nutritional factors, while using agro-industrial residues as animal feed. De-tannification of feed by tannase enzyme treatment can significantly improvise the quality of animal feed.

The biorefineries of seaweeds biomass in recent years has attracted attention, in this case, red seaweeds are a source of sulphated galactans such as agar and carrageenan that are relevant polysaccharides commonly applied as powerful gelling agents and stabilizers, particularly for food and nutraceutical purposes and currently are also studied as a rich source of sugars for biofuels as bioethanol production. Moreover, recent reports have focused on the wide biological activities of these polysaccharides as antithrombotics, antimetastasics, antivirals, anti-inflammatories and anticoagulants agents, based on the molecular weight and sulphation degree. Galactan hydrolases specific for polyanionic and insoluble polysaccharides are mainly classified as agarases and carrageenases. The extraction and production by different technologies of these enzymes opens the way to a new field of applications in terms of the biorefinery concept for food and biofuels applications.

Food plays a significant role in maintaining the health and wellness of the human being. The food we eat has a direct influence on the human gut microbiome. Stress also influences the gut health and is responsible for inflammatory reactions in the gut. Any dysbiosis in the gastrointestinal microbiota leads to several gut-related diseases like diarrhoea, irritable bowel syndrome and even colorectal cancer. Functional foods have the ability to rejuvenate and enrich the beneficial gut microorganisms, thereby alleviating the symptoms of various gut-related diseases and reducing the risk of cardiovascular diseases and improves health and well-being. Microbial enzyme processes use fermentation technology to engineer or enhance production of certain naturally occurring dietary substances to boost physiological benefits. These are functional foods and functional food ingredients. Functional foods enriched with probiotic, prebiotics, synbiotics and cobiotics as well as other plant and animal-related food components have the ability to improve the consumer's health and well-being. Enzyme catalysed the degradation of phytates to enhance bioavailability of iron in cereal-based foods is an example. Certain lactic acid bacteria produce glucose, galactose and oligosaccharides (prebiotics) due to transgalactosidal and lactose hydrolysis activities of beta-galactosidase. Other examples include non-starch polysaccharides (NSP) from cereals such as β-glucan and arabinoxylan as dietary fibre constituents or for producing prebiotic compounds.

One more chapter on food grade pigments is also included in this book. Food industry worldwide is mainly dependent upon various colours to make the food appealing to the consumers. Chemically synthesized colourant as food additives poses the risk of hazardous effects and toxicity to the consumers whereas the application of natural pigments as food additives is safer and in demand worldwide. In the present scenario, researchers have shown a great interest in the processing of various waste for fermentation processes in the development of value-added products like microbial pigments. Effective use of cheaply available agro-industrial

residues for production of microbial pigments can make the process cost-effective and environmentally friendly.

The present book provides a detailed overview of these topics organized in 21 chapters.

Chapter 2
Production of Microbial Proteases for Food Industry

Irem Deniz

Abstract Microbial proteases have become more and more attractive in the food industry regarding to its specific properties, such as high production yield, specificity for a certain substrate, and high activity as well as being environmentally friendly. Proteases have also activity in a wide range of temperature (20–80 °C) and pH values (3–13), which increases the fields of application. Chymosin and papain are the well-known proteases and recently novel enzymes and production techniques are studied due to the increase in application areas. Proteases are available in a broad diversity of microorganisms, plants, and animals. However microbial protease productions offer numerous benefits in terms of technical and economic properties such as higher yields in less time and less cost with a higher overall productivity. In this chapter, the studies on microbial protease productions for industrial applications are briefly overviewed. Trend microorganisms and bioreactor configurations are presented together with their potential uses in food industry.

Keywords Proteases · Microbial enzymes · Food industry
Bioreactor configurations

2.1 Proteases for Food Industry

Proteases can be considered as the one of the most value-added industrial enzymes that can be utilize in food industry. They hydrolyze the cleavage of peptide bonds in other proteins as well as within proteins (Singh et al. 2016). Based on their active site, proteases are mainly referred to as aspartic, cysteine, serine, and metallo proteases (dos Santos Aguilar and Sato 2017). Proteases also divide into two main groups based on their optimum pH value: alkaline and neutral proteases. The

I. Deniz (✉)
Bioengineering Department, Engineering Faculty, Manisa Celal Bayar University, Muradiye-Manisa, Turkey
e-mail: iremdenz@gmail.com

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_2

former is used generally in detergent industry whereas, the latter (EC 3.4.24.27/28) have a wide broad of applications in food industry (Sawant and Nagendran 2014).

Although proteases can be extracted from animals, plants and microorganisms, microbial sources are referred to the most preferred resource in industrial applications due to their easy accessibility, economic advantages, and faster growth rates. Proteases are preferred in food industry for a various type of applications. Microbial proteases are commonly used in food industry as a digestibility improvement enzyme, flavor, stabilizer, tenderizer of meat, and allergenicity reducer. They also helps to adjust coagulation, emulsification, foaming, gel strength, fat binding, etc., of food proteins (Pardo et al. 2000).

Lactate dehydrogenase as a protease is used in cheese manufacturing to hydrolyze specific peptide bonds to produce casein and macropeptides in dairy industry (Rao et al. 1998). The milk coagulation enzymes are animal rennets, microbial milk coagulants, and genetically engineered chymosin. These enzymes belong to the class of aspartate proteases and have molecular weights between 30,000 and 40,000 kDa. In cheese manufacturing, the most important role of proteases is to hydrolyze the specific peptide bond to produce para-k-casein and macropeptides (Allen et al. 1986).

In meat industry, the proteases, such as papain, are used for their ability to hydrolyze connective tissues and muscle fiber proteins for tenderization of meat (Kumar and Takagi 1999). Alkaline proteases take place in the formation of soy sauce and other soy products.

Proteases are also useful in baking industry to provide uniformity to dough, to reduce dough consistency, to maintain gluten strength in bread, and to improve flavor in baking products (Heredia-Sandoval et al. 2016). Wheat flour contains gluten, which is responsible for the characteristics of bakery doughs. Proteases produced by *Aspergillus oryzae* have been used to qualify wheat gluten. Enzymatic treatment of the dough reduces the mixing time and leads to enhanced loaf volumes. Microbial proteases are utilized to improve the extensibility and strength of the dough (Argos 1987).

Several hydrolytic enzymes, such as pectinase and glucose oxidase, have an important role in juice industry to degrade the turbidity coming from protein in fruit juices and alcohol based liquors (Souza et al. 2015).

2.2 Trend Microorganisms for Protease Production

Microbial proteases serve more benefits than their equivalents as animal or plant sources. These superiorities include lower production costs, higher production rates leading lower fermentation durations, higher chance for scaling-up, lower specific requirement. These characteristics make microbial enzymes applicable biocatalysts for several food industry processes.

Several microorganisms belonging to the *Bacillus* genus play an important role in food industry. Moreover, being GRAS (generally recognized as safe), for example *Bacillus subtilis* and *Bacillus licheniformis*, makes them more applicable for large-scale productions (Parrado et al. 2014). *B. licheniformis* has the ability to produce alkaline and neutral proteases. Amylases and proteases are used in food processes such as starch liquefaction. In a previous study, thermostable a-amylase and neutral proteases were produced using a thermophilic strain *Bacillus caldolyticus* DSM 405 and adding wastes to the fermentation medium had shown to be cost-effective with very enhanced enzyme activity (Jamrath et al. 2012). As another example, *Xanthomonas campestris* is used to generate xanthan gum which is a good stabilizer utilized in several applications (dos Santos Aguilar and Sato 2017).

There are several reports on genetically modified bacterial protease productions. Most recently, a new aspartic protease gene from the thermophilic fungus *Rhizomucor miehei* CAU432 was cloned and expressed in *Pichia pastoris* and the produced recombinant enzyme showed better meat tenderization than its conventional substitute papain enzyme (Sun et al. 2018). Also, lately three novel thermophilic xylanases produced by *Humicola* sp. have been described for their potential applications in the brewing industry (Du et al. 2013). Other novel microorganism and their products used in food applications are *A. oryzae*, *Bacillus* sp., and *B. subtilis* for alpha-amylase production (Dey and Banerjee 2012), *Saccharomyces* sp. and *Kluyveromyces marxianus* for inulinase production (Dilipkumar et al. 2013), *A. niger* and *S. cerevisiae* for invertase production (Al-Hagar et al. 2015), *Yarrowia lipolytica* for lipase production (Farias et al. 2014), marine fungi for naringinase production (Shehata et al. 2014), *Thermomyces lanuginosus* for phytase production (Berikten and Kivanc 2014).

2.3 Bioreactor Configurations for Microbial Protease Production

A generalized stirred tank bioreactor that is used for microbial protease production is illustrated in Fig. 2.1. The most bioreactor modes that have been used in food industry are batch productions due to their well-known nature and easy controllability. Stirred tank bioreactors and packed-bed system are preferred as they provide more efficiency. A recent study reported that the production of immobilized lipase by *R. miehei* could be most effectively worked in circulated batch packed-bed reactor (Veny et al. 2014). Fluidized bed bioreactors are also commonly used in food industry (Blamey et al. 2017). In the production of ethyl cinnamate which is used as an aroma and fragrance constituent, the kinetic studies showed that the overall reaction duration was 6.5 times faster in the fluidized bed bioreactor, compared to batch mode (Jakovetić et al. 2013). In some fermentation systems, fed-batch serves more efficiency than batch culture mode. In a previous study, the

protease activity was increased by 44% with the use of fed–batch bioreactor, compared to batch culture mode (Singh et al. 2004).

Submerged (SmF) and solid-state fermentations (SSF) have been studied in details for food industrial applications as it determines the microbial growth and enzyme productivity. In SmF, the microorganism grows in a highly free water, whereas in SSF it grow on solid substrates embedded in a very low water. For bacterial and fungal enzyme productions 2–4 and 3–7 days of fermentation durations are required, respectively (Sanchez and Demain 2017). Recently, protease production by *Penicillium citrinum* was reported in SSF with a maximal production of 94.30 U/mL alkaline protease (Xiao et al. 2015).

Generally, aeration and agitation are very important configurations for a bioreactor to produce microbial enzymes due to their contribution to both heat and mass transfer. In a previous study, *Brevibacillus laterosporus* was used to produce protease in lab-scale flasks with the incubation conditions of 180 rpm and they concluded that for more efficiency, the production should be scaled-up to bioreactors (Anbu 2016). *Bacillus sphaericus* was fermented at 300 rpm to produce alkaline proteases with a maximum protease activity of 680,000 U/mL in a 7 L bioreactor (Singh et al. 2004).

As a problem in industrial productions, researchers are in search of regaining cells from the medium. Recently, a spinning basket bioreactor was used to recycle cells in the bioreactor where the biomass is preserved within a closed sieve which is

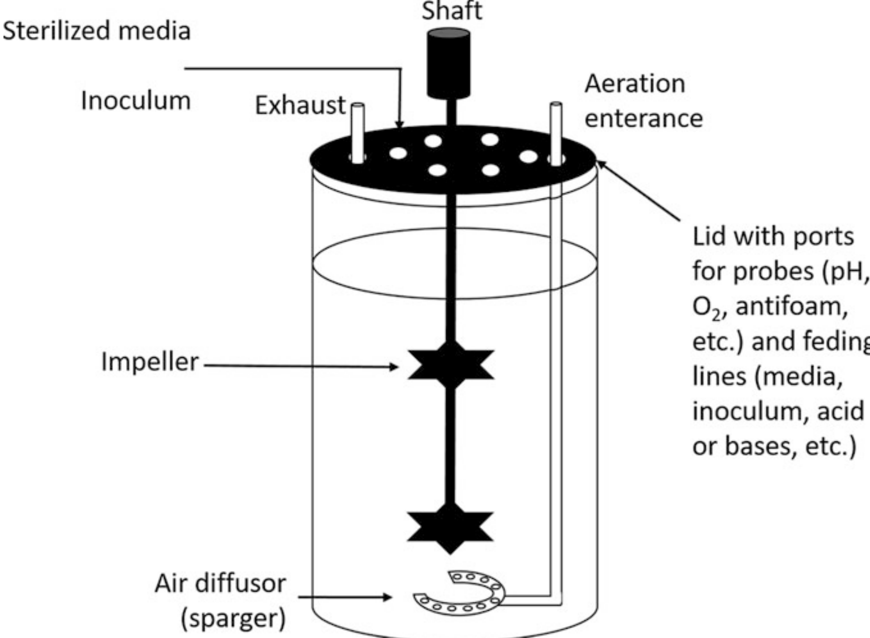


Fig. 2.1 Stirred tank bioreactor

only permeable for solubilized materials in the medium and its benefits to the yield were successfully shown as a model for degumming of rice bran oil with immobilized lecitase (Sheelu et al. 2008).

2.4 Conclusion

Microbial enzymes are very important and can be widely used in food industry. The main benefits of microbial enzymes are having higher bioconversion rates corresponding to higher volumetric productivities and higher yields. In order to increase the applications and production of these enzymes, novel bioreactor configurations are studied including recycling microorganisms, in situ product recovery mechanisms, integrated approach for bioconversion and separation.

References

Al-Hagar OE, Ahmed A, Hassan I (2015) Invertase production by irradiated *Aspergillus niger* OSH5 using agricultural wastes as carbon source. Br Microbiol Res J 6:135–146

Allen C, Stromberg VK, Smith FD, Lacy GH, Mount MS (1986) Complementation of an *Erwinia carotovora* subsp. carotovora protease mutant with a protease-encoding cosmid. Mol Gen Genet (MGG) 202:276–279

Anbu P (2016) Enhanced production and organic solvent stability of a protease from *Brevibacillus laterosporus* strain PAP04. Braz J Med Biol Res 49:1–7

Argos P (1987) A sensitive procedure to compare amino acid sequences. J Mol Biol 193:385–396

Berikten D, Kivanc M (2014) Optimization of solid-state fermentation for phytase production by *Thermomyces lanuginosus* using response surface methodology. Prep Biochem Biotechnol 44:834–848

Blamey JM, Fischer F, Meyer HP, Sarmiento F, Zinn M (2017) Enzymatic biocatalysis in chemical transformations: a promising and emerging field in green chemistry practice. In: Biotechnology of microbial enzymes, 1st edn. Academic Press (an imprint of Elsevier), UK

Dey T, Banerjee R (2012) Hyperactive α-amylase production by *Aspergillus oryzae* IFO 30103 in a new bioreactor. Lett Appl Microbiol 54:102–107

Dilipkumar M, Rajasimman M, Rajamohan N (2013) Enhanced inulinase production by *Streptomyces* sp. in solid state fermentation through statistical designs. 3 Biotech 3:509–515

dos Santos Aguilar JG, Sato HH (2017) Microbial proteases: production and application in obtaining protein hydrolysates. Food Res Int 103:253–262

Du Y, Shi P, Huang H, Zhang X, Luo H, Wang Y, Yao B (2013) Characterization of three novel thermophilic xylanases from *Humicola insolens* Y1 with application potentials in the brewing industry. Biores Technol 130:161–167

Farias M, Valoni E, Castro A, Coelho M (2014) Lipase production by *Yarrowia lipolytica* in solid state fermentation using different agro industrial residues. Chem Eng Trans 38:301–306

Heredia-Sandoval NG, Valencia-Tapia MY, Calderón de la Barca AM, Islas-Rubio AR (2016) Microbial proteases in baked goods: modification of gluten and effects on immunogenicity and product quality. Foods 5:59–69

Jakovetić SM, Luković ND, Bošković-Vragolović NM, Bezbradica DI, Picazo-Espinosa R, Knežević-Jugović ZD (2013) Comparative study of batch and fluidized bed bioreactors for lipase-catalyzed ethyl cinnamate synthesis. Ind Eng Chem Res 52:16689–16697

Jamrath T, Lindner C, Popović MK, Bajpai R (2012) Production of amylases and proteases by *Bacillus caldolyticus* from food industry wastes. Food Technol Biotechnol 50:355–361

Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561–594

Pardo MF, López LM, Canals F, Avilés FX, Natalucci CL, Caffini NO (2000) Purification of balansain I, an endopeptidase from unripe fruits of *Bromelia balansae* Mez (Bromeliaceae). J Agric Food Chem 48:3795–3800

Parrado J, Rodriguez-Morgado B, Tejada M, Hernandez T, Garcia C (2014) Proteomic analysis of enzyme production by *Bacillus licheniformis* using different feather wastes as the sole fermentation media. Enzym Microb Technol 57:1–7

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635

Sanchez S, Demain AL (2017) Useful microbial enzymes—an introduction. In: Biotechnology of microbial enzymes, 1st edn. Academic Press (an imprint of Elsevier), UK

Sawant R, Nagendran S (2014) Protease: an enzyme with multiple industrial applications. World J Pharm Sci 3:568–579

Sheelu G, Kavitha G, Fadnavis NW (2008) Efficient immobilization of lecitase in gelatin hydrogel and degumming of rice bran oil using a spinning basket reactor. J Am Oil Chem Soc 85:739–748

Shehata AN, El Aty A, Abas A (2014) Optimization of process parameters by statistical experimental designs for the production of naringinase enzyme by marine fungi. Int J Chem Eng 2014:1–10

Singh J, Vohra R, Sahoo DK (2004) Enhanced production of alkaline proteases by *Bacillus sphaericus* using fed-batch culture. Process Biochem 39:1093–1101

Singh R, Mittal A, Kumar M, Mehta PK (2016) Microbial proteases in commercial applications. J Pharm Chem Biol Sci 4:365–374

Souza PM, Bittencourt MLA, Caprara CC, Freitas M, Almeida RPC, Silveira D, Fonseca YM, Ferreira Filho EX, Pessoa Junior A, Magalhães PO (2015) A biotechnology perspective of fungal proteases. Braz J Microbiol 46:337–346

Sun Q, Chen F, Geng F, Luo Y, Gong S, Jiang Z (2018) A novel aspartic protease from *Rhizomucor miehei* expressed in *Pichia pastoris* and its application on meat tenderization and preparation of turtle peptides. Food Chem 245:570–577

Veny H, Aroua MK, Sulaiman NMN (2014) Kinetic study of lipase catalyzed transesterification of jatropha oil in circulated batch packed bed reactor. Chem Eng J 237:123–130

Xiao YZ, Wu DK, Zhao SY, Lin WM, Gao XY (2015) Statistical optimization of alkaline protease production from *Penicillium citrinum* YL-1 under solid-state fermentation. Prep Biochem Biotechnol 45:447–462

Chapter 3
Aspartic Proteases in Food Industry

Indu C. Nair and K. Jayachandran

Abstract Green processes are gaining more importance as it is very much essential to maintain the quality of the environment and sustained life. Enzymes, the green catalysts, play a prominent role in food industry because they are inevitable tools in many of the processing stages of food products. Enzymatic conversions are unique as it requires less energy and as they are having high specificity. Amylase, cellulase, protease, pectinase, lipase, etc. are common enzyme choices for catalysing the various stages of processing in food industries. Prospecting for new enzymes and their sources are essential to support the green trend in this sector. Aspartic proteases, which play a major role in the degradation of proteinaceous materials, comprise a small group of enzymes including cathepsin, rennin and pepsin. The enzymes carry two aspartate residues in its active site and act in association with bound water molecule in acidic pH. They are highly specific on dipeptide with hydrophobic residues and beta-methylene group. Their applications are well established in the processing and manufacturing of both traditional and novel food products. They are extensively used in beverage clarification, cheese manufacturing and also in the preservation of wine. Detailed knowledge regarding the mechanism of action, influencing factors and the structure of the enzyme is sure to bring further meaningful utilisation of this enzyme in food industry.

Keywords Green tools · Proteases · Aspartic proteases · Applications
Food industry

I. C. Nair (✉)
Department of Biotechnology, SAS SNDP Yogam College, Konni, Pathanamthitta, Kerala, India
e-mail: inducnair73@gmail.com

K. Jayachandran
School of Biosciences, Mahatma Gandhi University, P.D. Hills, Athirampuzha, Kottayam, Kerala, India
e-mail: jayansbs@gmail.com

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_3

3.1 Introduction

In the living organisms, enzymes drive the biochemical reactions and constitute an essential machinery of metabolism. The production and transportation of enzymes to specific sites are well-controlled processes in a cell. Enzymes are highly evolved functional molecules and through the process of evolution, they get perfectly organised to carry out specific functions. As biological catalysts, enzymes form a well-studied group of macromolecules with great diversity and specificity. But some enzymes show broad specificity such as enzymes in the degradation of hydrocarbons. Enzymes are generally proteinaceous in nature and hence susceptible to variations in physical conditions. They possess active sites where substrates are accommodated to be converted to products. Many enzymes exhibit proper functioning only with the association of cofactors and coenzymes. They support the architectural stability and catalytic interactions of enzymes. During enzymatic reactions, substrates are converted to products by lowering the activation energy. Enzymes are not consumed in the reactions and often remain susceptible to the action of various inhibitors. Enzymes can be highly modified to regulate the whole series of reactions in a metabolic pathway as allosteric enzymes in glycolysis. The emergence of enzymatic catalysis leads to molecular level metabolic regulation and prevention of forming undesirable products (Wackett and Hershberger 2001).

As more and more enzymes were discovered, it became very complex to identify and refer to a particular enzyme. To avoid the ambiguity in enzyme classification, International Union of Biochemistry and Molecular Biology typed and named enzymes. Each enzyme has a unique Enzyme Commission Number which is represented as a four-numbered code. The first digit assigns enzymes into six major classes. The next two levels type the members of major classes on the basis of cofactors, group transfer, etc. The fourth level provides information about substrate specificity. This classification threw light into the fact that some enzymes can catalyse more than one reaction which came to be known as enzyme promiscuity. Promiscuous enzymes are considered as intermediates in the evolution of enzyme functions.

The industrial dislodging into the biosphere perturbs the environmental parameters adversely affecting the survival of human life to a great extent. The recognition that harmful chemicals affect the environmental quality and sustainability led to the development of green processes. Green processes are sustainable processes that involve the design of products and process minimising the use and generation of hazardous substances. This is generally accepted as the most fundamental approach for sustainable development. Progressively, many countries have now developed process and product regulations for maintaining the quality of the environment. Biological agents like microbes do a good turn in accomplishing green processes. Enzymes are renewable natural material and are important tools in the context of green chemistry (Shoda et al. 2016). As proteinaceous, they are completely biodegradable and can be recycled through microbial decomposition. They are nontoxic and do not affect the components of ecosystem and are

categorised as nonhazardous. Another attractive feature is that they are active in mild conditions which make the processes inexpensive. Because of the high substrate specificity, stereoselectivity, natural occurrence, water solubility and activity under ambient conditions enzymes are considered as ideal tools for green process. They generate less waste and therefore are sustainable (Manahan 2005).

Invention of new biocatalysts, upgradation of the enzymes with protein engineering and new biotechnological applications have raised the acceptance of enzyme as promising green process tools. Many industrial applications of enzymes are attributed to enzyme engineering. Through directed evolution, successful biotransformation can be achieved more effectively even for the recalcitrant molecules (Kirk et al. 2002). In an emerging biobased economy, along with protein engineering, new trends in bioreactor designing and advanced techniques of enzyme immobilisation have improved the performance of enzymes as biocatalyst (Roger and Joln 2018). The enzymes are found to be contributing to most of the proposed principles of green chemistry. However, the scope of enzyme-mediated green process is still broad and much of these possibilities are yet to be exploited.

3.2 Proteases

Enzymes can be utilised to increase the production of a metabolite, accelerate the reaction, get a better metabolite, replace the chemical catalyst and also catalyse certain stereospecific conversion. Some of the specific enzymes used as green tools in industrial processes involve proteases, amylases, pectinases, monooxygenases, lipases, laccases, hydroxylases, glycosidases and hydrogenases. Of these, proteases are extensively used in the industry and hold a major share of the global market of enzymes.

Proteases are degradative enzymes that cleave peptide bonds of other proteins. They are of plant, animal and microbial origin. Proteolytic enzymes represent more than 2% of the total functional genes in human beings. Enzymes such as papain, bromelain and keratinases are of plant origin, whereas trypsin, chymotrypsin, pepsin and rennin are of animal origin. Bacterial species usually produce alkaline and neutral proteases. Fungal strains are capable of producing acid proteases too. Proteases find applications in food, beverage and detergent industries, medical treatment, diagnosis, and research and development activities and also in leather industries making them the largest segment in the whole enzyme industry (Table 3.1).

3.2.1 Types of Proteases

The whole proteases can be classified as exo- and endopeptidases. Exopeptidases generally include aminopeptidases, carboxypeptidases and omega-peptidases.

Table 3.1 Important applications of proteases

Applications	Significance in the green activity	Reference
Milk clotting	Utilised in cheese manufacture	Theron and Divol (2014)
In the manufacturing of natto	Enhances flavour by hydrolysing the soy proteins	Borah et al. (2012)
As a therapeutic agent	Proteases are involved in the life cycle of a number of disease-causing microbes facilitating pathogen inactivation by acting as ideal targets for protease inhibitors	Motyan et al. (2013)
In medical diagnosis	Facilitate rapid detection of many diseases like neurodegenerative disorders	Khan (2013)
In research and development sectors	Peptide synthesis, nucleic acid purification, antibody preparation, recombinant DNA techniques	Khan (2013)
As a supplement to treat digestive disorders	Acting as a digestive tool by bringing selective hydrolysis	Khan (2013)
In detergent industry	Most important industrial application	Feijoo-Siota and Villa (2011)
In leather industry	In dehairing, bating and tanning of leather	Feijoo-Siota and Villa (2011)
Beverage industry	Beer and wine manufacture Fruit juice clarification	Theron and Benoit Divol (2014)

Aminopeptidases include dipeptidases and tripeptidases, whereas carboxy peptidases include serine-type and cysteine-type also. Endopeptidases include serine, cysteine and aspartyl proteases besides metalloproteases.

Serine proteases are divided into 20 families mainly on the basis of their structural aspects. They are having serine group in their active site and a glycine residue in the close proximity. Based on the structural similarities, they have been classified into 20 families. These enzymes have broad substrate specificity and are active at alkaline and neutral pH. Cysteine proteases, common in all organisms, carry cysteine and histidine as important residues in their active site. Usually, these enzymes are active only in the presence of reducing agents and are mostly specific on neutral pH. Metalloproteases, requiring divalent metal ion for catalysing the reaction, are the most diverse of the protease enzyme group. They are classified into about 30 families.

Aspartic proteases, the acid proteases obtained from a range of organisms, have been extensively used for many industrial applications. The enzyme depends on two aspartic acid residues in the active site for their enzyme action besides a cysteine residue for the protease action. The mechanism of enzyme action includes the direct involvement of water molecule offering nucleophilic reaction.

3.2.2 *Aspartic Proteases*

Aspartic proteases show a universal distribution in plants including algae, animals, fungi and virus. In MEROPS database, they are grouped into six clans and classified into 14 families. The grouping is based on amino acid sequence homology. The enzyme is synthesised as a preproenzyme and made into active form by a cascade of proteolytic events. This removes the signal peptide and proenzyme fragment. This may give rise to monomeric and heterodimeric substances. The heterodimeric forms are again cleaved to remove the PSI fragment to get the active form. The heterodimer consists of two different subunits of varying size. Beta-strands are prominent in the secondary structure. The enzyme needs two crucial aspartic residues at the active site to form activated water molecule. At the catalytic centre, an Asp-Thr-Gly motif is commonly observed. The enzyme is universally inhibited by pepstatin A (Silva et al. 1997).

3.2.2.1 Mechanism of Action

Aspartic proteases form a group of protein-degrading enzymes which act on the peptide bonds. The catalytic aspartic residues are seen in the beta-strand cleft. These motifs are considered to be one among the largest of beta-strand motifs. Another feature is the presence of a flap-like structure in the active site which is also formed of beta-secondary structures.

The peptide linkage is broken by the action of a nucleophile. The water molecule has major role in performing the catalysis and this was observed connected to the asp residues through the hydrogen bond. The nucleophile is a water molecule polarised by the two aspartic residues. One aspartate absorbs a proton and the water remains charged and it attacks the carbonyl carbon of the substrate. As a result of this, a tetrahedral oxyanion is formed which is an important intermediate in the mechanism. The two aspartic residues are conserved in high degree. The enzyme breaks dipeptide bond with hydrophobic groups and beta-methylene residues (Theron and Divol 2014). The protonation states of the aspartic residues are important in determining the catalytic rate. The isoelectric point of the enzyme is 3–4.5.

3.2.2.2 Sources

Fungi

Many numbers of fungi are found to produce aspartic proteases. Mendieta et al. (2006) describe aspartic protease from *Phanerochaete chrysosporium.* It has a molecular mass of 38 KDa and shows optimum activity at 50 °C and pH 4.5. It is stable at a pH range of 3–8. Besides this organism, several species of *Aspergillus*. *Candida* and *Rhizomucor* are reported to yield high amount of the enzyme

(Table 3.2). Two species of microorganisms associated with grapes, *Metschnikowia pulcherrima* and *Candida apicola* also show the presence of aspartic proteases.

Bacteria

When compared with fungi, aspartic proteases are not much common in bacteria. However, there are reports of *Bacillus amyloliquefaciens* producing the enzyme. Different strains of *Bacillus subtilis* could also produce aspartic protease.

Table 3.2 Microorganisms producing aspartic proteases

Organisms	Enzyme	Activity/applications	Reference
Fungi			
Rhizomucor	Aspartic protease	Meat tenderization and preparation of turtle peptides	Sun et al. (2018)
Aspergillus oryzae *Aspergillus fumigates* *Aspergillus saitoi* *Aspergillus awamori* *Aspergillus niger*	Acid, neutral, alkaline proteases	Broad substrate specificity	Rao et al. (1998) Theron and Divol (2014)
Mucor miehei *Mucor pusillus*	Aspartic protease	Production of various types of cheeses	Mamo and Assefa (2018)
Candida albicans *Candida parapsilosis* *Candida tropicalis*	Aspartic proteases	Involved in candidiasis	Theron and Divol (2014)
Botrytis cinerea	Aspartic protease	Winemaking	Van Sluyter et al. (2013)
Phanerochaete chrysosporium	Aspartic protease	Milk clotting	da silva (2017)
Bacteria			
Bacillus amyloliquefaciens *Bacillus subtilis* *Bacillus subtilis var.natto*	Acid protease	Making of Cheddar type cheese Cheese making Milk clotting	An et al. (2014) Rao et al. (1998) Chen et al. (2010)
Enterococcus faecalis	Acid protease	Cheese making	Mamo and Assefa (2018)

Plants

Plant aspartic proteases are commonly known as phytepsins. They have many functional roles in a plant cell like ageing and apoptosis. They are involved in the hydrolysis of stored proteins and stress response. Simoes and Faro (2004) reports the role of aspartic protease in plant sexual reproduction (Garg and Virupaks 1970). Mendieta et al. (2006) discuss the antimicrobial functions due to aspartic proteases associated with *Solanum tuberosum* which can induce spore killing in the pathogens, *Fusarium solani* and *Phytophthora infestans*.

Aspartic proteases in plants show wide distribution including monocots, dicots and gymnosperms (Table 3.3). The presence of aspartic protease in grains was reported by Simoes and Faro (2004) (Simoes and Faro 2004). In the plant cells, aspartic proteases are present in storage vacuoles showing an intracellular occurrence. Nepenthesins are aspartic proteases seen in several species of Nepenthes, Drosera, Dionaea and Cephalotus. There are extracellular proteases reported in plant cells like tobacco and tomato.

Animals

Most commonly used aspartic proteases including rennet for cheese making are obtained from animals. The main component of calf rennet which is collected from the abomasums of 30 days calf is chymosin. Calf rennet is considered to be excellent in cheese making because of its limited proteolytic activities. Proteases from porcine and bovine origin exhibit unrestricted hydrolysis, but used in cheese production. Pepsin from animal source is also used in cheese industry, but they also show vast hydrolysis which affects the texture and nutritional content.

Further, many specific aspartic proteases are reported to be synthesised by variety of bacterial and fungal strains (Table 3.4). All these enzymes are capable of

Table 3.3 Plants as source of aspartic proteases

Ficus carica	Krishnaswamy et al. (1961)
Calotropis procera	Aworh and Muller (1987)
Oryza sativum	Asakura et al. (1997)
Centaurea calcitrapa	Domingos et al. (2001)
Cynara cardunculus L	Macedo et al. (1993)
Silybum marianum L Gaertn.	Vairo-Cavalli et al. (2005)
Cynara scolymus. L	Llorente et al. (2004)
Helianthus annuus	Egito et al. (2007)
Albezzia lebeck	Egito et al. (2007)
Ficus racemosa	Devraj et al. (2008)
Sideroxylon obtusifolium	Silva et al. (2013)
Onopordum acanthium	Benkahoul et al. (2016)

Table 3.4 Specific aspartic proteases and its source (Mamo and Assefa 2018)

Specific aspartic protease	Source
Saccharopepsin	*Saccharomyces cerevisae*
Yapsin	*Saccharomyces cerevisae*
Barrier pepsin	*Saccharomyces cerevisae*
Endothiapepsin	*Endothis parasitica*
Aspergillopepsin I	*Aspergillus niger*
Aspergillopepsin II	*Aspergillus orizae*
Penicillopepsin	*Penicillium janthinellum*
Mucorpepsin	*Mucor pusillus*
Candidapepsin	*Candida albicans*
Rhodotorulapepsin	*Rhodotorula glutinis*
Pycnoporopepsin	*Pycnoporus sanguineus*
Scytalidopepsin A	*Scytalidium lignicolum*
Scytalidopepsin B	*Scytalidium lignicolum*
Xanthomonapepsin	*Xanthomonas compestris*
Thermopsin	*Sulfolobus acidocaldarius*
Prepilin peptidase	*Pseudomonas aeruginosa*

bringing out unique proteolytic action which are having the potential to be exploited as tools for many green processes.

3.2.2.3 Industrial Applications

Microbial proteases have a pivotal role in industrial processes, especially in food industry. Aspartic proteases are widely distributed in organisms but microbial sources are convenient for large-scale preparations. The enzyme is capable of restricted catalysis with leucine, arginine, lysine, tyrosine and phenylalanine. The property of restricted catalysis makes the enzyme a favourite candidate for cheese production. Milk clotting ability of the enzyme is also remarkable in cheese making. In cheese production, the cleavage of casein in milk is an essential requirement. Casein is a phosphoprotein and is organised as micelles in the milk. Micelles are colloidal aggregates formed by the interaction of hydrophobic groups. The aggregates are dissociated by the action of proteases. As a result of this, the hydrophobic groups become accessible to calcium. Under the influence of calcium, further disaggregation leads to the formation of liquid and curd.

Further uses in baking include the hydrolysis of proteins of the flour during the preparation of biscuits, pastries, wafers, cookies, etc. The degradation of the protein improves the digestibility and nutritional value of the product. The production of experimental gluten-free pasta was attempted using sourdough fermentation by fungal proteases. The essential amino acid content in this was higher and showed better nutritional index. Acid proteases also find application in variety of fields such

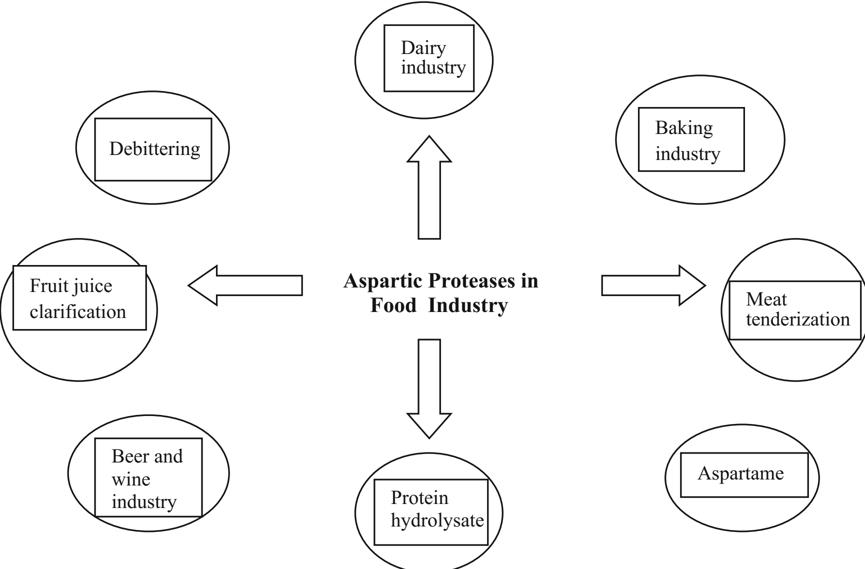


Fig. 3.1 Applications of aspartic proteases

as dairy industry, wine production, debittering, fruit juice clarification and also in the preparation of many protein hydrolysates (Fig. 3.1).

Dairy Industry

In dairy industry, cheese making greatly depends on the availability of aspartic proteases in rennet which exhibit restricted hydrolysis of proteins especially casein. Aspartic peptidases break peptide linkage between hydrophobic residues and their limited action render cheese with ideal texture and composition which has high demand in the world market. In the preparation of acid cheese like quarg, rennet is used in a low concentration. This is introduced either during fermentation when pH is not reaching acidic levels or after the addition of starter culture. In the acidic cheese named cottage, the cheese yields easier cutting at an acidic pH (4.6). The coagulation of milk by enzyme is a two-step process. In the first step, casein is split into a macropeptide and another polypeptide called para-k-casein. Clotting of micelles of casein occurs in the second phase (Claverie and Hernandez 2007).

Feta cheese is prepared from sheep's milk. Rennet is added to milk and after coagulation the segmented curd is dipped in brine and dried. When chymosin is used, the cheese produced is soft with limited loss of fat content. When pepsin is used, milk clotting takes place effectively but proteolysis is extended making the cheese less compact (Garg and Johri 1994).

Plant coagulants are used as milk clotting agents even from ancient times. Restricted proteolysis of milk protein is an attractive feature in dairy industry. But plant proteases are generally highly proteolytic. Even then many plant proteases are used as milk coagulants as they can hydrolyse all alpha-, beta- and gamma caseins. This makes the cheese softer and thus gives an added up flavour (Silva et al. 1997). A Spanish patent reports that flower extracts of C*yanara* sp. can be used in milk clotting. In the Mediterranean basin, cheese production using plant clotting agent is commonly practised traditionally and industrially. The main coagulants are dried flowers of plants like *Cyanara humilis*, *Cyanara scolymus* and *C. Cardunculus*. The extracts of dried flowers are used as milk clotting agents and their proteolytic activity results in high-quality cheese which is remarkably accepted worldwide (Llorente et al. 2004).

Globe artichoke (*C. scolymus* L) aspartic protease was purified by Llorente et al. (2004) with maximum milk clotting activity at a pH of 5. Flower was the main source of enzyme and was inhibited by pepstatin. An aspartic protease which is highly stable at 60 °C was studied by Devraj et al. (2008). The enzyme has a single polypeptide and depicted an optimum pH range of 4.5–6.5.

Claverie and Hernandez (2007) reported the use of a vegetable rennet isolated from *Calotropis procera* (Sodom apple). This enzyme preparation induces vast hydrolysis in milk but the cheese is harder and more compact. The protein and fat content were also reduced.

Paneer is considered to be an Indian variant of soft cheese produced from the milk of cow and buffalo. In India, which is one of the largest producers of milk, about 5% of total milk produced per year is used for paneer preparation. Paneer has rich protein and fat content with attractive flavour and texture so that it is a substitute for meat (Kumar et al. 2014). A plant coagulant conventionally used in India for cheese making is the fruit of *Withania coagulans* belonging to the family Solanaceae. The fruit pulp of the plant is enriched with plant rennin which shows aspartic protease activity. The husk of *W. coagulans* is also reported to have aspartic protease activity, and the plant is common in the arid parts of India, Afghanistan, Iran and Pakistan. The dried fruit preparations are capable of hydrolysing casein at a pH range of 4–6. At pH 7, more than 85% of enzyme activity was lost (Kazemipour et al. 2017).

Microbial enzymes are treated as alternative to chymosin as there are increasing demands of rennet. The strain of *P. chrysosporium*, extracellular enzyme, was produced and the medium was specially formulated with yeast extract and salts. Pepstatin A could completely inhibit the enzyme (Mendieta et al. 2006). Effective milk coagulation was observed in several strains of *B. subtilis* which can be applied in cheese making. Natto is a fermented food preparation from soybean, and many strains of *B. subtilis* capable of producing aspartic protease were isolated from this.

Chymosin manufactured through recombinant DNA technology is used today in industrial cheesemaking. Recombinant chymosin was first introduced in 1998 on an experimental scale. Maxiren and chymogen are two commercial recombinant enzymes prepared by DSM and Christian Hansen, respectively. Fermentation-produced chymosin (FPC) is prepared by introducing the sequence of calf

chymosin in microorganisms. Camel chymosin was also reported to be produced through recombinant techniques and its commercial production was also achieved. This is referred to as fermentation-produced camel chymosin (FCC). Use of FCC results in cheese with rich fat content in a ripening period of 150 days. It depicts the desirable property of limited hydrolysis yielding soft and chewier product with very low bitterness (Bansal et al. 2009).

Baking Industry

During the baking process, the batter or dough is acted upon by a range of enzymes. The macromolecules are hydrolysed to yield different products altering the texture and properties like flavour, aroma and softness. The incorporation of protease to the flour enables effective protein digestion and lessens the time of maturing. The presence of enzyme increases the dough consistency and uniformity. Proteases can reduce gluten elasticity and dough shrinkage. It becomes easier to mould the flour in different shapes to prepare cookies, biscuits, etc. (Mamo and Assefa 2018).

In baking industry, the composition and properties of wheat flour have an important role. The texture of the dough is mainly dependent on the presence of gluten, a wheat protein, and gluten forms the basic network structure. The degradation of gluten provides enhanced smoothness to the dough and avoids allergy problems associated with gluten. In a report using chemically acidified dough (CAD) as a control, the authors point out that the quantity of free amino acids was enhanced in the enzyme-treated dough. The amounts of water-soluble peptides were also increased. Wheat flour when treated with *A. niger and A. oryzae* showed less immunogenic gluten-derived peptides. A higher rate hydrolysis by acid proteases from *A. niger and A. oryzae* could efficiently degrade gluten. The increased concentration of amino acids in the treated flour supported this fact (Heredia-Sandovan et al. 2016).

Beer and Wine Industry

Grape makes the largest share of crop fruits and forms the basis of wine industry. A major problem in keeping wine and beer is the formation of haze. Hazy wine is an undesirable condition which reduces the clarity and acceptability of the wine. Many factors including shipping conditions affect the quality of wine, hence, attaining stability before bottling is essential. The microbial instability, tartrate instability and protein instability are three major reasons behind the production of haze.

Microbial instability and tartrate instability can be addressed by many methods like sulphur dioxide treatment and electrodialysis. Protein instability is controlled by the addition of bentonite. Bentonite can bind to the protein as a cation exchanger and precipitate bentonite sediments. The sedimented bentonite–protein complex comprises about 3–10% volume of wine. Wine is separated by using vacuum

filtration or centrifugation. These processes are mechanical, and the quality of wine is affected further by these operations. Van Sluyter et al. (2015) point out that bentonite precipitation leading to quality degradation and wine volume reduction accounts for about a loss of US S 1 billion per year. Bentonite addition has other adversary effects like occupational hazards due to inhalation, disposal problems and slip hazards by slurry.

The presence of haze in the beer affects its appearance. The clarity of beer is decreased by haze, and it influences the colour and nature of the foam. Clear, white foam denotes fresh preparations which are more palatable. Commonly, two types of hazes are encountered: chill haze (cold break) and age-related haze. Chill haze appears at very low temperatures near 0 °C and as temperature increases, it gradually disappears. Chill haze is therefore reversible. The age-related haze is irreversible and related to the quality of beer. Proteins are the main components of the haze with polyphenols and carbohydrates composing less than 20%. Chill haze is formed by the interaction of proteins and polyphenols noncovalently, while in the irreversible haze, covalent bond formation stabilises the peptide and polyphenol complexes. On increasing shelf life, the gradual enhancement in molecular interaction makes the permanent haze more visible.

The haze formation in wine is divided into three stages: protein unfolding, protein aggregation and aggregate cross-linking. The haze-forming proteins in grapes show variations in quantity and expression according to cultivar, seasonal changes, vintage, etc. The major groups include thaumatin like proteins, chitinases and a small percentage of pathogenesis-related proteins (PR proteins). PR proteins are highly expressed in ripened proteins.

The TLP and chitinases are unstable near 40 °C and undergo denaturation. The analysis of the structure of these proteins and hazing experiments reveals that they show aggregation potential at normal temperatures. In the wine, the above-mentioned proteins gradually unfold according to an increase in temperature. The unfolded proteins have a potential to aggregate together. Polyphenol is also involved in the aggregate formation along with proteins. A suggested mechanism involves the unfolding of the protein at higher storage temperatures exposing the hydrophobic inner core with a loop like structure. The disulfide bridge is reduced due to elevated temperature and exposed hydrophobic region can interact with the neighbouring proteins.

The formation of haze is inhibited in many ways. Silica adsorbents were used to adsorb the proteins. Removal of polyphenols which interact with proteins is also effective and a promising way is to treat the proteins with proteases. During fermentation, the addition of acid protease from *Saccharomyces fibuligera* and *Torulopsis magnolia* could inhibit the formation of hazes and acidic proteases from *A. niger* produced almost haze free beer. The breaking down of large polypeptides produces smaller components which are more soluble and provide higher yield of ethanol (Mamo and Assefa 2018).

Protein Hydrolysates

Various proteases from plant and microbial sources are extensively used for protein degradation and preparation of protein hydrolysates. The application of the protein hydrolysates depends upon the type of the substrate selected and the nature of enzyme used. Protein hydrolysates play an important role as supplements in infant food, health products and soft drinks. The hydrolysates may be prepared from casein, whey, soy protein, gelatine, meat protein and fish proteins. Even though alkaline proteases are predominantly used for the preparation of protein hydrolysate, neutral and acid proteases are also being exploited (Sun et al. 2018; Karamac et al. 2016). Aspartic protease from *Rhizomucor miehei* on expressing *Pichia* retained its protein hydrolysing activity. The allergic reactions caused by gluten in wheat-based food can be reduced by acid protease treatment. Acid proteases have thorough hydrolysing capability by which gluten-related peptides can be completely degraded to nullify their immunogenic effects (Heredia-Sandovan et al. 2016). In the animal farms, edible flesh is generated, the remains of which are accumulated in bulk. These are underutilised sources of protein hydrolysates and their processing by enzyme is an ideal example of green recycling. Aspartic proteases from microbes with their extensive proteolytic activities can act as suitable candidates for the generation of value-added products from farm-generated waste (Hou et al. 2017).

Aspartic proteases can also be used as flavour enhancers and also for the removal of bitter taste in protein hydrolysates. Bitter taste of these hydrolysates is due to hydrophobic amino acids and also proline. A suitable combination of endopeptidase and aminopeptidase is generally used for reducing the bitter taste of the protein hydrolysate.

Synthesis of Aspartame

Aspartic proteases are also being used in the preparation of Aspartame, the non-calorific artificial sweetener. This dipeptide is composed of L-aspartic acid and L-phenylalanine. The sweetness is attributed to the two L-amino acids. The precursor to aspartame is generally prepared through chemical methods and is found to be non-specific and the yield obtained was generally less. The enzymatic synthesis of the aspartame precursor using protease is a green method and was found to be highly specific. The process takes place under mild conditions, and the yield is found to be satisfactory. Aspartame can be prepared enzymatically using immobilised preparation of thermolysin from *Bacillus proteolyticus* (Miyanaga et al. 1995).

Fruit Juice Clarification

Fruit juices usually carry turbidity because of the various types of proteins present in it. Specific acid proteases have been used for the clarification of many fruit juices. These aspartic proteases include Aspergillopepsin I, Aspergillopepsin II, Enzeco protease, Novoenzyme, Zumizyme and Papain. Better fruit juice clarification is affected on treating with a combination of proteases along with heat treatment. Treatment of fruit juices with proteases after centrifugation and cold treatment has also been found to be active. Acid proteases can reduce the amount of proteins in fruit juices and make them clearer. The clarification of currant juice was achieved by the addition of five different proteases. The aspartic protease from *Aspergillus niger* was the best in removing these proteins. In cherry juice clarification, protease derived from *Aspergillus* sp. was found to be effective (Meyer et al. 2010).

Meat Tenderisation

Tenderness of meat is prerequisite for meat industry and tenderisation makes the meat soft and more palatable. It improves the digestibility of meat and cutting, chopping, etc. becoming easier. Tenderisation is achieved by many methods. Traditional methods mainly depend on plant extracts containing proteolytic enzymes. But sometimes this provides over tenderisation caused by high degree of hydrolysis. Microorganism-derived proteases are also being exploited for meat tenderisation. An aspartic protease from *R. miehei* could provide high yield and could enhance the tenderness of meat (Sun et al. 2018).

Future Trends

In food industry, there is always a search for new enzymes with digestive and processing properties. Thermostable and psychrophilic enzymes are significant in industrial processes as they can be applied to different temperature conditions without denaturation. Screening and isolation processes have been in progress for novel and stable enzymes with high specificity and turn over. Another approach is to clone the desirable gene in suitable hosts for enzyme synthesis. Design of protein structure with bioinformatics tools is being practised according to the needs of the industry. A promising trend in enzyme industry is directed evolution to engineer the enzyme properties for a desirable function.

References

An Z, He X, Gao W, Zhao W, Zhang W (2014) Characteristics of miniature cheddar type cheese made by microbial rennet from *Bacillus amyloliquefaciens*: a comparison with commercial calf rennet. J Food Sci 79(2):214–221

Asakura T, Watanabe H, Keiko A, Solichi A (1997) Oryzasin as an aspartic proteinase occurring in rice seeds: purification, characterisation and application to milk clotting. J Agric Food Chem 45:1070–1075

Aworh OC, Muller HG (1987) Cheese making properties of vegetable rennet from Sodom apple (*Calotrpes procera*). Food Chem 26:71–79

Bansal N, Piraino P, Harboe M, McSweeny PLH, Drake MA, Broe M, Fox PF (2009) Suitability of recombinant camel (*Cameles dromedaries*) chymosin as a coagulant for cheddar cheese. Int Dairy J 19(9):510–517

Benkahaul M, Benchiheub M, Bellil I, Khelifi I, Mechara AM (2016) Physical and chemical properties of the acid protease from *Onopordum acanthium*: comparison between electrophoresis and HPLC degradation casein profiles. Afr J Biotechnol 15(9):331–340. https://doi.org/10.5897/ajb2015.14810

Borah D, Yadav RNS, Sangra A, Shahin L, Chaubey AK (2012) Production, purification and characterisation of nattokinase from Bacillus subtilis, isolated from tea garden soil samples of Dibrugarh, Assum. Asian J Pharmacol Clin Res 3:124–125

Chen YM, Lu TY, Weng TM (2010) Comparison of milk clotting activity of proteinase produced by *Bacillus subtilis* var. Natto and *Rhizopus oligoporus* with commercial rennet. J Anim Sci 23 (10):1369–1379

Claverie M, Hernandez MC (2007) Aspartic protease used in cheese making. In: Industrial enzymes: structure, function and applications. Springer, Berlin, pp 207–219

da Silva RR, de Oliveira LC, Juliano MA, Juliano L, de Oliveira AH, Rosa JC, Cabrod H (2017) Biochemical and milk clotting properties and mapping of catalytic subsites of an extra cellular aspartic peptidase from *Basidiomucete fungus*, *Phanerochete chrysosporium*. Food Chem 225:45–54

Devraj KB, Gowda LR, Prakash V (2008) An unusual thermostable aspartic protease from the latex of *Ficus racemosa* (L). Phytochemistry 69(3):647–655

Domingos A, Xue Z, Cardoso CP, Pais M (2001) Purification, cloning and autoproteolytic processing of an aspartic proteinase from *Centaurea calcitrapa*. Eur J Biochem 267(23):6824–6831

Egito AS, Girardet JM, Laguna LE, Porison C, Molle D, Miclo L, Humbert G, Gaillard JL (2007) Milk clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k-casein. Int Dairy J 17:816–825

Feijoo-Siota L, Villa TG (2011) Native and biotechnologically engineered plant proteases with industrial applications. Food Bioprocess Technol 4:1066–1088

Garg SK, Johri BN (1994) Application of recombinant calf chymosin in cheese making. J Appl Anim Res 7:105–114

Garg GK, Virupaks TK (1970) Acid protease from germinated sorghum: Purification and characterisation of enzyme. Eur J Biochem 17(1):4–12

Heredia-Sandovan NG, Valencia-Tapia NY, Barca AMC, IslasRubio AR, Marioth M (2016) Microbial proteases in Baked goods. Modification of gluten and effects on immunogenicity and product quality. Foods 53:59. https://doi.org/10.3390/food5030059

Hou Y, Wu Z, Dai Z, Wang G, Wu G (2017) Protein hydrolysate in animal nutrition; industrial production, bioactive peptides and functional significance. J Anim Sci Biotechnol 8:24. https://doi.org/10.1186/S40104-017-0153-9

Karamac M, Kosinka-Cagnazzo A, Kulczyk A (2016) Use of different proteases to obtain flaxseed protein hydrolysates with antioxidant activity. Int J Mol Sci 17(7):1027

Kazemipour N, Inchebron MS, Valizadeh J, Sepehrimanesh M (2017) Clotting characteristics of milk by *Withania coagulans*. Proteomic and biochemical study. Int J Food Prop 20(6):1290–1301. https://doi.org/10.1080/10942912.2016.1207664

Khan F (2013) New microbial proteases in leather and detergent industries. Innov Res Chem 1:1–6

Kirk O, Borchert TV, Fuglsang C (2002) Industrial enzyme applications. Curr Opin Biotechnol 13 (4):345–351

Krishnaswamy MA, Johar DS, Subrahmanian V, Thomas SP (1961) Manufacture of cheddar cheese with the milk clotting enzyme from *Ficus carica* (vegetable rennet). Food Technol 15 (11):482–485

Kumar S, Raj DC, Niranjan K, Bhat ZF (2014) Paneer—an Indian soft cheese variant: a review. J Food Sci Technol 51(5):821–831

Llorente BE, Brutti CB, Caffini NO (2004) Purification and characterisation of a milk clotting aspartic proteinase from globe artichoke *Cyanarascolymus* L. J Agric Food Chem 52 (26):8182–8189

Macedo IQ, Faro CJ, Pires EM (1993) Specificity and kinetics of the milk clotting enzyme from cardoon (*Cyanara cardunculus* L) toward bovine. kappa.–casein. J Agric Food Chem 41 (10):1537–1540

Mamo J, Assefa F (2018) The role of microbial aspartic protease enzyme in food and beverage industries. J Food Qual. Article ID 7957269. https://doi.org/10.1155/2018/7957269

Manahan SE (2005) Green chemistry and ten principles of sustainability. Chem Car Research, Inc.

Mendieta JR, Pagano MR, Munoz FF, Daleo GR, Guevara MG (2006) Antimicrobial activity of potato aspartic proteases (StAPs) involves membrane permeabilization. Microbiology 152(Pt 7):2039–2047

Meyer AS, Zeuner B, Jimenez M (2010) Juice clarification by protease and pectinase treatments indicates new role of pectin and protein in cherry juice turbidity. Food Bioprod Process 88(2–3):259–265

Miyanaga M, Tanaka T, Sakiyama T, Nakanishi K (1995) Synthess of aspartame precursor with a immobilised thermolysin in mixed organic solvents. Biotechnol Bioeng 46(6):631–635

Motyan JA, Toth F, Tozser J (2013) Research application of proteolytic enzymes in molecular biology. Biomolecules 3:923–942

Rao MR, Tankasale AM, Ghatgeand MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62(3):597–635

Roger AS, Joln MW (2018) Role of biocatalysts in sustainable chemistry. Chem Rev 118:801–838

Shoda S, Uyama H, Kadokawa J, Kimura S, Kobayashi S (2016) Enzymes as green catalysts for precision macromolecular synthesis. Chem Rev 116(4):2307–2413

Silva LG, Carcia O, Lopes MT, Salas CE (1997) Changes in protein profile during coagulation of latex from Caricapapaya. Brazelian J Med Biol Res 30(5):615–619

Silva AC, Nascimento TE, Silva SA, Herculano PN, Moreira KA (2013) Potential of quixaba (*Sideroxylon obtusifolium*) latex as a milk-clotting agent. Food Sci Technol 33(3):494–499

Simoes I, Faro C (2004) Structure and function of plant aspartic proteinases. Eur J Biochem 271 (11):2067–2075

Sun Q, Chen F, Genq F, Luo Y, Geng S, Jiang Z (2018) A novel aspartic protease from *Rhizomucor meihei* expressed in *Pichia pastoris* and its application on meat tenderisation and preparation of turtle peptides. Food Chem 245:570–577. https://doi.org/10.1016/j.foodchem.2017.10.113

Theron LW, Divol B (2014) Microbial aspartic proteases: current and potential applications in industry. Appl Environ Microbiol 98(21):8853–8868

Vairo-Cavalli S, Claver S, Priolo N, Natalucci CI (2005) Extractionand partial characterisation of a coagulant preparation from *Sylibum marianum* flowers. Its action on Bovine caseinate. J Dairy Sci 72:271–275

Van Sluyter SC, Warnock NI, Schmidt S et al (2013) Aspartic acid protease from *Botrytis cinerea* removes haze forming proteins during white wine making. J Agric Food Chem 61:9705–9711

Van Sluyter SC, McRae JM, Falconer RJ, Smith PA, Bacic A, Waters EJ, Marabgan M (2015) Wine protein haze: mechanism of formation and advance in prevention. J Agric Food Chem 63 (16):4020–4030

Wackett LP, Hershberger CD (2001) Biocatalysis and biodegradation microbial transformation of organic compounds. ASM Press, Washington DC

Chapter 4
Influence of Proteases on Functional Properties of Food

Bindhumol Ismail, Hanif Mohammed and A. Jayakumaran Nair

Abstract Proteases are a class of enzymes occupying an important role with respect to their applications in both physiological and commercial fields. They promote the cleavage of peptide bonds in other proteins and are one of the largest groups of enzymes falling in the group of exopeptidases and endopeptidases. Proteases fall into different classes and find applications in various industries. The stability and activity of some proteases in high acidic environment make them a very potential candidate in food processing. They can be obtained from various sources including plants, animals, and microbes, even though microbial source being the most prevalent source. Protein engineering techniques also have been utilized to obtain proteases of unique specificity and stability. Nowadays, a strong "omic" approach, degradomics permits a wide look into the biodiversity of proteases. Due to this wide diversity they find role as nutritional improvers, proteolytic tenderization, etc. and help in bio-functional changes.

4.1 Introduction

Proteases are a class of enzymes which are ubiquitous in nature and are found in wide variety of sources like plant, animals and microorganisms. They fall into the group of hydrolases mainly involved in the hydrolysis of peptides. Papain, bromelain, keratinase and ficinare some of the well-known proteases from plant sources while the most familiar proteases from animal sources are pancreatic trypsin, chymotrypsin, pepsin and rennins. Microorganisms are an excellent source of these enzymes because of their broad biochemical diversity and are easily susceptible to genetic manipulation. Nowadays, 40% of the total world-wide enzyme sale is from microbial proteases.

Proteases are classified as endopeptidases or exopeptidases enzymes. The peptide bond proximal to the amino or carboxy termini of the substrate is cleaved by

B. Ismail (✉) · H. Mohammed · A. J. Nair
Department of Biotechnology, University of Kerala, Thiruvananthapuram, India
e-mail: bindu.ismail@gmail.com

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_4

the action of exopeptidases. Their action at the N or C terminus determines them as aminopeptidases and carboxypeptidases. Endopeptidases act on the peptide bonds within the substrate. The endopeptidases are further classified based on the functional group present at the active site. They fall into four prominent groups viz; serine proteases, aspartic proteases, cysteine proteases and metallo-proteases (Rao et al. 1998; Jaswal et al. 2008). They are again classified as acidic, neutral or alkaline proteases based on the pH optima of the protease enzymes (Vishalakshi and Dayanand 2009; Sawant and Nagendran 2014).

Proteases perform their roles in various industries (Fig. 4.1) like food processing, beverage production, animal nutrition, leather, paper and pulp, textiles, detergents, etc. (Mahajan et al. 2015). Several clinical studies have thrown light into the benefits of proteases in oncology, inflammatory conditions, blood rheology control and immune regulation.

The main protease enzymes being used in food industry include Alcalase®, Neutrase®, Esperase®, Protamex™, Novozym®, VERON® HPP and VERON® S50. They improve the functional, nutritional and flavour properties of proteins. They help in improving the palatability and stability of available protein sources. The protease enzymes used in alcohol production helps in improving yeast growth. The proteins in flour for biscuits, crackers and cookies are degraded with the help of

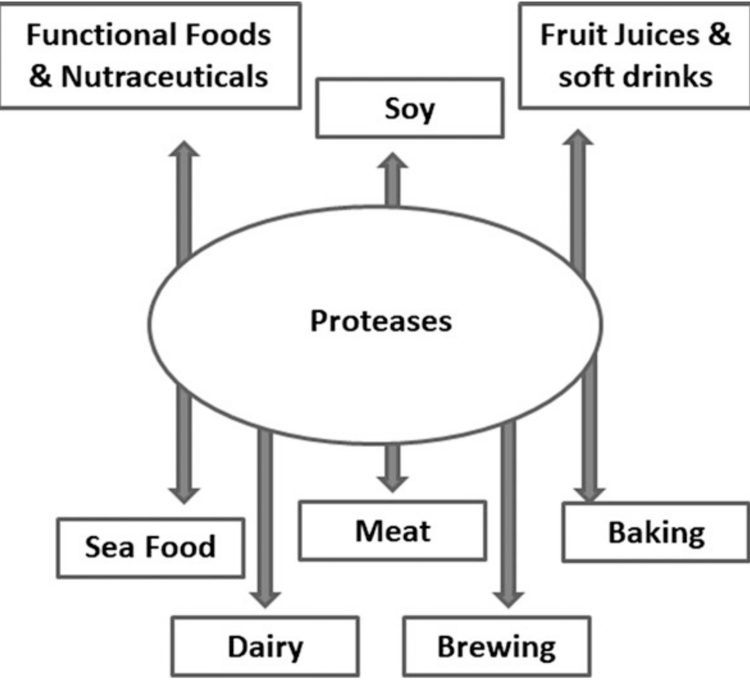


Fig. 4.1 Food industries employing proteases

Table 4.1 Proteases and their role in various food industries

Food industry	Application
Sea food	Deskinning and descaling, fish protein hydrolysate, fish sauce, salted fish ripening, sea food flavourings, caviar production
Meat	Tenderization
Dairy	Cheese coagulation, cheese ripening and debittering, whey protein hydrolysis, prevention of oxidized flavor in milk
Brewing	Clarification, malting
Baking	Gluten hydrolysis, texture and flavor improvement in bread, baked goods, crackers, pastries, biscuits and cookies
Soy	Soy protein hydrolysate, soy sauce
Functional foods and nutraceuticals	Production of bioactive peptides, infant food formulation
Fruit juices and soft drinks	Clarification, fortification of fruit juices and soft drinks

proteases. The desired level of nitrogen nutrients is attained with the help of proteases in brewing. They also aid in flavour modification in dairy products.

Proteases of various kinds are being used for cheese ripening and for modification of milk proteins to reduce the milk allergens (Afroz et al. 2015). Rennet is an enzyme belonging to proteases that has been used traditionally in the production of cheese. Similarly, tenderization of meats has been achieved with papain from the leaves and unripe fruit of *Carica papaya*. All these discoveries led to the development of important food applications for a wide range of available proteases (Table 4.1). This part of the book deals with the main roles of proteases in influencing the functional properties of various foods.

4.2 Green Processes for Protease Production

Of all the different sources of proteases, microorganisms form a very important source both in terms of economy as well as profit. Proteases can be obtained at industrial level from microbes using submerged and solid-state fermentation. Most of the microorganisms can be sources of more than one kind of proteases. The nature of proteases is also dependent on the composition of the media used. In such a scenario, the green protocol of using agro-industrial byproducts is an excellent idea. They are good and cheap sources of proteins, carbohydrates and minerals needed for the growth of these microorganisms and synthesis of microbial enzymes (Mukhtar and Haq 2013). Some of the common agro-industrial byproducts being used are wheat bran, soybean meal, sugar cane bagasse, corn stover, residues of coffee, paper and oil processing, cereal bran, husks and different defatted oil seed cakes. Nowadays, the eco-friendly approach of using agro-industrial byproducts for

the commercial production of proteases is increasing based on the enzyme level achieved using the process.

4.3 Proteases and Food Industries

4.3.1 Sea Food Industry

Sea food industry is a very important food industry where proteases from marine environment and other sources find wide applications in producing various food products and also as fish processing aids. Some of the main applications are as follows:

4.3.1.1 Deskinning and Descaling

Proteases are mainly used in sea food industry for deskinning and descaling. The process is mainly aimed to cause minimum damage to the flesh but currently mechanical procedures are being used which can result in an increased flesh damage. The increased flesh damage reduces the yield of the product (Fernandes 2016). Proteases serve as a good alternative for the purpose. Enzymatic deskinning is usually done by pepsin at low reaction temperatures. Deskinning aids in improving appearance and it prevents colour change, off-flavour and odour of processed fish products (Kim et al. 2002).

Similarly descaling could be attained with the help of enzymes replacing mechanical procedures. Mild acid treatment denatures the skin proteins and loosens the mucus layer and enzymatic action degrades the outer skin structures adhering the scales to the skin. Incubation in a mixture of enzymes at low reaction temperature helps in removing the scale without affecting the skin or flesh (Sikorski et al. 1995).

4.3.1.2 Caviar Production

Caviars are salt-cured, preserved eggs of aquatic animals. These eggs have been singled out and screened or separated from the supporting connective tissue. Fish eggs, when they are in skeins are commonly known as roe. The most widely recognized and valued caviar is made from sturgeon harvested from the Caspian Sea (Bledsoe et al. 2003). Caviar from paddlefish is also attaining significant interest nowadays. In addition to this catfish (*Ictalurus punctatus*) and Salmon or Red Caviar (Ikura) is also being used in the production of fish roe products.

Lumpfish Caviar, Tobiko or Flying Fish Roe, Imitation Tobiko, Whitefish Roe, Cod Roe, Shad Roe, Mullet Roe Products, Orange Roughy Roe, Herring Roe or

Kazunoko, Pollock Roe or Mentaiko, Hake Roe, Rock Sole Roe, Sea Urchin Roe (Uni), Sea Cucumber Roe and Roe from Crustaceans are the other widely used roe products. Fish roes have high concentration of lipid and protein, with lipid content ranging from 5 to 20% and protein 16–30%. They are also a rich source of vitamins (Eun et al. 1994).

Optimal maturity of roes is a critical factor in the preparation of caviar as immature roes produce caviars that are bitter in taste and overly matured ones may become soft and lose their elasticity. The preparation of caviar is initiated by a riddling process, consisting of separation of the roe from the roe sack (ovaries), should be taken from the fish at the moment of slaughter. The riddling process done either manually or mechanically destroys a large amount of roe while releasing the roe particles from the supportive connective tissue of the roe sac. This could be overcome with enzymes, collagenases to achieve a gentle separation of roe from connective tissue (Xu et al. 1996; Haard and Simpson 1994). Proteolytic enzymes as pepsin from poikilothermic organisms have also been used for the process. These proteases are highly active at low temperatures making it advantageous that the activity of the enzyme is easily deactivated by increasing temperature under protein denaturation temperature and also the enzymatic reaction at a low temperature prevents bacterial growth during enzymatic process. The separated roes are then brined or salted and then cured for 8 h or more at approximately 10 °C on inclined perforated plastic baskets or trays. The curing releases the excess surface and cellular fluid from the eggs and also permits the salt concentration within the egg to equilibrate and the eggs sheath to harden slightly.

4.3.1.3 Fish Sauce Production

Fish sauce is a traditional fermented product made from small pelagic species such as anchovy and sardine. Fish sauce, an amber solution in which salt and hydrolyzed fish protein are the major components, is mainly produced by mixing with sea salt and storing at ambient temperature in a storage tank for 6–12 months. Trypsin and chymotrypsin in fish intestine are major factors for protein degradation and solubilization during fish sauce fermentation, even if their activity is partly inhibited by the salt. Chymotrypsin is more important than trypsin during fermentation since chymotryptic activity is more active at neutral and weak acid conditions than trypsin (Heu et al. 1997).

Fish sauce production is a time-consuming process, which may take from months to years for completion (Faisal et al. 2015; Lee et al. 2015). Several methods were tried for the reduction of production time. Hydrolysis of fish tissue at lower salt content and at acid condition, fermentation at elevated temperatures and fermentation at alkaline condition with low salt content are the three methods tried. All the processes showed improved liquefaction time but the products showed inferior flavour and bitter taste. Plant proteases like bromelain, papain and ficin were also used to shorten fermentation time but the characteristic flavour of the products obtained was inferior on comparison to the traditional ones. The use of

marine proteases, squid hepatopancreatic proteases during fish sauce fermentation of capelin produced best results. The fermentation time of capelin sauce supplemented with squid hepatopancreas was shortened to 6 months and the product, Capelin sauce showed higher acceptability when compared to traditional fish sauce or other commercial protease supplements (Kim et al. 2002).

4.3.1.4 Fish Hydrolysate

Fish protein hydrolysates are prepared by extensive digestion of inexpensive pelagic fish and fish processing wastes such as bones, head, liver, skin, trimmings and viscera of fish flesh and of minces using proteolytic enzymes at adequate reaction conditions, leading to peptides with 2–20 amino acids (Fernandes 2016). They have been mainly used as food or feed ingredients. The traditional preparation of fish sauce involved the use of acids or alkali for hydrolysis. The operation is carried out at high temperatures and pressures. But the main disadvantages of the process are the destruction of the key aminoacid tryptophan and formation of other toxic compounds impairing the functionality of the hydrolysate(Kristinsson and Rasco 2000). These disadvantages were overcome with the technically and economically attractive enzymatic approach of using proteases at mild temperature, pressure and pH conditions (He et al. 2013). This process of fish protein hydrolysate production consists of protease treatment, inactivation of the enzymes, separation of soluble fraction, concentration and drying of the fraction. One of the key issues with the protein hydrolysates is their bitter taste reducing the consumer acceptancy. It can be removed by selective separation with chromatography and by enzymatic treatment inducing plastein synthesis and excessive protein hydrolysis to peptides and aminoacids. The most promising method was the use of exopeptidases and cold-adapted protease from fish was found to have a relatively high specificity for peptide bonds improving the level of pleasant-tasting active amino acid eliminating the bitter taste in protein hydrolysis (Haard 1992).

4.3.1.5 Salted Fish Ripening

The presence of salt induces some chemical and physicochemical changes in the flesh of some fatty fish species, leading to a process called ripening or “anchoado” (Czerner and Isabel 2013). Dry-salted fish is highly accepted by consumers due to its unique flavour and resistance to storage. The flavours are developed as a result of various reactions as lipolysis oxidation, protein degradation and Maillard reaction. Cathepsins, calpains, dipeptidases and aminopeptidases are the endogenous proteases that degrade proteins into polypeptides and free aminoacids in pickled products (Gallego et al. 2014; Toldra et al. 2000). Cathepsin B plays an important role in textural changes in fish tissue (Wua and Caoa 2018).

Among such products, salted-ripened anchovy (*Engraulis encrasicholus*) from Mediterranean countries has the highest international relevance. Salted-ripened

anchovy is a traditional product exhibiting typical sensorial characteristics as firm consistency, reddish colour, juicy texture and characteristic odour and flavour (Czerner et al. 2011).

4.3.1.6 Sea Food Flavourings

Exogenous and endogenous proteases play a very important role in the extraction of flavour compounds from fish and shellfish (Haard 1992). They are used as food additives in artificial crab, kamaboko, fish sausage and cereal-based extrusion products. The production process consists of degradation of raw material by enzymatic hydrolysis, thermal inactivation of enzymes, separation of bone and shells, filtration or centrifugation and concentration of flavour enhancers. A combination of protease and amylase also tried in some cases in which the peptides released by protease react with sugars released by amylase to generate a cooked meat aroma (Kim et al. 1996).

4.3.2 Meat Industry

Meat tenderness is a major factor affecting consumer satisfaction and eating quality. Meat tenderization is mainly governed by the degradation of key structural proteins and myofibrillar fragmentation. Tenderness can be improved by different physical, chemical and enzymatic methods. Enzymatic methods include infusion, marinating or injection with exogenous proteases from various sources (Table 4.2) as plants (ficin-*Ficus glabrata*, *Ficus anthelmintica*, and *Ficus laurifolia*, bromelain-pineapple, papain-*Carica papaya* and zingibain-Ginger), fruits (cucumisin-*Cucumis trigonus Roxburghii*, actinidin-*Actinidia deliciosa*) and microbes (collagenase, aspartic protease from *Aspergillus oryzae*, thermophile protease from *Bacillus strain*) (Bekhit et al. 2014). Bacterial proteases have relatively high specific activity and low inactivation temperatures making them useful for meat tenderization (Bekhit et al. 2014).

Table 4.2 Meat tenderizing enzymes and their sources

Enzyme	Source
Papain	*Carica papaya*
Bromelain	*Ananas comosus*
Ficin	*Ficus glabrata*, *Ficus anthelmintica*, *Ficus laurifolia*
Actinidin	*Actinidia deliciosa*
Cucumisin	*Cucumis trigonus Roxburghii*
Zingibain	*Zingiber officinale*
Aspartic protease	*Aspergillus oryzae*
Subtilisin, Neutral protease	*Bacillus subtilis*

The introduction of proteases into meat is an important factor in tenderization. A dipping method in which cut meat was dipped in a solution containing papain or proteinases from *Aspergillus* after contact-osmotic dehydration was found to be sufficient for meat tenderization (Gerelt et al. 2000). The method decreased the hardness in the meats treated with proteolytic enzymes and the sensory scores were also high. The papain-treated meat received the highest score in tenderness and rapid increase in fragmentation of myofibrils.

4.3.3 Dairy Industry

Growing urbanization has put a demand on processed dairy products and dairy industry is one of the oldest industries where proteases were of immense use. Proteases find their role in cheese production, whey hydrolysis and prevention of oxidized flavour in milk.

4.3.3.1 Cheese Coagulation

The protease enzyme, animal rennet (bovine chymosin) is conventionally being used as a milk-clotting agent for manufacturing quality cheeses of good flavour and texture. The increased demand for the calf rennet has put way to the search for an effective alternative as microbial rennet (Afroz et al. 2015). *Rhizomucor pusillus*, *Rhizomucor miehei*, *Endothia parasitica*, *Aspergillus oryzae* and *Irpexlactis* are being extensively used for rennet production in cheese manufacture. But two major drawbacks were exhibited by the microbial rennet (i) the presence of high levels of nonspecific and heat-stable proteases, which led to the development of bitterness in cheese after storage and (ii) poor yield (Rao et al. 1998). Extensive research has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases reducing bitterness. Nowadays, microbial rennet is being used for about 70% of USA cheese and 33% of cheese production world-wide.

Rennet helps in the hydrolysis of a specific peptide linkage in the k-casein protein present in milk in cheese making. It hydrolyses the bond between phenylalanine and methionine residues. The main function of k-casein is stabilization of the colloidal nature of the milk. Its hydrophobic N-terminal region associates with the lipophilic regions of the otherwise insoluble a- and b-casein molecules, while its negatively charged C-terminal region associates with water, preventing the casein micelles from growing too large. Hydrolysis of the labile peptide linkage between these two domains helps in the release of a hydrophilic glycosylated and phosphorylated oligopeptide (caseinomacropeptide) and the hydrophobic para-k-casein.

This removes the protective effect, allowing coagulation of the milk to form curds, which are then compressed and turned into cheese. The coagulation process is dependent on the presence of Ca^{2+} and temperature.

4.3.3.2 Cheese Ripening and Debittering

The commercial interest in cheese ripening owing to its quality maintenance overrules the high cost of ripening facilities. Accelerated cheese ripening for good flavour and textural development adding to the quality is obtained with the help of proteases. The intensity of cheese flavour is increased with a combination of individual neutral proteinases and microbial peptidases and its use with microbial rennet reduced the intensity of bitterness produced as a result of the peptides formed.

A combination of exogenous proteinases and Lactococcal cell-free extracts (rich in peptidases) accelerates ripening. The enzymes that have been used to accelerate cheese ripening include microbial serine proteinases, neutral proteinases, lactase and even lipases.

In the production of enzyme modified cheese (EMC) an extreme form of accelerated ripening is practiced. They are produced by using a cocktail of enzymes (proteinases, peptidases, lipases) and sometimes bacterial cultures to homogenized, pasteurized fresh curd or young cheese.

There is also a chance of developing unwanted bitter flavours as a result of the hydrolysis of cheese proteins by endopeptidases as animal and bacterial proteases. This is due to the accumulation of hydrophobic peptides. Debittering of the cheese can be obtained with Flavourpro 937MDP (by M/s. Biocatalysts Ltd., Wales), which is an exopeptidase preparation with low levels of endopeptidase activity. Flavorpro can be used to control bitterness by removing the bitter-tasting peptides.

4.3.3.3 Whey Protein Hydrolysis

The other proteins present in milk such as lactalbumin and lactoglobulin, with a molecular weight of 14.2 and 36 kDa respectively, are denatured with the help of proteases. Protease 2A and Trypsin could be used for whey protein hydrolysis. The other proteolytic enzymes as pancreatin and proteases from *Bacillus licheniformis*, *Aspergillus oryzae* and *Aspergillus sojae* were also used for the hydrolysis and proteases from *Bacillus licheniformis* found to be more effective than the others (Morais et al. 2014). Enzymatic hydrolysis of whey protein by selective proteases reduces the chances of undesirable side reactions or products. It also provides more moderate conditions of the process. The peptides produced by proteolysis are of smaller molecular sizes and less secondary structure and expected to have increased solubility, decreased viscosity and significant changes in foaming, gelling and emulsifying properties from those of original proteins(Severin and Xia 2006). This whey hydrolysis is essential for the production of creamier yogurts and for cheese production.

4.3.3.4 Prevention of Oxidized Flavour in Milk

Dairy products are prone to the spontaneous production of oxidized flavours. This flavour is implicated by the presence of several native milk enzymes. Peroxidases and Xanthin oxidase are the main enzymes which cause oxidative flavours (Browne 1993). Bovine trypsin and trypsin from cold-adapted fish help in the prevention of oxidized flavour. Trypsin from cold-adapted fish demonstrates more thermal stability than the bovine trypsin (Simpson and Haard 1984).

4.3.4 Brewing Industry

Proteases find their role in clarification by digestion of proteins and aid in malting in the brewing industry.

4.3.4.1 Clarification

In the brewing industry, proteins and polyphenols extracted from the disrupted plant tissue in beer and wine making results in the formation of haze (Lopez and Edens 2005). The haze-forming capability of proteins is directly linked to proline residues (Siebert 1999). Peptide bonds with proline residues are difficult to be cleaved and proteases of specific substrate specificity find their role in this cleavage. Proteases eliminate heat-unstable proteins, which cause heat-induced haze or precipitate in wine. It can be used at any stage of the wine-making process, but more advantageous to use at the beginning of fermentation before the generation of inhibitive factors.

Protease increase the degree of solubility of the proteins and accordingly lowers the viscosity of beer, in addition protease develops a good condition for yeast growth by satisfying the availability of free amino nitrogen.

4.3.4.2 Malting

During malting there occurs hydrolysis of proteins, during which a portion of insoluble proteins are being converted to soluble proteins for obtaining good brews (Jones and Budde 2005). The overall protease activity will be high during malting and a mixture of exo- and endoproteases is involved in the process.

4.3.5 Baking Industry

Proteases find their applications in baking industry mainly in gluten hydrolysis and texture and flavour improvement of bread, baked goods, crackers, pastries, biscuits and cookies.

4.3.5.1 Gluten Hydrolysis

Wheat gluten is often hydrolyzed enzymatically using endo and exopeptidases to release free aminoacids or small peptides. These peptides serve as flavour compounds and flavour precursors (Raksakulthai and Haard 2003). Gluten hydrolysis favours the development of gluten-free diet for people suffering from gluten allergy and also gluten hydrolysates are being used for their emulsifying and foaming properties. Several commercially available proteases as Alcalase 2.4L, PTN 6.0S, Pepsin, Pancreatin, Neutrase and Protamex™ are being used to hydrolyse wheat gluten. The protein recovery and hydrolytic efficiency was more with alcalase (Kong et al. 2007).

Flavourzyme™ is also an enzyme preparation usually being used for the hydrolysis of wheat gluten and other food proteins (Merz et al. 2015a). This enzyme preparation consists of seven proteolytic enzymes as well as amylase, esterase, phosphatase and glycosidase activity (Merz et al. 2015b).

4.3.5.2 Texture and Flavour Improvement

The insoluble protein present in wheat flour plays a very important role in determining the properties of bakery doughs. A wide range of products are being produced from wheat flour as enzymatic treatment of the dough facilitates its handling and machining. Mostly, endo- and exoproteinases from *Aspergillus oryzae* are being used to modify wheat gluten by limited proteolysis. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are also being used to improve the extensibility and strength of the dough (Rao et al. 1998). The action of proteases reduces gluten elasticity and thereby reduces shrinkage of dough. The elasticity of dough has considerable improving effect on the spread ration of cookies. The dough rheology, texture of bread and flavour is regulated by the gluten strength due to the modification by proteases (Hedayati and Sani 2016; Salleh et al. 2006).

Sourdough sweet-leavened baked goods as the Genoese dry biscuit, lagaccio, soft cake from north Italy, panettone and typical sourdough bread are also obtained by microbial protease fermentation from lactic acid bacteria (Moroni et al. 2009). *Lactobacillus* has a complex protease system capable of hydrolyzing various proline-rich peptides. Different species of *Lactobacillus* as *Lactobacillus alimentarius* 15 M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A and

Lactobacillus hilgardii 51B are being used during wheat flour fermentation (Di Cagno et al. 2004).

VERON® HPP, a high performing protease preparation and VERON® S50, a papain protease in powder form are new proteases being used in baking industry. The former is used to manufacture long-life confectionery products. VERON® HPP provides reduced dough resting times, with improved dough extensibility and dough handling in biscuit and cracker production. The final products obtained were of improved quality.

The dough properties of bakery products are improved by VERON® S50. The properties of baked biscuits and crackers as friability, browning, well-rounded edges, smooth surface, reduced cracking, uniform weight and size for packaging could be improved with VERON® S50.

4.3.6 Soy Industry

A high content of good quality protein in soy sauce and other soy products increases their demand in the food industry. Proteases, mainly alkaline and neutral proteases of fungal origin, play a crucial role in the preparation of soy products. They help in the improvement of functional properties of soy products and produce soluble hydrolysates with high solubility, good protein yield and low bitterness.

4.3.6.1 Soy Protein Hydrolysate

Soy protein hydrolysates find their role in protein-fortified soft drinks. It is also being used in the formulation of dietetic feeds (Rao et al. 1998). But the bitter taste of protein hydrolysates prevents their use in food products. The bitterness is attributed to the number of hydrophobic amino acids in the hydrolysate and the presence of a proline residue in the center of the peptide. Debittering could be reduced by cleaving hydrophobic amino acids and proline. Debitrase, aminopeptidases from lactic acid bacteria and Carboxypeptidase A have high specificity for hydrophobic amino acids and hence has a great potential for debittering. The use of an endoprotease for primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness.

4.3.6.2 Soy Sauce

Soy sauce is one of the mostly consumed oriental fermented products. It is a dark brown coloured liquid prepared by months of enzymatic brewing of a mixture of soybean, wheat and salt and is used as a flavouring agent. Mainly it is produced by a two-step fermentation process in which the first step is the fermentation for

production of proteolytic and amylolytic enzymes in the Koji using mold, followed by fermentation with yeast and bacteria, moromi (Tanaka et al. 2005). Koji is a source of proteolytic enzymes for converting soy bean proteins into peptides and amino acids. It is also a source of amylase for hydrolyzing gelatinized starch into simple sugars. The substances converted by the enzymes in Koji functions as nutrients for yeasts and lactic bacteria in the subsequent brine fermentation (Luh 1995).

4.3.7 Functional Foods and Nutraceuticals

Proteins being the main unit of health promoting molecules essential for normal growth, life maintenance and reproduction, they are of great concern in functional foods and nutraceuticals. They also contribute to the physicochemical and sensory properties of foods. Functional foods and nutraceuticals is one of the growing food industries where proteases are of great demand. Proteases are being used for the generation of bioactive peptides and infant food formulation in the industry.

4.3.7.1 Bioactive Peptides

Bioactive peptides are the one with specific biological properties produced as a result of partial digestion by proteases. They are produced from various sources of food proteins to function as an ingredient in functional food (Udenigwe and Aluko 2012). Nearly 1500 bioactive peptides have been reported in a database called 'Biopep' (Singh et al. 2014).

Bioactive peptides display various modes of actions as antimicrobial, immunomodulatory, antioxidative, opioid, mineral-binding, anti-thrombotic and antihypertensive (Sánchez and Vázquez 2017). Metabolic disorders and lifestyle diseases are prevented with the help of such peptide sequences (Mazorra-Manzano et al. 2017). The various food sources of bioactive peptides (Table 4.3) are milk, cheese, dairy products, gelatin, meat, eggs, various fish species as tuna, sardine, herring and salmon, wheat, maize, soy, rice, mushrooms, pumpkin, sorghum and amaranth (Mohanty et al. 2016; Pritchard et al. 2010; Choi et al. 2012; Lassoued et al. 2015; Kumagai 2010; Singh et al. 2014; Selamassakul et al. 2016; Moller et al. 2008; Silva-Sanchez et al. 2008; Mahdi et al. 2018).

The production of bioactive peptides and the different proteases being used in some of the important food sources commercially being used are explained.

Milk

Milk proteins possess immunoglobulins of immunoprotective effect, lactoferrin with antibacterial activity and growth factors and hormones, playing significant

Table 4.3 Bioactive peptides from various sources

Source	Peptide sequence	Function	Reference
Bovine milk			
	Asp-Lys-Ile-His-Pro-Phe, Tyr-Gln-Glu-Pro-Val-Leu	ACE inhibitory	Sánchez and Vázquez (2017)
	Val-Pro-Pro, Ile-Pro-Pro	ACE inhibitory	
	Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Thr-Met-Pro-Leu-Trp	Opioid, ACE inhibitory, immune-stimulatory	
	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met	Antioxidative	
	Tyr-Leu-Leu-Phe	ACE inhibitory	
	Lys-Val-Leu-Pro-Val-P(Glu)	ACE inhibitory	
	LKKISQRYQKFALPQY	Calmodulin binding	
Goat milk			
	LYQEPVLGPVRGPFPI	Antioxidative	Mahdi et al. (2018)
	YQEPVLGPVRGPFPIL	Antioxidative	
	VQSWMHQPPQPLSPT	Antioxidative	
Donkey milk			
	EWFTFLKEAGQGAKDMWR	Antioxidative	Chiozzi et al. (2016)
	GQGAKDMWR	Antioxidative	
	REWFTFLK	ACE inhibitory	
	MPFLKSPIVPF	ACE inhibitory	

(continued)

Table 4.3 (continued)

Source	Peptide sequence	Function	Reference
Fish			
Sardine	Leu-His-Tyr	Antioxidative	Bougatef et al. (2010)
	Leu-Ala-Arg-Leu	Antioxidative	
	Gly-Gly-Glu	Antioxidative	
	Gly-Ala-His	Antioxidative	
	Gly-Ala-Trp-Ala	Antioxidative	
	Pro-His-Tyr-Leu	Antioxidative	
	Gly-Ala-Leu-Ala-Ala-His	Antioxidative	
Shellfish	Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu	Antioxidative	Ngo and Kim (2013)
Egg			
	LAPSLPGKPKPD	Antidiabetic, antioxidative	Zambrowicz et al. (2015)
Meat			
	Arg-Pro-Arg, Lys-Ala-Pro-Val-Ala, Pro-Thr-Pro-Val-Pro	Antihypertensive	Escudero et al. (2012)
Fruits			
Plum	MLPSLPK, HLPLL, NLPLL, HNLPLL, KGVL, HLPLLR, HGVLQ, GLYSPH, LVRVQ, YLSF, DQVPR, LPLLR, VKPVAPF	Antioxidative, antihypertensive	González-García et al. (2014)

roles in post-natal development (Park and Nam 2015). These active peptides are released during gastrointestinal digestion or food processing (Table 4.3). Opioid peptides with pharmacological properties similar to morphine also exist in dairy and they play an active role in central nervous system (Haque et al. 2008).

Fermentation of milk proteins using lactic acid bacteria (LAB) is an effective method being followed to generate functional foods enriched with bioactive peptides from milk. They are produced by using different species of *Lactobacillus* as *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Lactobacillus delbueckii bulgaricus*, etc. In some cases, addition of pepsin and trypsin is also done for effective hydrolysis. The bioactive peptides thus released possess mineral-binding, opioid, ACE inhibitory, immunomodulatory, cytotoxicity, anti-carcinogenic, antibacterial and anti-thrombotic activities (Sánchez and Vázquez 2017). There will be some variation on the nature of bioactive peptide released based on the milk sources as buffalo, camel, goat, mare, sheep and yak milk.

Whey Protein Hydrolysate

Whey protein hydrolysate produced with proteases result in more digestible and functional peptides which on incorporation into food formulae bring out favourable contributions to texture and taste and reduction of allergenic effects. Trypsin, promatex, flavorzyme, protease N and crude commercial enzymes, 2709 alkaline protease and ASI.398 neutral protease have been used in the preparation of whey protein hydrolysates for the generation of bioactive peptides with antioxidant activity (Samuel and Rong-Rong 2011).

Soy Protein Hydrolysate

A variety of endoproteases as pronase, trypsin, Glu C protease, plasma proteases and kidney membrane proteases are being used to generate bioactive peptides from soy hydrolysate and the soy-fermented foods, natto and tempeh. A peptide with ACE inhibitory activity and a peptide with surface active properties were obtained from natto while hydrolyzing using pronase and a peptide with anti-thrombotic activity was produced with kidney protease. Soy protein hydrolysates with improved antioxidant properties can be generated using commercial proteases as neutral protease from *Bacillus subtilis* and validase from *Aspergillus oryzae* or Alcalase from *Bacillus licheniformis*. Soy protein hydrolysate with antioxidative property and improved functional properties was obtained by enzymatic hydrolysis using a protease preparation from *Chryseobacterium* sp. kr6 (de Oliveira et al. 2015).

4.3.7.2 Infant Food Formulation

Infant food formulation becomes an alternative for breast feeding when breast feeding is not possible or discontinued for some reasons. It should possess all the nutrients required for the adequate growth of infants and protein and amino acid requirement being higher than adults should be a good supplementary for proteins. Currently, cow's milk or soy proteins are being used in infant formula (Nguyen et al. 2015). But both vary in their aminoacid profile and digestibility. Soy protein has lower content of methionine, so needs to be added with methionine (Agostoni et al. 2006).

Cow milk is a potential allergen in infants and heat denaturation alone cannot be efficient in lowering allergic reactions. The main allergen in cow's milk protein is β-lactoglobulin. β-lactoglobulin is the most abundant protein in whey accounting for 50% of the total protein. Infant formula manufacturers are also adding whey proteins to infant formula as hydrolysed whey protein can be used to reduce allergenicity. They possess a superior taste, smell and amino acid profile compared to casein hydrolysates. Proteases, endo and exopeptidases, can be taken into consideration which along with heat denaturation reduces antigenicity and allergic reactions. Whey protein hydrolysates utilized in infant formula are generally either extensively hydrolysed or partially hydrolysed. Partial hydrolysis result in large peptides and extensive hydrolysis produces a mixture of large and small peptides and free aminoacids (Lee 1992).

Trypsin, chymotrypsin and pancreatin were being used to produce hypoallergenic whey protein hydrolysate for use in infant formula. But recently, non-animal protease Flavorpro™ 766MDP with both endopeptidase and exopeptidase activities was used to hydrolyse whey protein to produce a highly digestible whey protein hydrolysate, with a high degree of hydrolysis. The enzyme could efficiently hydrolyse beta-lactoglobulin and reduce allergenicity and produce whey protein hydrolysate with lower bitterness score.

4.3.8 Fruit Juices and Soft Drinks

In fruit juice and soft drink industry, proteases are widely being used for juice clarification and fortification.

4.3.8.1 Clarification

Clarification is mainly done to remove constituents that are responsible for the immediate turbidity and cloudiness in freshly produced juice. The clarification method also helps in the removal of substances (proteins and polyphenols) that may cause haze and sediment formation during storage as well as reconstitution of the concentrate or after bottling of the juice (Meyer et al. 2001). Industrially juice

clarification obtained by a combination of enzymatic depectinization, gelatin–silica sol, and/or bentonite treatment. But the gelatin-silica sol step is slow and downstream processing is also cumbersome. Alternatively, combination of enzymes pectinase and protease could be used.

Different treatment methods were tried for achieving juice clarification in various fruits. The addition of fungal acid protease could decrease the immediate turbidity and retard the haze formation during cold storage of kiwifruit juice (Dawes et al. 1994). Acid stable protease, pectinase and gallic acid combination proved successful in improving cherry juice clarity and diminished haze levels during cold storage. Cherry juice clarification was also achieved with addition of a pectinase, Pectinex Smash® and a protease, Enzeco, gallic acid and tannic acid as factors. Both these enzyme preparations are derived from *Aspergillus* sp. (Pinelo et al. 2010). Similarly black currant juice clarification and haze retardation was also achieved with certain fungal acid proteases. Two selected acid proteases (Enzeco and Novozyme 89L), a pectinase (Pectinex BE 3-L) and gallic acid were also used for the clarification of black currant juice (Landbo et al. 2006). A complex enzyme treatment of pectinase and protease was also favorable for pomegranate juice clarification (Hmid et al. 2016; Cerreti et al. 2017).

4.3.8.2 Fortification

Fruit drinks contain very little amount of protein as nutritional component, so needs to be fortified with proteins and carbohydrates to meet the specific needs. Most of the fruit juices are acidic in the range 3–4 and hence fortification of fruit drinks with protein is a challenge as proteins will become unstable in such an environment (Yadav et al. 2016). Protein hydrolysates (limited hydrolysis) can be an excellent alternative to fortify the foods as they possess greater and instant solubility, stability over wide range of pH and are easily digestible. Whey proteins are the one generally preferred for such fortification. Proteases role in the generation of whey protein hydrolysates is well known and can be made use for fortification also. Hydrolysates produced by acid proteases, Chymax and rennet from un-denatured whey protein concentrate exhibited greater solubility than the heat-denatured counterparts. The clarity of apple juices fortified with whey protein hydrolysate was higher (Goudarzi et al. 2015). High stability, solubility and good sensory acceptability were achieved when mango juice was also fortified with partially hydrolysed whey protein (Yadav et al. 2016).

4.4 Protein Engineering in Proteases

Protein engineering is mainly based on the available knowledge regarding the particular enzyme/protein, its structure, function, softwares used to analyse structure-function activity, bioinformatics database, recombinant strains producing

the particular enzyme and other materials that could change the enzyme and its interaction in the food environment improving the functional properties of foods (Kapoor et al. 2017). The advances in recombinant DNA technology, high throughput technology, genomics and proteomics have paved a way into the protein engineering of proteases. Owing to their particular roles under non-physiological conditions, proteases have been subjected to various protein engineering techniques as rational drug design or directed evolution method. The main aims of protein engineering of proteases were creation of diversity to alter specificity, developing *Escherichia coli* expression systems and specificity for post translationally modified aminoacids. These aspects were mainly made use in the development of cold adaptation of a mesophilic subtilisin-like protease using laboratory evolution techniques as proteases native to cold environments exhibited higher catalytic efficiency at low temperatures and greater thermosensitivity than the mesophilic ones (Wintrode et al. 2000). Programs like SOPMA, ProSA, SAVES, Procheck, ProsaII, etc. can be used for the structural evaluation and validation of the structure of proteases (Syed et al. 2012). One of the important application areas of protein engineering in food industry is the wheat gluten proteins. A wide variety of expression systems have been used for their expression studies and were compared for their protein structure-function relation.

4.5 Conclusion

Proteases due to their wide structural variety and substrate specificity find their roles in various food industries. The use of fermentation technologies with microorganisms created a boom in the enzyme industry. Along with this, the implementation of green protocols for protease production by fermentation has improved the enzyme industry in terms of economy and profit. Protein engineering strategies, "omics" approach and other techniques also will continue to generate proteases of improved functional properties in the food market. The changing consumer demands in the food industry will be met with the growing biotechnological ideas incorporating multiple benefits from proteases, more precisely food and health. The advances in all related fields will boost the protease market and directly the food industry.

References

Afroz QM, Khan KA, Ahmed P, Uprit S (2015) Enzymes used in dairy industries. Int J Appl Res 1 (10):523–527

Agostoni C, Axelsson I, Goulet O, Koletzko B, Michaelsen KF, Puntis J, Rieu D, Rigo J, Shamir R, Szajewska H, Turck D (2006) Soy protein infant formulae and follow-on formulae: a commentary by the ESPGHAN Committee on Nutrition. J Pediatr Gastroenterol Nutr 42 (4):352–361

Bekhit AA, Hopkins DL, Geesink G, Bekhit AA, Franks P (2014) Exogenous proteases for meat tenderization. Crit Rev Food Sci Nutr 54:1012–1031. https://doi.org/10.1080/10408398.2011.623247

Bledsoe GE, Bledsoe CD, Rasco B (2003) Caviars and fish roe products. Crit Rev Food Sci Nutr 43(3):317–356

Bougatef A et al (2010) Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinellaaurita*) by-products proteins. Food Chem 118:559–565

Browne RJ (1993) Dairy products. In: Nagodawithana T, Reed G (eds) Enzymes in food processing, 3rd edn. Academic Press, San Diego, pp 347–361

Cerreti M, Liburdi K, Benucci I, Spinelli SE, Lombardelli C, Esti M (2017) Optimization of pectinase and protease clarification treatment of pomegranate juice. LWT-Food Sci Technol 82 (1):58–65. https://doi.org/10.1016/j.lwt.2017.04.022

Chiozzi RZ, Capriotti AL, Cavaliere C, La Barbera G, Piovesana S, Samperi R, Lagana A (2016) Purification and identification of endogenous antioxidant and ACE-inhibitory peptides from donkey milk by multidimensional liquid chromatography and nano HPLC-high resolution mass spectrometry. Anal Bioanal Chem 408:5657–5666

Choi J, Sabikhi L, Hassan A, Anand S (2012) Bioactive peptides in dairy products. Int J Dairy Technol 65:1–12

Czerner M, Isabel M (2013) Bacterial contribution to salted anchovy (*Engraulis anchoita* Hubbs & Marinni, 1935) ripening process. J Aquat Food Prod Technol. https://doi.org/10.1080/10498850.2012.697537

Czerner M, Tomás MC, Yeannes MI (2011) Lipid oxidation and the development of color and other sensorial characteristics during the ripening of salted anchovy. J Sci Food Agric 91 (4):609–615

Dawes H, Struebi P, Keene J (1994) Kiwifruit juice clarification using a fungal proteolytic enzyme. J Food Sci 59:858–861

de Oliveira CF, Corrêa APF, Coletto D, Daroit DJ, Cladera-Olivera F, Brandelli A (2015) Soy protein hydrolysis with microbial protease to improve antioxidant and functional properties. J Food Sci Technol 52(5):2668–2678. https://doi.org/10.1007/s13197-014-1317-7

Di Cagno R, De Angelis M, Auricchio S, Greco L, Clarke C, De Vincenzi M, D'Archivio M, Landolfo F, Parrilli G, Minervini F et al (2004) Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac sprue patients. Appl Environ Microbiol 70:1088–1096

Escudero E, Toldra F, Sentandreu MA, Nishimura H, Arihara K (2012) Antihypertensive activity of peptides identified in the in vitro gastrointestinal digest of pork meat. Meat Sci 91:382–384

Eun JB, Chung HJ, Hernsberger JO (1994) Chemical composition and micro flora of Channel catfish (*Ictalurus punctatus*) roe and swim bladder. J Agric Food Chem 42:714–717

Faisal M, Islami SNE, Islam MN, Kamal M, Khan MNA (2015) Study on microbial and physical changes in fish sauce during fermentation. Res Agric Livest Fish 2:375–383. https://doi.org/10.3329/ralf.v2i2.25024

Fernandes P (2016) Enzymes in fish and seafood processing. Front Bioeng Biotechnol 4:59. https://doi.org/10.3389/fbioe.2016.00059

Gallego M, Mora L, Fraser PD, Aristoy M-C, Toldrá F (2014) Degradation of LIM domain-binding protein three during processing of Spanish dry-cured ham. Food Chem 149:121–128

Gerelt B, Ikeuchi Y, Suzuki A (2000) Meat tenderization by proteolytic enzymes after osmotic dehydration. Meat Sci 56(3):311–318. https://doi.org/10.1016/S0309-1740(00)00060-7

González-García E, Marina ML, García MC (2014) Plum (*Prunus Domestica* L.) by-product as a new and cheap source of bioactive peptides: extraction method and peptides characterization. J Funct Foods 11:428–437

Goudarzi M, Madadlou A, Mousavi ME, Emam-Djomeh Z (2015) Formulation of apple juice beverages containing whey protein isolate or whey protein hydrolysate based on sensory and physicochemical analysis. Int J Dairy Technol 68(1):70–78

Haard NF (1992) A review of proteolytic enzymes from marine organisms and their application in the food industry. J Aquat Food Prod Technol 1:17–35

Haard NF, Simpson BK (1994) Proteases from aquatic organisms and their uses in the seafood industry. In: Martin AM (ed) Fisheries processing: biotechnological applications. Chapman & Hall, London, pp 132–153

Haque E, Chand R, Kapila S (2008) Biofunctional properties of bioactive peptides of milk origin. Food Rev Int 25:28–43

He S, Franco C, Zhang W (2013) Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). Food Res Int 50:289–297. https://doi.org/10.1016/j.foodres.2012.10.031

Hedayati G, Sani AM (2016) Application of melon serine protease in food processes. Biotechnol Indian J 12(1):18–24

Heu MS, Kim HR, Cho DM, Godber JS, Pyeun JH (1997) Purification and characterization of cathepsin L-like enzyme from the muscle of anchovy, *Engraulis japonica*. Comp Biochem Physiol B: Biochem Mol Biol 18B:523–529

Hmid I, Elothmani D, Hanine H, Oukabli A (2016) Effects of enzymatic clarification of pomegranate juice by protease and pectinase treatments. J Bio Innov 5(4):506–515. ISSN 2277-8330 (Electronic)

Jaswal RK, Kocher GS, Virk MS (2008) Production of alkaline protease by *Bacillus circulans* using agricultural residues: a statistical approach. Indian J Biotechnol 7:356–360

Jones BL, Budde AD (2005) How various malt endoproteinase classes affect wort soluble protein levels. J Cereal Sci 41:95–106

Kapoor S, Rafiq A, Sharma S (2017) Protein engineering and its applications in food industry. Crit Rev Food Sci Nutr 57(11):2321–2329

Kim HR, Ahn CB, Jung KJ, Baek HH, Cadwallader KR (1996) Characterization and utilization of waste water from oyster processing plants as flavoring agents. IFT Annual Meeting, New Orleans, LA, USA

Kim H-R, Seo HJ, Byun DS, Heu M-S, Pyeun J-H (2002) Proteolytic enzymes from fish and their utilization. Fish Sci 68(2):1557–1562

Kong X, Zhou H, Qian H (2007) Enzymatic hydrolysis of wheat gluten by proteases and properties of the resulting hydrolysates. Food Chem 102(3):759–763. https://doi.org/10.1016/j.foodchem.2006.06.062

Kristinsson HG, Rasco BA (2000) Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr 40:43–81. https://doi.org/10.1080/10408690091189266

Kumagai H (2010) Wheat proteins and peptides. In: Mine Y, Li-Chan E, Jiang B (eds) Bioactive proteins and peptides as functional foods and nutraceuticals. Wiley-Blackwell, Oxford, pp 289–303

Landbo A-KR, Pinelo M, Vikbjerg AF, Let MB, Meyer AS (2006) Protease-assisted berry juice clarification. J Agric Food Chem 54(18):6554–6563

Lassoued I, Mora L, Barki A, Aristoy M-C, Nasri M, Toldra F (2015) Bioactive peptides identified in thornback ray skin's gelatin hydrolysates by proteases from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. J Proteomics 128:8–17

Lee YH (1992) Food-processing approaches to altering allergenic potential of milk-based formula. J Pediatr 121(5 Pt 2):S47–S50

Lee HW, Oh JY, Kang DI (2015) Method for manufacturing a fish sauce and a fish sauce prepared by the same. Patent Application US 20150296854 A1

Lopez M, Edens L (2005) Effective prevention of chill-haze in beer using an acid proline-specific endoprotease from *Aspergillus niger*. J Agric Food Chem 53:7944–7949

Luh BS (1995) Industrial production of soy sauce. J Ind Microbiol 14:467–471

Mahajan R, Chaudhari G, Chopadaa M (2015) Report on biotechnological applications of proteolytic enzymes from lattices of euphorbian plants. J Appl Biotechnol Rep 2(4):333–337

Mahdi C, Untari H, Padaga MC (2018) Identification and characterization of bioactive peptides of fermented goat milk as a sources of antioxidant as a therapeutic natural product. In: IOP

conference series: materials science and engineering, vol 299, p 012014. https://doi.org/10.1088/1757-899x/299/1/012014
Mazorra-Manzano MA, Ramírez-Suarez JC, Yada RY (2017) Plant proteases for bioactive peptides release: a review. Crit Rev Food Sci Nutr. https://doi.org/10.1080/10408398.2017.1308312
Merz M, Eisele T, Berends P, Appel D, Rabe S, Blank I, Stressler T, Fischer L (2015a) Flavourzyme, an enzyme preparation with industrial relevance: automated nine-step purification and partial characterization of eight enzymes. J Agric Food Chem 63:5682–5693
Merz M, Eisele T, Claassen W, Appel D, Rabe S, Stressler T, Fischer L (2015b) Continuous long-term hydrolysis of wheat gluten using a principally food-grade enzyme membrane reactor system. Biochem Eng J 99:114–123
Meyer AS, Köser C, Adler-Nissen J (2001) Efficiency of enzymatic and other alternative clarification and fining treatments on turbidity and haze in cherry juice. J Agric Food Chem 49:3644–3650
Mohanty D, Jena R, Choudhury PK, Pattnaik R, Mohapatra S, Saini MR (2016) Milk derived antimicrobial bioactive peptides: a review. Int J Food Prop 19:837–846
Moller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J (2008) Bioactive peptides and proteins from foods: indication for health effects. Eur J Nutr 47:171–182
Morais HA, Silvestre MPC, Amorin LL, Silva VDM, Silva MR, Simões e Silva AC, Silveira JN (2014) Use of different proteases to obtain whey protein concentrate hydrolysates with inhibitory activity toward angiotensin-converting enzyme. J Food Biochem 38(1):102–109
Moroni AV, Dal Bello F, Arendt EK (2009) Sourdough in gluten-free bread-making: an ancient technology to solve a novel issue? Food Microbiol 26:676–684
Mukhtar H, Haq I (2013) Comparative evaluation of agroindustrial byproducts for the production of alkaline protease by wild and mutant strains of *Bacillus subtilis* in submerged and solid state fermentation. Sci World J. https://doi.org/10.1155/2013/538067
Ngo DH, Kim SK (2013) Marine bioactive peptides as potential antioxidants. Curr Protein Pept Sci 14:189–198
Nguyen TTP, Bhandari B, Cichero J, Prakash S (2015) Gastrointestinal digestion of dairy and soy proteins in infant formulas: an in vitro study. Food Res Int 76(3):348–358
Park YW, Nam MS (2015) Bioactive peptides in milk and dairy products: a review. Korean J Food Sci Anim Resour 35:831–840
Pinelo M, Zeuner B, Meyer AS (2010) Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. Food Bioprod Process 88(2–3):259–265
Pritchard SR, Phillips M, Kailasapathy K (2010) Identification of bioactive peptides in commercial Cheddar cheese. Food Res Int 43:1545–1548
Raksakulthai R, Haard NF (2003) Exopeptidases and their application to reduce bitterness in food: a review. Crit Rev Food Sci Nutr 43:401–445
Rao MB, Tanksale AM, Ghatge MS, Despande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62(3):597–635
Salleh AB, Razak CNA, Rahman RNZRA, Basri M (2006) Protease: introduction. In: Salleh AB, Rahman RNZRA, Basri M (eds) New lipases and proteases. Nova Science Publishers, New York, pp 23–39
Samuel MK, Rong-Rong L (2011) The effect of enzymes and hydrolysis conditions on degree of hydrolysis and DPPH radical scavenging activity of whey protein hydrolysates. Curr Res Dairy Sci 3:25–35
Sánchez A, Vázquez A (2017) Bioactive peptides: a review. Food Qual Saf 1:29–46. https://doi.org/10.1093/fqs/fyx006
Sawant R, Nagendran S (2014) Protease: an enzyme with multiple industrial applications. World J Pharm Pharm Sci 3(6):568–579
Selamassakul O, Laohakunjit N, Kerdchoechuen O, Ratanakhanokchai K (2016) A novel multi-biofunctional protein from brown rice hydrolysed by endo/endo-exoproteases. Food Funct 7:2635–2644

Severin S, Xia WS (2006) Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. J Food Biochem 30:77–97
Siebert KJ (1999) Effects of protein-polyphenol interactions on beverage haze, stabilization, and analysis. J Agric Food Chem 47:353–362
Sikorski ZE, Gildberg A, Ruiter A (1995) Fish products. In: Reuiter A (ed) Fish and fisher product-composition, nutritive properties and stability. CAB International, Wallingford, pp 3154–3346
Silva-Sanchez C, de la Rosa APB, Leon-Galvan MF, de Lumen BO, de Leon-Rodriguez A, de Mejia EG (2008) Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. J Agric Food Chem 56:1233–1240
Simpson BK, Haard NF (1984) Purification and characterization of trypsin from the Greenland cod (*Gadus ogac*). 1. Kinetic and thermodynamic characteristics. Can J Biochem Cell Biol 62 (9):894–900. https://doi.org/10.1139/o84-114
Singh BP, Vij S, Hati S (2014) Functional significance of bioactive peptides derived from soybean. Peptides 54:171–179
Syed R, Sabeena RR, Masoodi TA, Shafi G, Alharbi K (2012) Functional analysis and structure determination of alkaline protease from *Aspergillus flavus*. Bioinformation 8(4):175–180
Tanaka F, Morita K, Mallikarjunan P, Hung YC, Ezeike G (2005) Analysis of dielectric properties of soy sauce. J Food Eng 71(1):92–97
Toldra F, Aristoy M-C, Flores M (2000) Contribution of muscle aminopeptidases to flavor development in dry-cured ham. Food Res Int 33:181–185
Udenigwe CC, Aluko RE (2012) Food protein-derived bioactive peptides: production, processing, and potential health benefits. J Food Sci 71:R11–R24
Vishalakshi N, Dayanand A (2009) Production of alkaline protease from *Streptomyces gulbargensis* and its application in removal of blood stain. Indian J Biotechnol 8(1):280–285
Wintrode PL, Miyazaki K, Arnold FH (2000) Cold adaptation of a mesophilicsubtilisin-like protease by laboratory evolution. J Biol Chem 275:31635–31640
Wua YY, Caoa SM (2018) Study on endogenous protease and protein degradation of dry-salted *Decapterus maruadsi*. CYTA-J Food 16(1):350–356. https://doi.org/10.1080/19476337.2017.1406006
Xu RA, Wong RJ, Rogers ML, Fletcher GC (1996) Purification and characterization of acidic proteases from the stomach of the deepwater finfish orange roughy (*Hoplostethus atlanticus*). J Food Biochem 20:31–48
Yadav DN, Vishwakarma RK, Borad S, Bansal S, Jaiswal AK, Sharma M (2016) Development of protein fortified mango based ready-to-serve beverage. J Food Sci Technol 53(10):3844–3852. https://doi.org/10.1007/s13197-016-2395-5
Zambrowicz A et al (2015) Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization. Amino Acids 47:369–380

Chapter 5
Recent Development in the Uses of Asparaginase as Food Enzyme

Shahenvaz Alam, Kumar Pranaw, Rameshwar Tiwari and Sunil Kumar Khare

Abstract Asparaginase (E.C.3.5.1.1), an important enzyme, is broadly disseminated in all the three domains of life and responsible for catalysing conversion of asparagine into aspartic acid and ammonia. It is one of the most utilized clinical enzymes used in the treatment of different types of cancers. However, there has been a renewed interest in other application of this enzyme especially for minimizing the acrylamide content in baked/fried starchy food products. Acrylamide is generated as a by-product of Maillard reactions between asparagine and reducing sugars. The reactions usually occur at temperature above 100 °C and account for colour and flavour developments in fried/baked starchy foods. In the year 1994, Acrylamide was first time classified in Group B2, i.e. as probably carcinogenic to humans by the International agency for research on cancer. Significant contents of acrylamide have been detected in range of food products including roasted potatoes, root vegetables, chips, crisps, toasts, cakes, biscuits, cereals and coffee. Extensive efforts have been made to reduce the formation of acrylamide during baking/toasting or frying by incorporating asparaginase enzyme as pretreatment. The present chapter encompasses all these aspects and sources of asparaginases, their enzymatic properties, engineered thermostable asparaginase and their various applications in food processing.

5.1 Introduction

Asparaginase (asparagine aminohydrolase; E.C.3.5.1.1) belongs to the amino acid amidohydrolase that catalyses the conversion of L-asparagine to L-aspartate and ammonia. This enzymatic reaction played a central role in a number of metabolic processes in diverse living organisms. Asparaginases have remarkable considerable attention from last few decades due to its application in amino acid metabolism,

S. Alam · K. Pranaw · R. Tiwari · S. K. Khare (✉)
Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, Hauz Khas, New Delhi 110016, India
e-mail: skkhare@chemistry.iitd.ac.in

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_5

human health and food industry. The global demand of asparaginase was 380 million USD in 2017 and market will reach upto 420 million USD by 2025 (Global Asparaginase Sales Market Report 2018). Hence, it is important to deal with the limitation to meet this huge global demand. Although, the enzyme is widely distributed in almost all clade of life including plant, animal, bacteria and archaea, however, increasing attention has been drawn to microbial asparaginases particularly from bacterial sources due to high yield, easy maintenance, versatile characteristics and suitability for the industrial application.

For last more than 4 decades, asparaginase enzyme was widely studied and used in the treatment of different type of cancer mainly non-Hodgkin's lymphoma, acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in children. It is one of the leading medically important enzymes because of its high efficiency and efficacy against leukemia and lymphoblastic leukemia (Swain et al. 1993). Asparagine (a free amino acid) found in foods and taken up by the lymphoma or myeloid cells as a nutrient. Asparaginases convert asparagine into aspartate and ammonia (Krishnapura and Belur 2016), which reduces the asparagine availability to myeloid cells (Fig. 5.1). Generally, myeloid cells do not have asparagine synthetase enzyme which is responsible for asparagine production and due to unavailability of asparagine, it leads to starvation of cell (Asselin and Rizzari 2015).

Apart from its medicinal use, in recent years, asparaginase was also explored in the food industry for the reduction of acrylamide formation in finished products. The International agency for research on cancer classified acrylamide in Group B2, i.e. as probably carcinogenic to humans (IARC 1994). Acrylamide is mainly

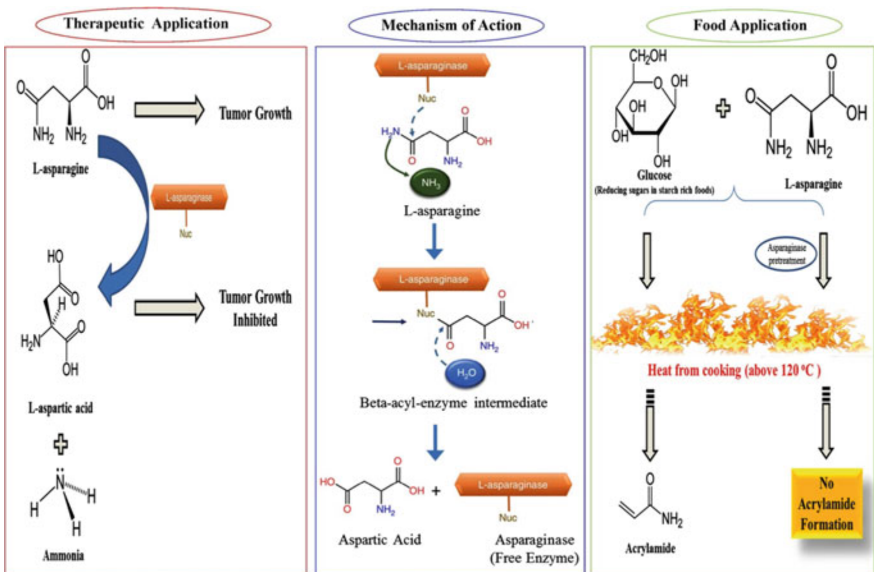


Fig. 5.1 Mechanism of action of asparaginase and its role in different applications

formed during baking/frying of starchy/high carbohydrate food items, when reducing sugar and asparagine reacts at high temperature more than 120 °C and with low water content. The reference dose (RfD) established for acrylamide is around 25–35 μg of acrylamide per day for a person weighing 68 kg by different agencies like the US EPA, United Kingdom (British Food Standard Agency; www.foodstandards.gov.uk), Norway (www.snt.no), Switzerland (Swiss Federal Office of Public Health 2002) and Germany (www.bfr.bund.de). Hence, the food industry is under pressure to reduce the formation of acrylamide in different food products. Various mitigation strategies like physicochemical methods, asparaginase pretreatment, etc were reported by several researchers. Physicochemical methods were not well accepted due to the formation of different secondary attributes in the finished food. Asparaginase played a major role in the food industry for reducing the acrylamide content without forming any secondary attributes like odour, souring etc (Fig. 5.1). Asparaginases enzyme hydrolysed the asparagine during pretreatment which leads to the inhibition of Maillard reaction and the amount of acrylamide was reduced significantly (Pedreschi et al. 2008; Ravi and Gurunathan 2018). Although the use of asparaginase in food industry has several limitations like high cost, low productivity and low thermal stability still remain a matter of concern.

Some researchers have worked upon thermal stability of enzyme by screening asparaginase from thermostable microbial sources (Pradhan et al. 2013). Moreover, engineering of asparaginase enzyme using different techniques like immobilization of asparaginase (Balcao et al. 2001) and specific site saturation mutagenesis (Feng et al. 2018) for thermal stability and high yield were also studied.

There are commercially available asparaginases which were efficiently utilized for acrylamide reduction, i.e. PreventASe™ from DSM (Heerlen, The Netherlands) and Acrylaway® from Novozymes A/S (Bagsvaerd, Denmark). For better enzymatic properties, and thermal stability, enzymes need to be isolated from novel sources with enhanced properties.

This book chapter highlights different microbial sources of asparaginase and their enzymatic characteristics. Asparaginase mechanism of action, sequence-based structural and functional relationship, chemical and molecular modifications of enzyme for enhancing enzymatic properties is also elaborated.

5.1.1 History of Asparaginase

The discovery of asparaginase as anticancer drug started first in the year 1953, when J. G. Kidd showed the regressive effect of guinea pig serum on transplanted lymphomas in rat and mice model. They had proposed certain active constituents in serum leads to the regression of lymphomas (Kidd 1953). Earlier to this, Clementi in 1922 had suggested the characteristics of guinea pig serum and claimed that they were enriched with asparaginase enzyme (Clementi 1922). Progressive studies by Broome in 1961 also showed the inhibitory effect of asparaginase present in guinea

pig serum on lymphoma 6C3HED cells (Broome 1963). Further, advanced studies by Mashburn and Wriston (1963) indicated the potential of microbial asparaginase as a mean of chemotherapeutic agent to inhibit tumour growth. Tumour cells require asparagine as nutrient source and the ability of asparaginase to deplete asparagine made it possible in the lost battle of fighting against malignant tumour and lymphoblastic lymphomas. Asparaginase from microbial sources came out as flawless weapon and explored widely for its medical application. Several trials were tested to treat Acute Lymphoblastic leukemia (ALL) and later it was proposed as an excellent approach for absolution in leukemic patients (Hill et al. 1967).

5.1.2 Types of Asparaginase

L-asparaginase of two types, i.e. type I and type II asparaginase, which are widely distributed in microorganisms, plants, vertebrates and animal tissues (Qeshmi et al. 2018). Type I asparaginases are found in the cytosol, and hence, these enzymes are named as cytosolic asparaginase which shows a low affinity towards L-asparagine and greater affinity towards L-glutamine. Hence, these enzymes are considered confound as they showed an affinity towards the unspecific substrate. Type II asparaginases are better studied and found at the vicinities of periplasmic spaces. They are produced extracellularly. They showed high affinity towards L-asparagine and less specific towards L-glutamine (Swain et al. 1993). Both enzymes were substrate specific but Type II enzymes were less immunogenic. Due to this, Type II asparaginase has been comprehensively used for medical applications, mainly in treatment of acute lymphoblastic leukemia (ALL) (Zuo et al. 2015). Type II asparaginase has also been implicated in the food industry for removal of asparagine from the primary ingredients before processing to reduce the acrylamide formation in the finished food items (Hendriksen et al. 2009).

5.1.3 Classification of Asparaginases

L-asparaginase is produced by all kingdoms of life: Plants, microorganisms and animals. Hence, enzymes are classified based on the sequences of amino acids and biochemical characteristics of the enzyme. It is classified and grouped into mainly three types: bacterial, plant and rhizobial type. Bacterial enzymes mainly from *E. coli* and *Erwinia chrysanthemi* were studied extensively because of their varied properties and has been used vigorously as chemotherapeutic agent in ALL treatment from last two decades (Duval et al. 2002; Shi et al. 2017).

(a) **Bacterial type asparaginase**

Most of the Type II enzymes are isolated from mesophilic sources consisting tetra dimers and their molecular masses were analysed in the range of 140–170 KDa, whereas Type I enzymes are mainly isolated from thermophilic sources and are homodimer structurally. The molecular mass of this enzyme was up to 70 KDa. Some of the microbes like *E. coli* produced both isozymes in which Type I enzymes are cytosolic and have a low affinity for L-asparagine and considered to be a constitutive enzyme. Type II asparaginase showed higher affinity for L-asparagine and considered periplasmic. These enzymes are expressed subject to stress conditions, aeration and changes in the media or nutrient sources like carbon and nitrogen sources, etc. One of the two types of asparaginase showed antitumour activity and these properties vary from strain to strain and culture conditions. *E. coli* and *P. geniculate* comprise of type II asparaginase and known for their antitumour properties. Whereas in *Mycobacterium tuberculosis*, it has both isozymes but only one enzyme has shown antitumour activity. These unlike activities differ due to changes in pH, temperature, clearance rate (Half-life inside serum), activity profile and significant activity towards L-asparagine (Qeshmi et al. 2018; Badoei-Dalfard 2015).

Asparaginase from *E. coli* and *E. chrysanthemi* was studied extensively, and hence, asparaginase enzymes from different species were always harmonized with these microbes. Genus *Bacillus* contains both isozymes and genome sequencing of ansZ gene showed functional asparaginase, with 59% similarity with *Erwinia* asparaginase and 53% similarity with *E. coli* Type II asparaginase. Also, Gene ansA in *Bacillus* sp. encodes Type I asparaginase. Asparaginase from *Bacillus* sp. was utilized expeditiously for removal of acrylamide content in food processing but its role in antitumour activity is a matter of research.

(b) **Plant-type asparaginase**

Asparagine hydrolysis in higher plants is done utilizing two different kinds of routes. In the first catabolic pathway, amino nitrogen was transaminated by the enzyme asparagine oxo acid amino transferase and produces 2-oxosuccinamic acid and an amino acid. The second route utilized L-asparaginase which hydrolysed the asparagine into aspartate and ammonia found mainly in temperate legumes. Plant-type asparaginase is produced as inactive precursor molecules and belongs to N-terminal hydrolases. High level of asparaginase was detected mainly in developing leaves or roots (Michalska et al. 2008). Plants contain two different kind of asparaginase—classified on the basis of reaction catalysis and their potassium dependence. These are immunological significant and separate from each other (Lea et al. 1984; Michalska and Jaskolski 2006).

Plants like *Sphagnum fallax* have two types of cytosolic L-asparaginase. Potassium ions have no effect on the overall stability of the enzyme but required for the synergistic higher activity of the enzyme (Heeschen et al. 1996). Comparatively, potassium-independent enzymes are highly stable than potassium dependent but potassium dependent enzymes have higher affinity for substrate (Chagas and Sodek

2001). L-asparaginase extracted from *Phaseolus vulgaris* has a low glutamine activity which removes unwanted side effects and the purpose of chemotherapy could be sorted in the treatment of ALL (Mohamed et al. 2016).

Plant asparaginases were studied exclusively on the basis of homology modeling comparison with the crystal structure of *E. coli* asparaginase (Michalska et al. 2008). *Sphagnum fallax* asparaginase has intermediate properties of microbial as well as plants of higher origin (Heeschen et al. 1996). Marine algae *Chlamydomonas* and *Pisum* reported to have better enzymatic activity in the light region. Two group of plant asparaginase showed higher sequence similarity with each other and it is unlike to the microbial asparaginase where they are distinct from each other (EcA I cytosolic and EcAII periplasmic).

(c) **Rhizobial-type asparaginase**

Rhizobial-type asparaginase has shown sequence similarity with L-asparaginase from *Rhizobium etli. R. etli* are symbiont bacteria lived with the association of leguminous plants (Borek and Jaskólski 2001; Shi et al. 2017). Peculiar feature of these bacteria was associated with asparagine as they utilized it as a single source of carbon and nitrogen for their living. Asparaginase and aspartase enzymes were employed for this purpose. There are two kinds of asparaginases were reported from *R. etli*; asparaginase I which were constitutive enzyme and thermostable while asparaginase II was thermolabile and expressed only when asparagine is present other than any carbon source. Both the enzyme had not shown any sequence similarity with bacterial asparaginase like *E. coli* and *E. chrysanthemi.* However, type II asparaginase from *R. etli.* was having activity free from glutaminase, which shows a better potential for chemotherapy (Moreno-Enríquez et al. 2012).

5.2 Microbial Sources of Asparaginase

Asparaginase from microbial sources added value to the enzyme as enzymes would inherit the properties of the sources from which they were isolated. Microbial source of asparaginase enzyme is considered as effective as well as an easy way for industrial. A wide range of microbes such as fungi, bacteria, actinomycetes, yeast and algae has been well characterized for the production of L-Asparaginase till date. Different sources of microbial enzyme with their property were listed here in Table 5.1.

Bacillus sp. and *Pseudomonas* sp. are well-known producers of bacterial asparaginase. The prevalence of *Bacillus* sp. in the different environments can be correlated with their typical nature of spore formation, which can be aerially distributed for long distance (Chubukov et al. 2014). The asparaginase production is greatly influenced by media composition as well as the method of production, i.e. solid-state fermentation (SSF) or submerged fermentation (SmF). In bacteria, when the primary carbon source is present along with high concentrations of a variety of

Table 5.1 Different microbial sources of asparaginase with their optimum pH and temperature

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
Bacteria						
1	*Bacillus* sp.	SmF	7	37	–	Moorthy et al. (2010)
2	*B. licheniformis*	SmF	6–10	70	Anticancerous Thermostable	Mahajan et al. (2014)
3	*B. brevis*	SmF	6.7	30	–	Narta et al. (2011)
4	*B. subtilis* BsAIIS40M BsAIID49M	SmF SmF	8 7	65 50	Thermostable	Onishi et al. (2011)
5	*B. subtilis* WB600	SmF	7.5	65	Thermostable GRAS category	Feng et al. (2017)
6	*B. megaterium*	SmF	7	40	Acrylamide reduction	Zhang et al. (2015)
7	*B. subtilis* KDPS1	SSF	5	37	–	Sanghvi et al. (2016)
8	*B. tequilensis* PV9W	SmF	8.5	35	Anticancerous	Shakambari et al. (2016)
9	*B. aryabhattai* ITBHU02	SmF	8.5	40	Antineoplastic activity	Singh et al. (2013)
10	*B. licheniformis* MTCC 429	SmF	8	37	Recombinant Glutaminase-Free l-Asparaginase (ansA3)	Sudhir et al. (2014)
11	*B. subtilis* B11−06	SmF	7.5	40	–	Jia et al. (2013)
12	*Enterobacter cloacae*	SmF	7–8	35–40	Glutaminase-Free l-Asparaginase	Husain et al. (2016a)
13	*E. aerogenes* MTCC111	SmF	8	25	–	Erva et al. (2017)
14	*Pseudomonas otitidis*	SmF	7.5	40	Glutaminase-Free l-Asparaginase	Husain et al. (2016b)
15	*P. aeruginosa* strain SN004	SmF	5.0	50	Asparaginase with low glutaminase activity	Badoei-Dalfard (2015)
16	*P. fluorescens*	SmF	6.3	34	Cloned into *E. coli* BL21 for large scale production	Kishore et al. (2015)
17	*P. aeruginosa* strain EGYII DSM 101801	SmF	8.5	45	Cloned and expressed in *E. coli* BL21(DE3) pLysS	El-Sharkawy et al. (2016)
18	*P. fluorescens*	SmF	7	37	–	Sinha et al. (2015)
19	*P. Aeruginosa* 50071	SSF	9	37	–	El-Bessoumy et al. (2004)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
20	*Erwinia carotovora*	SmF	–	–	Cloned and expressed in *E. coli*	Pourhossein and Korbekandi (2014)
21	*E. aroideae* NRRL B-138	SmF	8.2	45	–	Tiwari and Dua (1996)
22	*E. carotovora*	SmF	7.5	25	Biopharmaceutical properties	Sukhoverkov and Kudryashova (2015)
23	*E. carotovora*	SmF	8.6	35		Kamble et al. (2006)
24	*E. chrysanthemi* 3937	–	–	–		Gilbert et al. (1986)
25	*Escherichia coli* W3110 Free enzyme Immobilized enzyme		7.5 8.5	37 50	Efficiently immobilized in calcium alginate gelatin composites	Youssef and Al-Omair (2008)
26	*E. coli*		7	37	–	Vidya et al. (2014)
27	*E. coli* MTCC 739		7	37	–	Sajitha et al. (2015)
28	*Pectobacterium carotovorum*	SmF	8–10	40	–	Kumar et al. (2011)
29	*Thermococcus kodakaraensis*	SmF	9.5	85	Thermostable enzyme	Chohan and Rashid (2013)
30	*T. gammatolerans* EJ3	SmF	8.5	85	Thermostable enzyme	Zuo et al. (2014)
31	*Pyrococcus furiosus*	SmF	9	85	Thermostable enzyme	Bansal et al. (2010)
32	*Rhizobium etli*	SmF	9	37	Rhizobial-type family of L-Asparaginase	Moreno-Enriquez et al. (2012)
33	*Yersinia pseudotuberculosis* Q66CJ2	SmF	8	60	Cloned and expressed in *E. coli* BL21(DE3)	Pokrovskaya et al. (2012)
34	*Rhodospirillum rubrum*	SmF	9.2	54	–	Pokrovskaia et al. (2013)
35	*Photobacterium* sp. J15	–	7	25	–	Yaacob et al. (2014)
Fungi						
36	*Penicillium* sp.	SmF	7	37	–	Patro and Gupta (2012)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
37	*P. digitatum*	SmF	7	30	–	Shrivastava et al. (2012)
38	*P. brevicompactum* NRC829	SmF	8	37	Anticancerous	Elshafei et al. (2012)
39	*Nocardia levis* MK-VL_113	SmF	7	30	–	Kavitha and Vijayalakshmi (2012)
40	*Aspergillus oryzae* CCT 3940	SmF	8	50	–	Dias et al. (2016)
41	*A.* niger AKV-MKBU	SmF	7	30	–	Vala et al. (2018)
42	*A. aculeatus*	SmF	9	30	–	Dange and Peshwe (2011)
43	*Talaromyces pinophilus*	SmF	8	28	–	Krishnapura and Belur (2016)
44	*Trichoderma viride*	SmF	7	37	–	Lincoln et al. (2015)
45	*Cladosporium* sp.	SSF	6.3	30	–	Kumar and Manonmani (2013)
46	*Rhizomucor miehei*	SmF	7	45	For the food industry and in chemotherapeutics for leukemia	Huang et al. (2013)
47	*Mucor hiemalis*	SmF	7	37	–	Thakur et al. (2013)
Actinomycetes						
48	*Streptomyces* sp.	SmF/SSF	7.5	50	–	Basha et al. (2009)
49	*S. noursei* MTCC 10469	SmF	8	50	Isolated from Marine sponges	Dharmaraj (2011)
50	*S. gulbargensis*	SmF	8.5	40	–	Amena et al. (2010)
51	*S. fradiae* NEAE-82	SmF	8.5	40	Cytotoxicity and anticancer activities	El-Naggar et al. (2016)
52	*S. brollosae* NEAE-115	SSF	–	–	–	El-Naggar et al. (2017)
53	*Nocardiopsis alba* NIOT-VKMA08	SmF	8	37	–	Meena et al. (2015)
54	*Actinobacterial* sp.	SmF	8	35	–	Varma et al. (2016)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
Algae						
55	*Spirulina maxima*	SmF	8.5	37	–	Abd El Baky and Baroty (2016)
56	*Chlamydomonas* species	SmF	–	50	–	Paul (1982)
57	*Chlorella vulgaris*	SmF	–	–	Inducible intracellular l-asparaginase	Ebrahiminezhad et al. (2014)

amino acids under anaerobic conditions, the asparaginase production can be enhanced by 100-fold (Cedar and Schwartz 1968). Since the different metabolites of amino acids like asparagine can contribute directly into the citric acid cycle, and its presence in the medium can trigger the asparaginase gene expression. Although, the model carbon source, glucose may serve as a catabolic repressor for enzyme expression.

The next important and limiting factors for production of asparaginases are their respective microbes. The enzyme catalytic property is importantly influenced by pH, temperature, substrate specificity and other kinetic parameters. It may vary among the same genus and or species. L-asparaginase from most of the *Bacillus* sp. exhibited optimum pH in the range of 6–8 and optimum temperature in the range of 30–40 °C while *B. licheniformis* (Mahajan et al. 2014), *B. subtilis* BsAIIS40M (Onishi et al. 2011), *B. subtilis* WB600 (Feng et al. 2017) and *B. aryabhattai* ITBHU02 (Singh et al. 2013) showed optimum pH around 8.5 and optimum temperature around 60–70 °C. Thermostable asparaginases from bacterial source like *Thermococcus kodakaraensis*, *Thermococcus gammatolerans EJ3*, *Pyrococcus furiosus* etc. are having optimum pH and temperature of 9 and 85°C respectively and can be of interest for industrial applications (Chohan and Rashid 2013; Zuo et al. 2014; Bansal et al. 2010).

When bacterial L-asparaginase used for medicinal purpose, it was found that there is some side effect such as hypersensitivity, pancreatitis, thrombosis, etc., which is mainly due to glutaminase activity (Killander et al. 1976; Ramya et al. 2012). To minimize the side effect, the search of eukaryotic microorganism like yeast and fungi was taken up for production of the enzyme. Several researchers reported that fungi and yeast can produce reasonable production of the enzyme under both solid-state fermentation (SSF) and submerged fermentation (SmF) (Dias et al. 2016; Kumar and Manonmani 2013). The fungal genera such as Aspergillus (Dias et al. 2016; Vala et al. 2018; Dange and Peshwe 2011), Penicillium (Shrivastava et al. 2012; Elshafei et al. 2012; Patro and Gupta 2012) and Trichoderma (Lincoln et al. 2015) are vastly studied for the production of asaparaginases.

Oceans were the cradle of life and they still host an enormous biodiversity which is rather under-explored. Research to explore the marine environment for microorganisms has rapidly increased over the past two decades. Marine microbes majorly include bacteria, archaea and actinomycetes (Izadpanah et al. 2018). Marine actinomycetes were also explored for L-asparaginase production and their application. Genus Streptomyces is the most studied actinomycetes till date (Basha et al. 2009; Dharmaraj 2011; Meena et al. 2015; Varma et al. 2016; Amena et al. 2010; El-Naggar et al. 2016, 2017).

5.3 Application of Asparaginase as Food Enzyme

In recent years, L-asparaginase has been successfully used as food enzyme as it has found an application in reducing acrylamide formation in food system. Asparagine is present in most of the starchy foods, which reacts with their reducing sugar during thermal processing leads to formation of neo formed contaminants named acrylamide which is a bi-product of Maillard reaction (Mottram et al. 2002). It is a potent chemical carcinogen and neurotoxic which forms adduct with the haemoglobin when consumed in high dose and causes reproductive disability (Tyl et al. 2003). Acrylamide toxicity is dose dependent and when ingested in high amount leads to cytotoxicity and necrosis. Acrylamide cytotoxic studies were conducted by Kacar et al. (2018) where acrylamide's cytotoxicity and anti-proliferative properties were analysed on human cell lines A549 and acrylamide (IC_{50}) dose was attained in 24 h was 4.5 mM. Hence, such a small concentration of acrylamide had a deep and regressive effect on human body.

In a view of food system and prevention of human health hazards, acrylamide toxicity and reduction of its content in food systems were studied extensively. Several International Food related agencies like U.S. Food and Drug Administration (FDA), European Food Safety Authority (EFSA) initiated the battle to reduce acrylamide contents in deep fried and baked food products formed during heat processing after its surprise discovery by Swedish scientists in 2002 (Tareke et al. 2002). FDA had issued several magazines and scientific reports claiming the formation of acrylamide in different kinds of food products and formation of other contaminants in heat-processed system (Robin and Clanci 2007). EFSA had done extensive research work on the contents of acrylamide in different food products along with the toxicity levels on human. They had also reported the acrylamide toxicity and its metabolism pathway inside human body where acrylamide is metabolized into glycidamide (GA) which forms an adduct with haemoglobin and leads to genotoxicity and neurotoxicity (EFSA 2015; Bandarra et al. 2013).

Hence, there was a sudden rush to cope up with this major issue. In the wake of this, several studies were conducted and guidelines for cooking were finalized (Robin and Clanci 2007). After that, several parameters were studied like decreasing pH, lowering the temperature for frying and blanching of the potato chips before frying for decreasing the levels of acrylamide in French fries

(Pedreschi et al. 2007). Enzymatic pretreatment of food with asparaginase enzyme leads to reduction of acrylamide at significant levels apart from other treatment procedure (Meghavarnam and Janakiraman 2018). Different food products have been subjected to pretreatment with asparaginase and clearly shown the reduction of acrylamide in variety of food products. Some of the food products with high acrylamide contents and successful reduction of acrylamide using asparaginase enzyme will be listed here (Table 5.2).

Table 5.2 Strategy and use of asparaginase in acrylamide reduction in foods

S. No.	Food products	Enzyme source	Conditions for pretreatment	Acrylamide reduction	References
1	French fries	*Aspergillus oryzae*	Soaking time—1 min, enzyme load—10.5 ASNU/mL	60–85%	Hendriksen et al. (2009)
2	Sliced potato chips	*A. oryzae*	Blanching for 20 min, enzyme load—10.5 ASNU/mL	60%	Hendriksen et al. (2009)
3	Fried dough pastry	Commercial	Different enzyme load—100 U/kg, 500 U/kg and 1000 U/kg	Upto 90%	Kukurová et al. (2009)
4	Potato chips	Commercial	Blanching 85 °C for 3.5 min, enzyme load—10 ASNU/mL	Upto 90%	Pedreschi et al. (2011)
5	Gingerbread	Commercial	Pretreatment time −30 to 60 min, enzyme load—1000 U/kg of dough	97%	Ciesarova et al. (2009)
6	Potato	*Bacillus licheniformis*	30 IU/mL	80%	Mahajan et al. (2012)
7	Potato crisps	*B. subtilis*	Enzyme load—0–40 U	80%	Onishi et al. (2015)
8	French fries	*Thermococcus zilligii*	Enzyme load 0–20 U	80%	Zuo et al. (2015)
9	Fried Kochchi Kesel chips	*A. terreus*	5 U/mL, pretreatment-at 60 °C for 20 min	55%	Ravi and Gurunathan (2018)
10	Potato chips	*Fusarium culmorum*	300 U/L	85%	Meghavarnam and Janakiraman (2018)
11	Sweet bread	*F. culmorum*	300 U/L	78%	Meghavarnam and Janakiraman (2018)

ASNU is defined as the amount of asparaginase that produces 1 μmol of ammonia per min under the conditions of the assay (pH = 7 ± 0.005; 37 ± 0.5 °C) using Acrylaway®

5.4 Asparaginase Sequence Analysis Combined with Mode of Action

Many asparaginase gene sequences have been deposited into GenBank and crystal structure of functional enzymes also available in Protein Database (PDB) which comprises of various microbial sources. Type I asparaginase is from *Lactobacillus reuteri* (PDB ID: A5VMR3), *Escherichia coli* (PDB ID: 2HIM), *Yersinia pestis* (PDB ID: 3NTX), *Vibrio cholera* (PDB ID: 2OCD), *Thermococcus kodakaraensis* (PDB ID: 5OT0), *Pyrococcus furiosus* (PDB ID: 4Q0M) and *P. horikoshii* (PDB ID: 1WLS) while Type II asparaginases are from *E. coli* (PDB ID: 3ECA), *E. chrysanthemi* (PDB ID: 5F52), *Erwinia carotovora* (PDB ID: 2HLN), *Wolinella succinogenes* (PDB ID: 5K3O), *Pseudomonas* (PDB ID: 1DJP), *Helicobacter pylori* (PDB ID: 2WLT), *Campylobacter jejuni* (PDB ID: 3NXK) and *Acinetobacter glutaminasificans* (PDB ID: 1AGX). The phylogenetic relationship among the microbial asparaginase protein sequences retrieved from Protein Database (PDB) revealed high divergence among the Type I and Type II asparaginases (Fig. 5.2). For example, Type I and Type II asparaginase from *E. coli* shares only 24% of protein identity. The protein sequences of both Type I and Type II asparaginases consisted with larger N and smaller C terminal domains, connected with approximately 20 amino acid long linker region (Guo et al. 2017) (Fig. 5.3). The active sites of the asparaginase can be grouped into rigid and flexible regions (Nguyen et al. 2016). The rigid regions consisted of residues between first and third parallel β strands of N-terminal and residues of the C terminal of neighbouring monomer. The flexible region represent the residues involved in

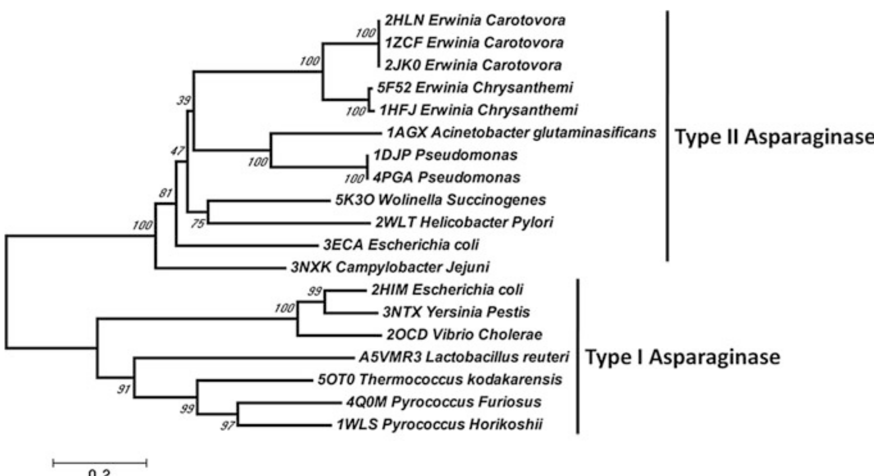


Fig. 5.2 Phylogenetic analysis of Type I and Type II asparaginase protein structures based on the protein sequences retrieved from Protein Database

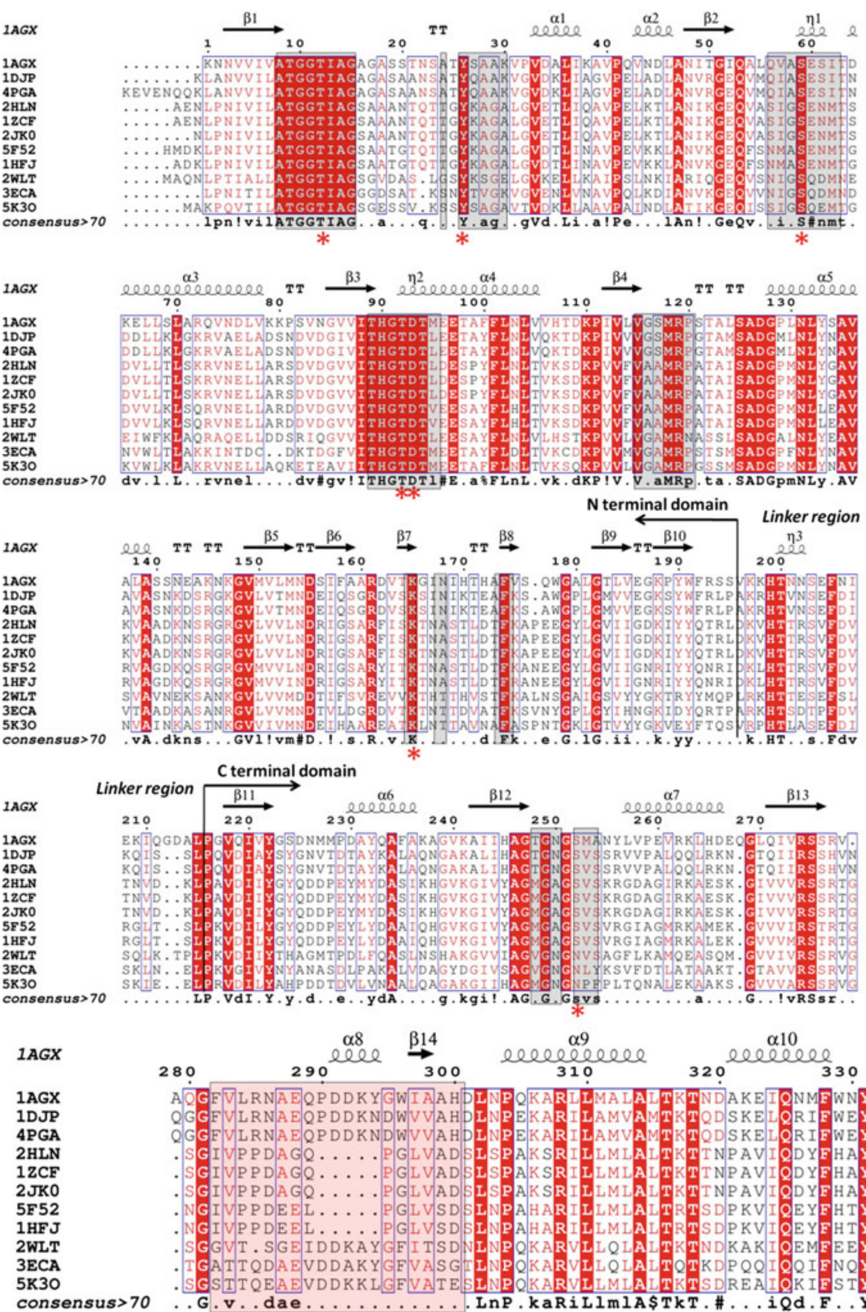


Fig. 5.3 Multiple sequence alignment of the asparaginase protein sequences showing the secondary structure characteristics. All the conserved residues are boxed and the fully conserved residues are coloured white with red background. The less conserved residues are coloured red. The alignment was performed using ESPript 3.0

substrate-enzyme binding pocket and nucleophilic initiation of the reaction, which is situated at the starting of the N-terminal region (Borek and Jaskólski 2001).

The protein sequence of asparaginase may reveal its mechanism of action which shows similarity with serine protease (Tomar et al. 2014). The 'catalytic triad' composed with a nucleophilic residue, a general base and acidic residue which connected by hydrogen bonds define the complete mechanism of asparaginase. As per the reported crystal structures of asparaginases, the active site has two catalytic triad named as Triad I and Triad II. The Triad I is composed with Thr-Tyr-Glu residues are present at flexible region and act as nucleophilic (Thr), a base (Tyr) and an acidic (Glu) residue (Singh et al. 2015). In a similar way, Triad II is represented by Thr (nucleophilic)-Lys (base)-Asp (acid) residues (Bansal et al. 2012). These two triads function intensively for the conversion of substrate into the product in the presence of water as second nucleophile.

The reaction mechanism of asparaginase composed of two-step sequential 'ping-pong' nucleophilic mechanism (Ehrman et al. 1971; Yao et al. 2005). This reaction is initiated by the activation of the enzyme's nucleophile via strong H bonding to the nearby basic residue which attacks the carbon atom of amide bearing substrate (Fig. 5.1). The rearrangement of tetrahedral transition state leads to the formation of acyl-enzyme intermediate product. In transition state, the negative charge on the O atom of amide group is stabilized by the H bond donors known as 'oxyanion hole'. In the second step, activated water is acted as the second nucleophile and attacked on C atom of ester group and again a transition state with oxyanion hole is developed and finally converted into free enzyme and product. These two steps reactions are common in Type I and Type II asparaginase with some structural differences. The 'open and closed' conformations for substrate binding can be defined by a highly flexible β-hairpin at N-terminal domain (Aghaiypour et al. 2001). The enzyme to ligand binding induces the transition of nucleophilic Thr residue which allows the change in the conformation from open to close. This binding of substrate and induced conformational change of Thr residue for the closer of flexible N-terminal loop also revealed the substrate selection of L-asparagine or L-glutamine (Nguyen et al. 2016). The involvement of other residues in enzyme mechanism has also been discussed by various researchers as the sequence variation among various microorganisms leads to change the catalytic proximity of asparaginase.

5.5 Improvement in Asparaginase Characteristics

The biocatalyst gained its properties from the native living source like microorganism, plant, animal, etc. However, it is essential to alter the native properties of the biocatalyst within the purview of its utilization in applied sectors like industrial, agricultural or medical. Microbial production of asparaginase displayed several bottlenecks like less enzyme activity and narrow range of kinetic parameters like substrate specificity, reusability and thermal stability. These incompatibilities of

microbial asparaginase need to be addressed for robust industrial physiochemical conditions. Several attempts have been made to optimize the asparginase enzyme at the molecular level which leads to higher productivity combined with improved properties favourable for industrial or medical applications. For asparaginase enzyme, these selected modifications were carried out by chemical or genetic modifications. The following sections specifically describe the most recent studies, advantages and disadvantages of both modifications.

5.5.1 Chemical Modifications of Asparaginase

The chemical modifications are important to rationalize the immunological responses as well as other characteristics. The immobilization of microbial asparaginase on nanofibers (Ghosh et al. 2012), nanoparticles (Ulu et al. 2018), PEG (Zhang et al. 2004), Sepharose CL-6B (Kotzia and Labrou 2007) agarose-glutaraldehyde (Balcao et al. 2001) and aluminium oxide pellets (Agrawal et al. 2018) which shows advancement of various chemical modifications and its contribution to the asparaginase desired properties. Most of the research has been carried out to possess the asparaginase activity for therapeutic applications (Table 5.3). However, few reports are also available which revealed that the asparaginase immobilization can induce higher reduction of asparagine content that decreases the acrylamide formation in heat-treated food materials. Studies show that immobilized asparaginase-based reduction of asparagine was significantly higher as compared with free asparaginase enzyme. Aiswarya and Baskar (2018) revealed that asparaginase immobilized on magnetic nanoparticle possess 75% mitigation of acrylamide formation in fried potato chips. Similar result was also obtained by Agrawal et al. (2018) and Hendriksen et al. (2016), where asparaginase immobilized on aluminium oxide pellets and silica-based carrier also reduce the asparagine content in fried food materials, respectively. The studies were also suggested that immobilization of asparaginase not only improve the kinetic parameters but thermostability was also shown to be enhanced which is essential of food applications. Different immobilization carrier materials including organic, inorganic and hybrid materials have been used in the studies. However, no study has been reported on the impact of these immobilized asparaginases or other carrier materials on the food quality, texture and flavours. Hence, the research has to be extended in a view of food sample specific carrier materials used for asparaginase immobilization.

5.5.2 Genetic Modifications of Asparaginase

The developments of biotechnological and bioinformatics techniques allowed to attain various approach to improve and assess diverse range of biocatalysts with desired characteristics. These tools can be used to rational or random alteration in

Table 5.3 Chemical and fusion proteins based modifications of microbial asparaginase with improved properties

S. No.	Microbial source	Type of chemical modification	Improved property	References
1	Commercial enzyme	Thiol silane functionalized magnetic Fe_3O_4@MCM-41 core–shell nanoparticles	• 63% higher thermostability • 1.15 fold lower K_m	Ulu et al. (2018)
2	*E. coli*	Poly (2-vinyl-4,4-dimethylazlactone)-functionalized magnetic nanoparticles	• Retain 95.7% activity after 10 repeated uses • Maintain 72.6% activity after 10 weeks storage	Mu et al. (2014)
3	*E. coli*	Covalent immobilization onto agarose-glutaraldehyde	• Prevents enzyme inactivation by subunit dissociation • Improved thermostability	Balcao et al. (2001)
4	*E. coli*	Covalently immobilized on aluminium oxide pellets	• Lowering enzyme level in potato chips • No activity loss after nine cycles	Agrawal et al. (2018)
5	*Bacillus circulans*	Polyaniline nanofibers	2.05-fold lower K_m	Ghosh et al. (2012)
6	*E. coli*	Encapsulated within erythrocytes	• Reduction in the number and severity of allergic reactions • Less coagulation disorders	Domenech et al. (2011)
7	*E. coli*	Variable region antibody fragment (ScFv) conjugated asparaginase	• Resistance to proteolysis • Increased biological half-life	Guo et al. (2000)
8	*Cladosporium* sp.	*N*-Bromosuccinimide-modified asparaginase	Prolonged half-life and proteolytic digestion	Kumar et al. (2013)
9	*E. coli*	Conjugated with modified polyethyleneglycol (mPEG2)	• Improved half-life • Complete abolishment of immunogenicity	Zhang et al. (2004)
10	*Erwinia chrysanthemi*	Immobilized on epoxy-activated Sepharose CL-6B	• Retaining 82% of its activity • Resistance to proteolysis	Kotzia and Labrou (2007)
11	*Aspergillus terreus*	Immobilized on nanomagnetic particle	• No reduction on activity after three cycles • Higher reduction in acrylamide content in fried potato chips	Aiswarya and Baskar (2018)
12	*Pyrococcus furiosus*	Immobilization on silica-based carrier	• Reduced level of asparagines in heat-treated food materials	Hendriksen et al. (2016)

the genetic information embedded in the native organism or recombinant host. Several asparaginase genes from microbial sources like *Bacillus subtilis* (Jia et al. 2013), *Thermococcus kodakaraensis* (Chohan and Rashid 2013), *Pseudomonas fluorescens* (Kishore et al. 2015), *Nocardiopsis alba* (Meena et al. 2016), *Thermococcus litoralis* (Washio and Oikawa 2018), *E. coli* (Vidya et al. 2011), *Streptomyces* (Sudhir et al. 2012), etc. were extracted, cloned and overexpressed by recombinant DNA technology. The molecular analysis of asparaginases has opened up a new avenue for exploring such information for the development of structure–function relationship, substrate binding and selectivity phenomenon, mechanism reestablishment and other identical physicochemical properties. Hence, this information can also be used to rearrange the molecular structure of asparaginase to obtain previously described characteristics. Improvement in thermostability of asparaginase has already been proven and most crucial factor which can broaden its application in therapeutics as well as food for eliminating the toxic content of acrylamide. Therefore, many researchers have attempted to alter the molecular state of native asparaginase proteins by three different and validated strategies: rational design, directed evolution or preparation of synthetic gene construct. Rational design through protein engineering can be defined as hypothesis-driven manipulation of the protein sequence that can change its biophysical properties in a controlled manner. However, the complete crystal structure of validated protein sequence with high resolution and enzyme–ligand binding modeling information is essential for rational protein engineering. The rational hypothesis can be made by algorithmically simulated and optimized designing of available natural sequences for desired protein properties. Few examples are available showing rational designing of asparaginase for the improvement of yield (Verma et al. 2014; Gervais and Foote 2014), substrate specificity (Nguyen et al. 2016) and thermostability (Long et al. 2016; Vidya et al. 2014). Most of the research has been made on the improvement of thermostability of asparaginase. Previous reports concluded that the thermostability of asparaginase can be improved by enhancing the hydrophobicity, H bond and salt bridge, low Ser/Cys content, high Tyr/Arg/Pro content, extra loop deletion, and increased surface polarity and area. Table 5.4 displayed different examples of the rational designing of asparaginase enzyme for the desired properties.

Table 5.4 Rational designing based protein engineering of asparaginase for improved characteristics

S. No.	Enzyme source	Modifications	Conditions and improvement	Reference
1	*E. coli* Asparaginase II	Site-directed mutagenesis	Enhanced stability with activity as compared with wild type	Verma et al. (2014)
2	*E. coli* Asparaginase II	Site-directed mutagenesis	Variants are designed replacement of two positively charged residues (K139 and K207) on the surface loops with neutral and reverse charges; thermally stable	Vidya et al. (2014)
3	*Bacillus subtilis* B11-06	Site-directed mutagenesis	Variant mutants G107D, T109Q, T109S and S166A; G107D displayed superior thermal tolerance and activity	Long et al. (2016)
4	L-asparaginase	Protein-polyelectrolyte complex (PPC) containing L-Asnase and poly-l-lysine (polyK); precipitation-redissolution method	Tolerance towards thermal and shaking stress, protection from oxidation stress and stabilize Asnase from physicochemical stress	Maruyama et al. (2015)
5	*Helicobactor pylori*	Site-directed mutagenesis	HpASNase (T16, T95 and E289); E289A reduced catalytic activity but increase thermo-tolerance	Maggi et al. (2015)
6	*Erwinia chrysanthemi*	Two mutations with single deamination site; (N41D and N281D)	N281D have lowest glutaminase activity; specific activity increase in both mutations	Gervais and Foote (2014)
7	*Bacillus subtilis*	Quadruple mutant NTFS (S180 N/D289T/E260F/E292S) Site-directed mutagenesis	8.1 fold increased Half-life at 65 °C	Feng et al. (2018)

5.6 Conclusion

The book chapter comprehends to the evaluation of microbial asparaginase in terms of its utilization for food industries for the elimination of toxic acrylamide content. Getting insight into the precise mode of action will find the exploration of new microbial sources for mining novel asparaginase. The integration of Omics approaches needs to be explored to mine new candidates of asparaginase from the environments especially extreme environment. The sequence-based diverse interpretation of structure–function relationship has to be utilized for its future existence in dramatically improving the food products and generation of sequence-based rational protein engineering technology can help to decipher improved functional characteristics to meet the industrial desirability.

References

Abd El Baky HHE, Baroty GS (2016) Optimization of growth conditions for purification and production of L-asparaginase by *Spirulina maxima*. Evid Based Complement Alternat Med

Aghaiypour K, Wlodawer A, Lubkowski J (2001) Do bacterial L-asparaginases utilize a catalytic triad Thr-Tyr-Glu? BBA-Protein Struct M 1550(2):117–128

Agrawal S, Sharma I, Prajapati BP, Suryawanshi RK, Kango N (2018) Catalytic characteristics and application of l-asparaginase immobilized on aluminum oxide pellets. Int J Biol Macromol 114:504–511

Aiswarya R, Baskar G (2018) Enzymatic mitigation of acrylamide in fried potato chips using asparaginase from *Aspergillus terreus*. Int J Food Sci Tech 53(2):491–498

Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K (2010) Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. Braz J Microbiol 41 (1):173–178

Asselin B, Rizzari C (2015) Asparaginase pharmacokinetics and implications of therapeutic drug monitoring. Leuk Lymphoma 56(8):2273–2280

Badoei-Dalfard A (2015) Purification and characterization of L-asparaginase from *Pseudomonas aeruginosa* strain SN004: production optimization by statistical methods. Biocatal Agric Biotechnol 4(3):388–397

Balcao VM, Mateo C, Fernández-Lafuente R, Malcata FX, Guisan JM (2001) Structural and functional stabilization of L-asparaginase via multisubunit immobilization onto highly activated supports. Biotechnol Prog 17(3):537–542

Bandarra S, Fernandes AS, Magro I, Guerreiro PS, Pingarilho M, Churchwell MI, Gil OM, Batinic-Haberle I, Gonçalves S, Rueff J, Miranda JP, Marques MM, Beland FA, Castro M, Gaspar JF, Oliveira NG (2013) Mechanistic insights into the cytotoxicity and genotoxicity induced by glycidamide in human mammary cells. Mutagenesis 28(6):721–729

Bansal S, Gnaneswari D, Mishra P, Kundu B (2010) Structural stability and functional analysis of L-asparaginase from *Pyrococcus furiosus*. Biochem (Mosc) 75(3):375–381

Bansal S, Srivastava A, Mukherjee G, Pandey R, Verma AK, Mishra P, Kundu B (2012) Hyperthermophilic asparaginase mutants with enhanced substrate affinity and antineoplastic activity: structural insights on their mechanism of action. FASEB J 26(3):1161–1171

Basha NS, Rekha R, Komala M, Ruby S (2009) Production of extracellular anti-leukaemic enzyme L-asparaginase from marine actinomycetes by solidstate and submerged fermentation: Purification and characterization. Trop J Pharm Res 8(4):353–360

Borek D, Jaskólski M (2001) Sequence analysis of enzymes with asparaginase activity. Acta Biochim Pol 48:893–902

Broome JD (1963) Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. J Exp Med 118(1):99–120

Cedar H, Schwartz JH (1968) Production of L-asparaginase II by *Escherichia coli*. J Bacteriol 96 (6):2043–2048

Chagas EP, Sodek L (2001) Purification and properties of asparaginase from the testa of immature seeds of pea (*Pisum sativum*). Braz Arch Biol Technol 44(3):239–245

Chohan SM, Rashid N (2013) TK1656, a thermostable L-asparaginase from *Thermococcus kodakaraensis*, exhibiting highest ever reported enzyme activity. J Biosci Bioeng 116(4):438–443

Chubukov V, Gerosa L, Kochanowski K, Sauer U (2014) Coordination of microbial metabolism. Nat Rev Microbiol 12(5):327–340

Ciesarova Z, Kukurova K, Bednáriková A, Marková L, Baxa S (2009) Improvement of cereal product safety by enzymatic way of acrylamide mitigation. Czech J Food Sci 27:96–98

Clementi A (1922) The enzymatic deamidation of asparagine in different animal species and the physiological significance of its presence in the body. Int Arch Physiol 19(4):369–398

Dange VU, Peshwe SA (2011) Production, purification and characterization of fungal L-asparaginase. Bionano Front 4:162–167

Dharmaraj S (2011) Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa*. Iran J Biotechnol 9(2):102–108

Dias FFG, Ruiz ALTG, Della Torre A, Sato HH (2016) Purification, characterization and antiproliferative activity of L-asparaginase from *Aspergillus oryzae* CCT 3940 with no glutaminase activity. Asian Pac J Trop Biomed 6(9):785–794

Domenech C, Thomas X, Chabaud S, Baruchel A, Gueyffier F, Mazingue F, Auvrignon A, Corm S, Dombret H, Chevallier P, Galambrun C, Huguet F, Legrand F, Mechinaud F, Vey N, Philip I, Liens D, Godfrin Y, Rigal D, Bertrand Y (2011) L-asparaginase loaded red blood cells in refractory or relapsing acute lymphoblastic leukaemia in children and adults: results of the GRASPALL 2005-01 randomized trial. Br J Haematol 153(1):58–65

Duval M, Suciu S, Ferster A, Rialland X, Nelken B, Lutz P, Benoit Y, Robert A, Manel AM, Vilmer E, Otten J, Philippe N (2002) Comparison of *Escherichia coli*-asparaginase with *Erwinia*-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. Blood 99(8):2734–2739

Ebrahiminezhad A, Rasoul-Amini S, Ghoshoon MB, Ghasemi Y (2014) *Chlorella vulgaris*, a novel microalgal source for L-asparaginase production. Biocatal Agric Biotechnol 3(2):214–217

EFSA Panel on Contaminants in the Food Chain (CONTAM) (2015) Scientific opinion on acrylamide in food. EFSA J 13(6):4104

Ehrman M, Cedar H, Schwartz JH (1971) l-Asparaginase II of *Escherichia coli* studies on the enzymatic mechanism of action. J Biol Chem 246(1):88–94

El-Bessoumy AA, Sarhan M, Mansour J (2004) Production, isolation, and purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. BMB Rep 37(4):387–393

El-Naggar NEA, Deraz SF, Soliman HM, El-Deeb NM, El-Ewasy SM (2016) Purification, characterization, cytotoxicity and anticancer activities of L-asparaginase, anti-colon cancer protein, from the newly isolated alkaliphilic *Streptomyces fradiae* NEAE-82. Sci Rep 6:32926

El-Naggar NEA, Moawad H, Abdelwahed NA (2017) Optimization of fermentation conditions for enhancing extracellular production of L-asparaginase, an anti-leukemic agent, by newly isolated *Streptomyces brollosae* NEAE-115 using solid state fermentation. Ann Microbiol 67 (1):1–15

Elshafei AM, Hassan MM, Abd M, Abouzeid E, Mahmoud DA, Elghonemy DH (2012) Purification, characterization and antitumor activity of L-asparaginase from *Penicillium brevicompactum* 2 NRC 829. Brit Microbiol Res J 2(3):158–174

El-Sharkawy AS, Farag AM, Embaby AM, Saeed H, El-Shenawy M (2016) Cloning, expression and characterization of aeruginosa EGYII L-Asparaginase from *Pseudomonas aeruginosa* strain EGYII DSM 101801 in *E. coli* BL21 (DE3) pLysS. J Mol Catal B Enzym 132:16–23

Erva RR, Goswami AN, Suman P, Vedanabhatla R, Rajulapati SB (2017) Optimization of L-asparaginase production from novel *Enterobacter* sp., by submerged fermentation using response surface methodology. Prep Biochem Biotechnol 47(3):219–228

Feng Y, Liu S, Jiao Y, Gao H, Wang M, Du G, Chen J (2017) Enhanced extracellular production of L-asparaginase from *Bacillus subtilis* 168 by *B. subtilis* WB600 through a combined strategy. Appl Microbiol Biotechnol 101(4):1509–1520

Feng Y, Liu S, Jiao Y, Wang Y, Wang M, Du G, Chen J (2018) Improvement of L-asparaginase thermal stability by regulating enzyme kinetic and thermodynamic states. Process Biochem https://doi.org/10.1016/j.procbio.2018.05.002

Gervais D, Foote N (2014) Recombinant deamidated mutants of *Erwinia chrysanthemi* L-asparaginase have similar or increased activity compared to wild-type enzyme. Mol Biotechnol 56(10):865–877

Ghosh S, Chaganti SR, Prakasham RS (2012) Polyaniline nanofiber as a novel immobilization matrix for the anti-leukemia enzyme l-asparaginase. J Mol Catal B Enzym 74(1–2):132–137

Gilbert HJ, Blazek R, Bullman HM, Minton NP (1986) Cloning and expression of the *Erwinia chrysanthemi* asparaginase gene in *Escherichia coli* and *Erwinia carotovora*. Microbiology 132(1):151–160

Global Asparaginase Sales Market Report (2018) https://www.marketinsightsreports.com/reports/0709674345/Global-Asparaginase-Sales-Market-Report-2018?source=thefreenewsman&Mode=15

Guo L, Wang J, Qian S, Yan X, Chen R, Meng G (2000) Construction and structural modeling of a single-chain Fv–asparaginase fusion protein resistant to proteolysis. Biotechnol Bioeng 70 (4):456–463

Guo J, Coker AR, Wood SP, Cooper JB, Chohan SM, Rashid N, Akhtar M (2017) Structure and function of the thermostable L-asparaginase from *Thermococcus kodakarensis*. Acta Cryst D 73(11):889–895

Heeschen V, Matlok J, Schrader S, Rudolph H (1996) Asparagine catabolism in bryophytes: purification and characterization of two L-asparaginase isoforms from *Sphagnum fallax*. Physiol Plant 97(2):402–410

Hendriksen HV, Kornbrust BA, Østergaard PR, Stringer MA (2009) Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from *Aspergillus oryzae*. J Agric Food Chem 57(10):4168–4176

Hendriksen HV, Puder K, Olsen AG (2016) Method for reducing the level of asparagine in a food material. U.S. Patent Application No. 14/781,760

Hill JM, Roberts J, Loeb E, Khan A, MacLellan A, Hill RW (1967) L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. JAMA 202(9):882–888

Huang L, Liu Y, Sun Y, Yan Q, Jiang Z (2013) Biochemical characterization of a novel L-Asparaginase with low glutaminase activity from *Rhizomucor miehei* and its application in food safety and leukemia treatment. Appl Environ Microbiol 80(5):1561–1569

Husain I, Sharma A, Kumar S, Malik F (2016a) Purification and characterization of glutaminase free asparaginase from *Enterobacter cloacae*: in-vitro evaluation of cytotoxic potential against human myeloid leukemia HL-60 cells. PLoS ONE 11(2):e0148877

Husain I, Sharma A, Kumar S, Malik F (2016b) Purification and characterization of glutaminase free asparaginase from *Pseudomonas otitidis*: induce apoptosis in human leukemia MOLT-4 cells. Biochimie 121:38–51

IARC (International Agency for Research on Cancer) (1994) Acrylamide. IARC monographs on the evaluation of carcinogenic risks to humans. Ind Chem 60:389–434 (France: Lyon)

Izadpanah F, Homaei A, Fernandes P, Javadpour S (2018) Marine microbial L-asparaginase: Biochemistry, molecular approaches and applications in tumor therapy and in food industry. Microbiol Res 208:99-112

Jia M, Xu M, He B, Rao Z (2013) Cloning, expression, and characterization of L-asparaginase from a newly isolated *Bacillus subtilis* B11–06. J Agric Food Chem 61(39):9428–9434

Kacar S, Vejselova D, Kutlu HM, Sahinturk V (2018) Acrylamide-derived cytotoxic, anti-proliferative, and apoptotic effects on A549 cells. Hum Exp Toxicol 37(5):468–474

Kamble VP, Rao RS, Borkar PS (2006) Purification of L-asparaginase from a bacteria *Erwinia carotovora* and effect of a dihydropyrimidine derivative on some of its kinetic parameters. Indian J Biochem Biophys 43(6):391–394

Kavitha A, Vijayalakshmi M (2012) A study on L-asparaginase of *Nocardia levis* MK-VL_113. Sci World J Article ID 160434. https://doi.org/10.1100/2012/160434

Kidd JG (1953) Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum: I. course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. J Exp Med 98(6):565–582

Killander D, Dohlwitz A, Engstedt L, Franzen S, Gahrton G, Gullbring B, Holm G, Holmgren A, Höglund S, Killander A, Lockner D (1976) Hypersensitive reactions and antibody formation during L-asparaginase treatment of children and adults with acute leukemia. Cancer 37(1):220–228

Kishore V, Nishita KP, Manonmani HK (2015) Cloning, expression and characterization of l-asparaginase from *Pseudomonas fluorescens* for large scale production in *E. coli* BL21. 3 Biotech 5(6):975–981

Kotzia GA, Labrou NE (2007) L-Asparaginase from *Erwinia chrysanthemi* 3937: cloning, expression and characterization. J Biotechnol 127(4):657–669

Krishnapura PR, Belur PD (2016) Partial purification and characterization of L-asparaginase from an endophytic *Talaromyces pinophilus* isolated from the rhizomes of *Curcuma amada*. J Mol Catal B Enzym 124:83–91

Kukurová K, Morales FJ, Bednáriková A, Ciesarová Z (2009) Effect of L-asparaginase on acrylamide mitigation in a fried-dough pastry model. Mol Nutr Food Res 53(12):1532–1539

Kumar NM, Manonmani HK (2013) Purification, characterization and kinetic properties of extracellular L-asparaginase produced by *Cladosporium* sp. World J Microbiol Biotechno 29 (4):577–587

Kumar S, Dasu VV, Pakshirajan K (2011) Purification and characterization of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Bioresour Technol 102 (2):2077–2082

Kumar NM, Ramasamy R, Manonmani HK (2013) Production and optimization of L-asparaginase from *Cladosporium* sp. using agricultural residues in solid state fermentation. Ind Crops Prod 43:150–158

Lea PJ, Festenstein GN, Hughes JS, Miflin BJ (1984) An immunological and enzymological survey of asparaginase in seeds of Lupinus. Phytochemistry 23(3):511–514

Lincoln L, Niyonzima FN, More SS (2015) Purification and properties of a fungal L-asparaginase from *Trichoderma viride* pers: sf grey. J Microbiol Biotechnol Food Sci 4(4):310

Long S, Zhang X, Rao Z, Chen K, Xu M, Yang T, Yang S (2016) Amino acid residues adjacent to the catalytic cavity of tetramer L-asparaginase II contribute significantly to its catalytic efficiency and thermostability. Enzyme Microb Technol 82:15–22

Maggi M, Chiarelli LR, Valentini G, Scotti C (2015) Tackling critical catalytic residues in *Helicobacter pylori* L-asparaginase. Biomolecules 5(2):306–317

Mahajan RV, Saran S, Kameswaran K, Kumar V, Saxena RK (2012) Efficient production of L-asparaginase from *Bacillus licheniformis* with low-glutaminase activity: optimization, scale up and acrylamide degradation studies. Bioresour Technol 125:11–16

Mahajan RV, Kumar V, Rajendran V, Saran S, Ghosh PC, Saxena RK (2014) Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from *Bacillus licheniformis*: in vitro evaluation of anti-cancerous properties. PLoS ONE 9(6):e99037

Maruyama T, Izaki S, Kurinomaru T, Handa K, Kimoto T, Shiraki K (2015) Protein-poly(amino acid) precipitation stabilizes a therapeutic protein L-asparaginase against physicochemical stress. J Biosci Bioeng 120(6):720–724

Mashburn LT, Wriston JC Jr (1963) Tumor inhibitory effect of L-asparaginase. Biochem Biophys Res Commun 12(1):50–55

Meena B, Anburajan L, Dheenan PS, Begum M, Vinithkumar NV, Dharani G, Kirubagaran R (2015) Novel glutaminase free l-asparaginase from *Nocardiopsis alba* NIOT-VKMA08: production, optimization, functional and molecular characterization. Bioprocess Biosyst Eng 38(2):373–388

Meena B, Anburajan L, Vinithkumar NV, Shridhar D, Raghavan RV, Dharani G, Kirubagaran R (2016) Molecular expression of l-asparaginase gene from *Nocardiopsis alba* NIOT-VKMA08 in *Escherichia coli*: a prospective recombinant enzyme for leukaemia chemotherapy. Gene 590 (2):220–226

Meghavarnam AK, Janakiraman S (2018) Evaluation of acrylamide reduction potential of L-asparaginase from *Fusarium culmorum* (ASP-87) in starchy products. LWT—Food Sci Technol 89:32–37

Michalska K, Jaskolski M (2006) Crystal structure of plant asparaginase. J Mol Biol 360(1):05–116

Michalska K, Borek D, Hernández-Santoyo A, Jaskolski M (2008) Crystal packing of plant-type L-asparaginase from *Escherichia coli*. Acta Cryst D 64(3):309–320

Mohamed SA, Elshal MF, Kumosani TA, Aldahlawi AM, Basbrain TA, Alshehri FA, Choudhry H (2016) L-Asparaginase isolated from *Phaseolus vulgaris* seeds exhibited potent anti-acute lymphoblastic leukemia effects in-vitro and low immunogenic properties *in-vivo*. Int J Environ Res Public Health 13(10):1008

Moorthy V, Ramalingam A, Sumantha A, Shankaranaya RT (2010) Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of *Bacillus* sp. Afr J Microbiol Res 4(18):1862–1867

Moreno-Enríquez A, Evangelista-Martínez Z, González-Mondragón EG, Calderón-Flores A, Arreguín R, Pérez-Rueda E, Huerta-Saquero A (2012) Biochemical characterization of recombinant L-asparaginase (AnsA) from *Rhizobium etli*, a member of an increasing rhizobial-type family of L-asparaginases. J Microbiol Biotechnol 22(3):292–300

Mottram DS, Wedzicha BL, Dodson AT (2002) Acrylamide is formed in the Maillard reaction. Nature 419(6906):448

Mu X, Qiao J, Qi L, Dong P, Ma H (2014) Poly(2-vinyl-4, 4-dimethylazlactone)-functionalized magnetic nanoparticles as carriers for enzyme immobilization and its application. ACS Appl Mater Interfaces 6(23):21346–21354

Narta U, Roy S, Kanwar SS, Azmi W (2011) Improved production of L-asparaginase by *Bacillus brevis* cultivated in the presence of oxygen-vectors. Bioresour Technol 102(2):2083–2085

Nguyen HA, Su Y, Lavie A (2016) Structural insight into substrate selectivity of *Erwinia chrysanthemi* L-Asparaginase. Biochem 55(8):1246–1253

Onishi Y, Yano S, Thongsanit J, Takagi K, Yoshimune K, Wakayama M (2011) Expression in *Escherichia coli* of a gene encoding type II L-asparaginase from *Bacillus subtilis*, and characterization of its unique properties. Ann Microbiol 61(3):517–524

Onishi Y, Prihanto AA, Yano S, Takagi K, Umekawa M, Wakayama M (2015) Effective treatment for suppression of acrylamide formation in fried potato chips using L-asparaginase from *Bacillus subtilis*. 3 Biotech 5(5):783–789

Patro KR, Gupta N (2012) Extraction, purification and characterization of L-asparaginase from *Penicillium* sp. by submerged fermentation. Int J Biotechnol Mol Biol Res 3(3):30–34

Paul JH (1982) Isolation and characterization of a Chlamydomonas L-asparaginase. Biochem J 203(1):109–115

Pedreschi F, Kaack K, Granby K, Troncoso E (2007) Acrylamide reduction under different pre-treatments in French fries. J Food Eng 79(4):1287–1294

Pedreschi F, Kaack K, Granby K (2008) The effect of asparaginase on acrylamide formation in French fries. Food Chem 109(2):386–392

Pedreschi F, Mariotti S, Granby K, Risum J (2011) Acrylamide reduction in potato chips by using commercial asparaginase in combination with conventional blanching. LWT-Food Sci Technol 44(6):1473–1476

Pokrovskaia MV, Pokrovskiĭ VS, Aleksandrova SS, Anisimova N, Adrianov RM, Treshchalina EM, Ponomarev GV, Sokolov NN (2013) Recombinant intracellular *Rhodospirillum rubrum* L-asparaginase with low L-glutaminase activity and antiproliferative effect. Biomed Khim 59(2):192–208

Pokrovskaya MV, Aleksandrova SS, Pokrovsky VS, Omeljanjuk NM, Borisova AA, Anisimova NY, Sokolov NN (2012) Cloning, expression and characterization of the recombinant Yersinia pseudotuberculosis L-asparaginase. Protein Expr Purif 82(1):150–154

Pourhossein M, Korbekandi H (2014) Cloning, expression, purification and characterisation of *Erwinia carotovora* L-asparaginase in *Escherichia coli*. Adv Biomed Res 3:82

Pradhan B, Dash SK, Sahoo S (2013) Screening and characterization of extracellular L-asparaginase producing *Bacillus subtilis* strain hswx88, isolated from Taptapani hotspring of Odisha, India. Asian Pac J Trop Biomed 3(12):936–941

Qeshmi IF, Homaei A, Fernandes P, Javadpour S (2018) Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. Microbiol Res 208:99–112

Ramya LN, Doble M, Rekha VPB, Pulicherla KK (2012) L-Asparaginase as potent anti-leukemic agent and its significance of having reduced glutaminase side activity for better treatment of acute lymphoblastic leukaemia. Appl Biochem Biotechnol 167(8):2144–2159

Ravi A, Gurunathan B (2018) Acrylamide mitigation in fried *Kochchi Kesel* chips using free and immobilized fungal asparaginase. Food Technol Biotechnol 56(1):51

Robin LP, Clanci S (2007) Acrylamide, Furan, and the FDA. Food Saf Mag 13(3):17–21

Sajitha S, Vidya J, Binod P, Pandey A (2015) Cloning and expression of L-asparaginase from *E. coli* in eukaryotic expression system. Biochem Eng J 102:14–17

Sanghvi G, Bhimani K, Vaishnav D, Oza T, Dave G, Kunjadia P, Sheth N (2016) Mitigation of acrylamide by L-asparaginase from *Bacillus subtilis* KDPS1 and analysis of degradation products by HPLC and HPTLC SpringerPlus 5(1):533

Shakambari G, Birendranarayan AK, Lincy MJA, Rai SK, Ahamed QT, Ashokkumar B, Saravanan M, Mahesh A, Varalakshmi P (2016) Hemocompatible glutaminase free L-asparaginase from marine *Bacillus tequilensis* PV9W with anticancer potential modulating p53 expression. RSC Adv 6(31):25943–25951

Shi R, Liu Y, Mu Q, Jiang Z, Yang S (2017) Biochemical characterization of a novel L-asparaginase from *Paenibacillus barengoltzii* being suitable for acrylamide reduction in potato chips and mooncakes. Int J Biol Macromol 96:93–99

Shrivastava A, Khan AA, Shrivastav A, Jain SK, Singhal PK (2012) Kinetic studies of L-asparaginase from *Penicillium digitatum*. Prep Biochem Biotechno 42(6):574–581

Singh Y, Gundampati RK, Jagannadham MV, Srivastava SK (2013) Extracellular L-asparaginase from a protease-deficient *Bacillus aryabhattai* ITBHU02: purification, biochemical characterization, and evaluation of antineoplastic activity in vitro. Appl Biochem Biotechnol 171 (7):1759–1774

Singh J, Srivastava A, Jha P, Sinha KK, Kundu B (2015) L-Asparaginase as a new molecular target against leishmaniasis: insights into the mechanism of action and structure-based inhibitor design. Mol BioSyst 11(7):887–1896

Sinha RK, Singh HR, Jha SK (2015) Production, purification and kinetic characterization of L-asparaginase from *Pseudomonas fluorescens*. Int J Pharm Pharm Sci 7(1):135–138

Sudhir AP, Dave BR, Trivedi KA, Subramanian RB (2012) Production and amplification of an l-asparaginase gene from actinomycete isolate *Streptomyces ABR2*. Ann Microbiol 62 (4):1609–1614

Sudhir AP, Dave BR, Prajapati AS, Panchal K, Patel D, Subramanian RB (2014) Characterization of a recombinant glutaminase-free L-asparaginase (ansa3) enzyme with high catalytic activity from *Bacillus licheniformis*. Appl Biochem Biotechnol 174(7):2504–2515

Sukhoverkov KV, Kudryashova EV (2015) PEG-chitosan and glycol-chitosan for improvement of biopharmaceutical properties of recombinant L-asparaginase from *Erwinia carotovora*. Biochem (Mosc) 80(1):113–119

Swain AL, Jaskólski M, Housset D, Rao JK, Wlodawer A (1993) Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc Natl Acad Sci USA 90(4):1474–1478

Tareke E, Rydberg P, Karlsson P, Eriksson S, Törnqvist M (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J Agric Food Chem 50(17):4998–5006

Thakur M, Azmi W (2013) Extraction and purification of β-carotene from filamentous fungus *Mucor azygosporus*. Ann Phytomed 2:79–84

Tiwari N, Dua RD (1996) Purification and preliminary characterization of L-asparaginase from *Erwinia aroideae* NRRL B-138. Indian J Biochem Biophys 33(5):371–376

Tomar R, Sharma P, Srivastava A, Bansal S, Kundu B (2014) Structural and functional insights into an archaea l-asparaginase obtained through the linker-less assembly of constituent domains. Acta Cryst D 70(12):3187–3197

Tyl RW, Friedman MA (2003) Effects of acrylamide on rodent reproductive performance. Reprod Toxicol 17(1):1–13

Ulu A, Noma SAA, Koytepe S, Ates B (2018) Magnetic Fe3O4@ MCM-41 core–shell nanoparticles functionalized with thiol silane for efficient l-asparaginase immobilization. Artif Cells Nanomed Biotechnol 1–11

Vala AK, Sachaniya B, Dudhagara D, Panseriya HZ, Gosai H, Rawal R, Dave BP (2018) Characterization of L-asparaginase from marine-derived *Aspergillus niger* AKV-MKBU, its antiproliferative activity and bench scale production using industrial waste. Int J Biol Macromol 108:41–46

Varma RA, Kanapala S, Naga SBV, Bodaiah B, Poda S (2016) Partial purification, characterization and optimization of anti-leukemic enzyme L-asparaginase from mangrove soil actinobacteria. J Pharma Res 10(7):502–511

Verma S, Mehta RK, Maiti P, Röhm KH, Sonawane A (2014) Improvement of stability and enzymatic activity by site-directed mutagenesis of *E. coli* asparaginase II. Biochim Biophys Acta (BBA)-Proteins Proteomics 1844(7):1219–1230

Vidya J, Vasudevan UM, Soccol CR, Pandey A (2011) Cloning, functional expression and characterization of L-asparaginase II from *E. coli* MTCC 739. Food Technol Biotech 49 (3):286–290

Vidya J, Ushasree MV, Pandey A (2014) Effect of surface charge alteration on stability of l-asparaginase II from *Escherichia* sp. Enz Microbiol Technol 56:15–19

Washio T, Oikawa T (2018) Thermostable and highly specific L-aspartate oxidase from *Thermococcus litoralis* DSM 5473: cloning, overexpression, and enzymological properties. Extremophiles 22(1):59–71

Yaacob MA, Hasan WANW, Ali MSM, Rahman RNZRA, Salleh AB, Basri M, Leow TC (2014) Characterisation and molecular dynamic simulations of J15 asparaginase from *Photobacterium sp*. strain J15. Acta Biochim Pol 61(4)

Yao M, Yasutake Y, Morita H, Tanaka I (2005) Structure of the type I L-asparaginase from the hyperthermophilic archaeon *Pyrococcus horikoshii* at 2.16 Å resolution. Acta Cryst D 61 (3):294–301

Youssef MM, Al-Omair MA (2008) Cloning, purification, characterization and immobilization of L-asparaginase II from *E. coli* W3110. Asian J Biochem 3(6):337–350

Zhang JF, Shi LY, Wei DZ (2004) Chemical modification of L-asparaginase from *Escherichia coli* with a modified polyethyleneglycol under substrate protection conditions. Biotechnol Lett 26 (9):753–756

Zhang S, Xie Y, Zhang C, Bie X, Zhao H, Lu F, Lu Z (2015) Biochemical characterization of a novel l-asparaginase from *Bacillus megaterium* H-1 and its application in French fries. Food Res Int 77:527–533

Zuo S, Xue D, Zhang T, Jiang B, Mu W (2014) Biochemical characterization of an extremely thermostable l-asparaginase from *Thermococcus gammatolerans* EJ3. J Mol Catal B Enzym 109:122–129

Zuo S, Zhang T, Jiang B, Mu W (2015) Reduction of acrylamide level through blanching with treatment by an extremely thermostable l-asparaginase during French fries processing. Extremophiles 19(4):841–851

Chapter 6
Applications of Asparaginase in Food Processing

G. Baskar, R. Aiswarya and S. Renganathan

Abstract Asparaginase is significantly utilized in various pharmaceutical and food industries. Asparaginase, an intercellular enzyme, has gained wide attention in food processing industries for the reduction of acrylamide in foods. Engineering of enzymes finds an effective pathway in the design of application according to the specificity and the structural changes. The rational design of enzymes helps in evaluating the purpose of enzymes in food processing industries and bulk chemicals. The asparaginase isolated from fungal species was regarded to be safe with high specificity with minimum activity towards glutamine for the mitigation of acrylamide in food. The enzyme asparaginase has been declared as safe and favourable additive by various food committee experts. Presently, asparaginase is from various bacterial and fungal sources. Free and immobilized asparaginases are used for the mitigation of acrylamide level in food during the blanching process. The immobilized asparaginase is repeatedly reused without any loss in the activity of the enzyme. The main drawback on using asparaginase lies in the commercialization of the product at various countries due to the issues associated at the industrial level. Incorporation of asparaginase in food industries needs extensive research on the enzymatic effect and pre/post-processing conditions. The purification of enzyme needs an extensive attention as they influence in the mitigation of acrylamide. The adverse effect using asparaginase on sensory properties of food and commercialization of the enzymatic approach towards the mitigation process at various sectors of food industries is the next future scope in the food processing industries.

Keywords Asparaginase · Pretreatment · Acrylamide · Food toxins
Food processing

G. Baskar (✉) · R. Aiswarya
Department of Biotechnology, St. Joseph's College of Engineering, Chennai 600119, India
e-mail: basg2004@gmail.com

S. Renganathan
Centre for Biotechnology, A. C. Technology, Anna University, Chennai 600025, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_6

6.1 Introduction

Enzymes known as protein molecules find their application in the production of many value-added products (Bruggink et al. 1998; Estell et al. 1985; Jensen and Rugh 1987; Sedlaczek 1988). Enzymes have been impeded widely owing to its potential properties in terms of its stability and specificity (Elleuche et al. 2014). The enzymes are mainly screened from natural sources and during random mutations for its wide applications. Enzymes are engineered according to the structural information and substrate ranges for the production of various intermediates (Bornscheuer et al. 2012). The structural and mechanistic approach is needed for the utilization of enzymes in various industries. The emergence of rational design and computational methods based on the direct evolution plays a vital role in enhancing the efficiency of enzyme (Kumar and Singh 2013; Siegel et al. 2010). The production of enzyme using microorganisms such as bacteria, yeast and fungi helps in for improving the taste and texture of food. This offers an added advantage for an effective and consistent production of various products (Sindhu et al. 2018).

The process of cooking helps in the elimination of bacteria and other contaminants that are harmful to human (Dearfield et al. 1995). The establishment of fast foods has brought a lot of behavioural changes towards the consumption of foods. This has also led to improper diet on daily consumption of foods. The nutritional value of foods was also changed due to the intervention attained during the processing of food (Allal et al. 2014). In 2002, the Swedish National Food Administration jointly with University of Stockholm declared acrylamide as probable human carcinogenic compound. The compound acrylamide is mainly formed during conventional cooking process such as frying, baking and roasting. The formation of acrylamide is noted highly in carbohydrate-rich foods including potatoes, cereals, peanuts and lentil. This compound is formed in the presence of asparagine and reducing sugar (Dybing et al. 2005; Jin et al. 2013). The potential risk on acrylamide consumption has made various agencies such as WHO/FAO/NCFST to focus consistent research on eliminating the toxic compound (Lofstedt 2003). The mitigation of acrylamide is classified into three types of which researchers mainly focus on the processing parameters. Many changes were proposed by the scientist during the post and preprocessing conditions which includes monitoring of temperature and the addition of additives. The processing variables should be studied during the frying process as they directly influence the formation of acrylamide. It was reported that pretreatment conditions have improved the mass transfer and diffusion rate during the Maillard reaction. The use of asparaginase is one of the enzymatic methods adopted for the mitigation of acrylamide which cleaves asparagine to aspartic acid and ammonia (Jaeger et al. 2010; Baskar and Aiswarya 2018). This chapter mainly focuses on the enzymatic approach on acrylamide mitigation and also explains about the importance of enzyme engineering and recombinant strain towards the design of enzyme application.

6.2 Enzymes in Food Processing

The demand for high-quality food in terms of taste and flavours in developing countries has created a shift from staple source of foods. This demand has triggered the need for the extensive approach towards the food industries (Li et al. 2012). Food enzymes are widely used in baking, brewing, wine industries and in the manufacturing of fruit juices. The enzymes are added to improve the flavour, digestibility and nutritional value of the food. The enzymes are added according to the regulation which is distinguished as additives and processing aids. The difference between additives and processing aids is significantly noted as the regulation context differs for different countries. The processing aids added during the process do not possess any functional demand the final stage of the product whereas the enzymes used during the food processing contain certain metabolites which act as stabilizers. The addition of enzymes has to be apparently monitored during the manufacturing process, and it is also important that they should fall with the guidance and regulation framed under good manufacturing practices (Fernandes 2010).

The key role during the enzyme preparations is the safety assessment of enzyme as they are produced mainly from microbial strains. FDA has recognized recombinant microorganism including bacterial and fungal species as Generally Recognized As Safe (GRAS). Certain modifications were done at the production level with the help of wild-type host microorganism (Koushki 2011; Olempska-Beer et al. 2006). The nutritional aspects of food in the food processing industries are directly related to the functional properties of foods (Akoh et al. 2008). According to the Global Industry Analysis (GIA), prebiotic market has high demand in recent times as they utilize the simple process for high production yield at low investment. Inulin uses the cheapest substrate such as sucrose for the production of DiFructose Anhydride (DFA) through the coupled enzyme reaction with the yield of 10% (Hang et al. 2013). Galacto-oligosaccharides, a prebiotic product, are produced by ß-galactosidases with lactose. The production yield of 30–35% w/w was reported on using yeast and fungal sources (Rodriguez-Colinas et al. 2011; Vera et al. 2012; Park and Oh 2010). The microbial-derived asparaginase enzymes are derived from specifically selected or genetically modified. This asparaginase enzyme finds a special class in pharma and nutraceutical industries. This enzyme acts as anticancer agent in humans, which helps in the depletion of asparagine affecting the growth of cancerous cells. It is also used during the processing of foods such as frying of oil and baking which forms acrylamide from asparagine. The depletion of asparagine has been found effective in the mitigation of acrylamide by enzymatic treatment (Mohan Kumar et al. 2014; Krishnapura et al. 2016).

6.3 Recombinant Enzymes for Food Applications

Safety evaluation of enzymes in food processing is an essential study and has been extensively studied by various researchers (Pariza and Johnson 2001). Various regulatory authorities and organizations such as Scientific Committee for Food formulated guidelines for the safety assessment of enzymes from recombinant microorganism. The assessment was mainly carried out to analyse the pathogenic and toxicological properties. It was found that certain fungi produced a low level of toxic secondary metabolites under fermentation condition. In case of recombinant enzyme production strain selection and construction of host microorganism has to be selected cautiously as they improve the vital enzymatic properties.

Industrial production of recombinant enzymes typically follows various stages for the successful production of recombinant strain. The recombinant enzymes used in the industries follow the following stages for efficient formulation and applications:

- Development of host strain,
- Construction of host strain,
- Transformation of best recombinant strain,
- Identification of best recombinant strain and
- Characterization of produced strain.

Most of the host microorganisms secrete enzymes to the fermentation media. The microorganisms such as *Escherichia coli K-12* and *Pseudomonas fluorescens* accumulate heterologous enzyme in the fermentation media. This accumulation of enzymes requires high purification steps than the secreted enzymes in the media. The wild-type strains produce extracellular enzyme which finds its application in the final stage of enzyme preparation which is used during the processing of food. Fungal species such as *A. oryzae*, *F. venenatum* and *Aspergillus niger* are considered to be nontoxigenic according to the submission to FDA (Olempska-Beer et al. 2006; Jonas et al. 1996).

For several decades, *B. subtilis* 168 is used for processing of foods. The recombinant enzyme from *B. subtilis* is considered to be safe during the food processing as suggested by FDA (Boer et al. 1994). The other bacterial species that are considered to be safe for processing of foods include *B. licheniformis*, *B. amyloliquefaciens* and *Geobacillus stearothermophilus* (Naizna et al. 2001; Pedersen et al. 2002; De Boer and Diderichsen 1991). It was reported that chymosin derived from *E. coli* K-12 is widely used for milk clotting. This enzyme is the first regulated recombinant enzyme used for the processing of cheese and other dairy products (Flamm 1991).

The filamentous fungi *A. oryzae and A. niger* are widely used in food production. They are used in baking, brewing and in fermentation of soy sauce. The strains of *A. oryzae* A1560 and 1FO 4177 are used in the production of Koji moulds with the help of $BEch_2$ gene during the production of Kojic acid. The recombinant enzymes used in food processing are derived from characterized microorganism

with the development of modern efficient screening technique. The optimized properties of thermophilic enzymes are widely used in baking and starch processing (Machida et al. 2005).

The assessment of enzyme preparation used for processing of food is noted to be at low levels. The main assessment of enzymes of food processing is based upon the calculation of Total Organic Solids (TOS). The various strain improvement strategies help in creating an efficient and safer enzyme production strain with a wide knowledge of genetic makeup of microorganism (FCC 2004).

6.4 Recent Trends in Enzyme Engineering

The current trend in enzyme engineering is the combination of structure-based rational design and computational methods. This combination decreases the size of library by increasing the success rate to random mutagenesis. DNA shuffling by SCHEMA is one of the computational methods that are available for the estimation of DNA recombination (Silbeg et al. 2004; Heinzelman et al. 2009). The compact structure and number of charge interaction in case of extremophiles are studied with respect to the stability of enzymes at both structural and functional level (Van den Burg 2003; Illanes et al. 2012). In order to study the targeting site and design, various statistical methods such as ProSAR, CASTing and HITS are developed for the schematic screening and analysis of sequence activity. Conjugation of ProSAR with direct evolution was successfully used to enhance the catalytic activity and enantioselectivity by ketoreductase and R-selective transaminase (ATA-117) (Evran et al. 2012; Huisman et al. 2010; Savile et al. 2010; Acker and Auld 2014). Recent development in the measurement of enzyme activity and expression level is done by genetic circuits (Choi et al. 2014; Kim et al. 2013). Computational modelling along with Molecular Dynamics (MD) helps in the prediction of unstable residues during the estimation of enzyme activity (Krieger et al. 2002; Khare et al. 2012; Joo et al. 2011; Lee et al. 2010; Seo et al. 2014). Multienzyme along with channelling of intermediates helps in the establishment of an efficient synthetic pathway using various scaffold molecules (Fu et al. 2014; Zhang 2011; Rollin et al. 2013).

6.5 Significance and Source of Asparaginase Enzyme

L-asparaginase aminohydrolase (l-asparaginase, EC 3.5.1.1) has gained attention in recent years due to its important applications the treatment of cancer cells such as leukaemia, malignant diseases of the lymphoid system and Hodgkin’s lymphomas. This enzyme also finds application in food industry for the reduction of acrylamide in foods formed at higher temperature (Appel et al. 2007; Medeiros et al. 2012).

The hydrolysis process occurs in two steps through an intermediate: beta-acyl-enzyme. The nucleophilic residue of the enzyme is activated by a strong base with an intermediate for the formation of beta-acyl-enzyme. The second reaction step involves the activation by water molecule. The mechanism of asparaginase is similar to that of serine protease where the activity depends on amino group known as catalytic triads. This trait consists of one nucleophilic amino group, serine (Ser), Histine (His) and Asparagine (Asp), connected by hydrogen bonds (Verma et al. 2007).

Asparaginase is mainly produced by submerged fermentation with the help of microbial strains such as *Pseudomonas fluorescens*, *Serratia marcescens*, *Escherichia coli*, *Erwinia carotovora*, *Proteus vulgaris*, *Saccharomyces cerevisiae*, *Karnatakensis Streptomyces*, *Streptomyces venezuelae* and several genres of fungi such as *Aspergillus*, *Penicillium* and *Fusarium*. L-asparaginase is produced from various sources such as animal cells, serum of rodents, chicken liver, plant cells, algaes and microbes such as bacteria, actinomycetes, fungi and yeast. L-asparaginase from bacteria has various therapeutic value to treat acute lymphoblastic leukaemia. Although L-asparaginase from *Erwinia carotovora* is serologically and biochemically distinct from the L-asparaginase of *E. coli*, its antineoplastic activity and toxicity are similar. L-asparaginase production from *E. carotovora* and the influence of dipyrimidine on the kinetic parameters were studied effectively (Kamble et al. 2006).

L-asparaginase production by *Nocardia* sp. was reported for high yield of enzyme production (Gunasekaran et al. 1995). Production of intracellular and extracellular asparaginase from *Streptomyces longsporus flavus* (F-15) has been studied effectively. *Streptomyces* sp. isolated from the gut of the fish *Therampon jarbua* and *Villorita cyprilloids* has L-asparaginase activity (Dhevendaran et al. 2002). L-asparaginase production from agricultural waste by solid-state fermentation using *A. niger* was reported for enzyme production. The solid-state fermentation is highly preferred over the submerged fermentation process for production of L-asparaginase due to the high yield of enzyme (Abha 2006). Cytotoxic property of L-asparaginase from endophytic fungi from Thai medicinal plant and oxidant property of L-asparaginase from *Aspergillus flavus* KUFS20 using orange peel was also studied (Teerayut et al. 2009). In addition, yeast has become the other alternative source for asparaginase production. *Rhodosporidium toruloides* and *Rhodotorula* sp., a red imperfect yeast, were reported for enzyme production (Ramakrishnan and Joseph 1996). L-asparaginase specific for L-Asparagine has been purified from a marine C*hlamydomonas* sp. which is the first enzyme to be purified from microalgae. This L-asparaginase showed a limited antitumour activity in an anti-lymphoma assay in vivo. Properties of the enzyme were contrasted with those of asparaginases from prokaryotic and eukaryotic sources (Paul and Cooksey 1981).

6.6 Acrylamide in Processed Food

Acrylamide, a low molecular weight compound is found to be colourless and crystalline in nature (Friedman 2003). This compound is considered as toxic causing damage to humans when exposed on long run. Acrylamide is formed by Maillard Reaction which is highly influenced by various processing variables. This chemical reaction influences the colour formation and has high impact on nutritional properties of food. The undesired products are mainly formed at the surface of food matrix during the thermal treatment at high temperature (Tilson 1981). It was reported that certain anti-nutritive compounds and multiple reaction products are formed at the surface of food contributing various mutagenic effects that affect the total metabolism. Acrylamide is mainly formed during roasting, baking and frying whereas process such as drying, pasteurization remains unfavourable for the formation of acrylamide. The mechanism of Maillard reaction undergoes three major stages. The first step involves the formation of acrolein by the condensation of amine and reducing sugars. The second step results in the formation of aldehydes and ammonia by degradation of amino acids at high temperature and pressure. The third step involves the formation of brown coloured nitrogenous compound known as acrylamide along with acrylic acid (Keramat et al. 2011; Van Boekel 2006). The mechanism of acrylamide formation is explained in detail in Fig. 6.1.

Fig. 6.1 Mechanism of acrylamide formation (Vleeschouwer et al. 2007)

It was reported that the formation of acrylamide does not occur in raw food products naturally unless the food containing carbohydrates are exposed to high temperature. The plant-derived food products contain high amount of glucose, fructose and asparagine which result in the formation of acrylamide. The introduction of dietary acrylamide is a major breakthrough in the management of acrylamide consumption but later it was found that they also contribute a certain negative impact on human health. During the processing of potato chips, frying temperature, time and oil used play a significant role in the initiation of reaction between asparagine and reducing sugars. Acrylamide formation was reported mainly in potato related products as they contain a high amount of carbohydrates (Sanny et al. 2012; Rommens et al. 2008). In case of wild potatoes, acrylamide formation was reported to be 5%. The western countries are highly exposed to acrylamide consumption due to daily intake of cereals, a major contributor in acrylamide formation. The strong colour and flavours are noted in bakery products as they are highly influenced by Maillard Reaction (Pedersen et al. 2012). Acrylamide formation was investigated in roasted almonds, almonds and bakery products where it was found that roasted almonds contained high acrylamide content than bakery products (Arvanitoyannis and Dionisopoulou 2014). The acrylamide content in espresso coffee and coffee blends were found to be 11.4–36.2 μg l^{-1} and 200.8–229.4 μg l^{-1}, respectively (Murkonic and Derler 2006).

6.7 Enzymatic Mitigation of Acrylamide in Fried Foods

The intracellular L-asparaginase enzyme is obtained from a variety of microorganisms where production from *E. coli* was reported with low yield due to complex formation and non-availability of optimum medium conditions. Hence, major research was focused on the optimal production method where Response Surface Methodology was adopted for optimization and was achieved with tenfold increase in the production of enzyme (Kenari et al. 2011). Asparaginase is declared as favourable food additives by the committee members of FAO as it regarded as 'generally recognized safe' by US government. It was reported that commercial asparaginase from Aldrich (A2925 *Erwinia chrysanthemi*) significantly reduced the acrylamide to 88% in mashed potato slurry of 60 g. The dosage of enzyme used in this study was 50 U. The same dosage of enzyme was used for microwaved mashed potato snack in which 99% of acrylamide reduction was achieved (Zyzak et al. 2003). It was reported that 60–90% of acrylamide reduction was noted at initial concentration achieved by the hydrolysis of asparagine to aspartic and ammonia (Mohan Kumar et al. 2014). The effect of enzymatic mitigation of acrylamide using asparaginase and other combination is given in Table 6.1.

The use of commercial asparaginase enzyme known as Acrylaway was reported for the foods such as gingerbread, crispbread, French fries and crisps. The asparaginase enzyme was listed with various sources such as *B. licheniformis*, *Cladosporium* sp., *Rhizomucor miehei*, *B. subtilis and Thermococcus zilligii*. The

Table 6.1 Effect of asparaginase in different fried foods

Food source	Type of mitigation	Optimized conditions	% of acrylamide reduction (%)	Reference
Potato slices	Asparaginase	Blanched in distilled water Temp.—75 °C Time—10 min Frying temp.—170 ° C Frying time—15 min	62 82	Pedreschi et al. (2008), Aiswarya and Baskar (2017b)
Potato crisps and French fries	Asparaginase	Soaking time—20 min	59	Xu et al. (2014)
French fries and potato crisps	Asparaginase	Frying temperature and time—180 °C for 2.5 min	80	Mahajan et al. (2012)
Potato crisps	Asparaginase (Acrylaway)	Temperature—60 °C	60	Pedreschi et al. (2008)
Banana (Kochchi kesel)	Asparaginase with chitosan	Frying temperature and time—180 °C and 25 min	85	Aiswarya and Baskar (2017a)
Carrot slices	Asparaginase with	Roasting temperature and time—170 °C and 10 min	88	Aiswarya and Baskar (2018)

use of asparaginase enzyme in French fries using *T. zilligii* was noted with the enzyme dosage of 0–20 U at the optimized temperature of 175 °C for 5 min with 80% in the acrylamide concentration (Zuo et al. 2015). The *B. subtilis* with the dosage of 0–140 U in potato crisps at a temperature of 170 °C for 90 s achieved 80% of reduction in the acrylamide concentration (Onishi et al. 2015). The amount of asparagine in foods should be considered during the optimization of enzyme concentration. The use of enzyme–substrate ratio and the dosage of the enzyme should be determined properly for easy mitigation of acrylamide in food. The usage of this enzyme at industrial level in a continuous process is a great challenge due to the cost of the enzyme. The enzyme substrate ratio and the dosage of enzyme should be determined properly for the effective mitigation of acrylamide (Anese et al. 2011). The enzymatic mitigation of acrylamide using asparaginase from *Candida utilis* ATCC9950 in potato crisps was studied effectively (Momeni et al. 2015). The effect of additives such as calcium chloride and sodium chloride solutions at various pH conditions was evaluated. The potato slices were blanched in water, and further, they were pretreated with commercial asparaginase for the reduction of acrylamide. The reduction of acrylamide by 58% was observed when

potato crisps were blanched in hot water prior to frying (Torang et al. 2016; Abboudi et al. 2016). Asparaginase isolated from *Cladosporium* sp. was found to be effective in acrylamide reduction at the optimized conditions of 220 °C and 25 min. The reduction of 97% was achieved in sweet bread crust at the enzyme concentration of 50–300 U (Mohan Kumar et al. 2014).

Asparaginase was reported to be isolated from different sources. The asparaginase isolated from *Bacillus licheniformis* which showed 80% reduction in potato strips. Asparaginase isolated from *R. miehei* was named as RmAsnase at pH of 7 at 40 °C. In order to increase the specificity of the asparaginase, the isolated enzyme was cloned and expressed in *E. coli*. It was observed that 80% reduction was achieved when treated in bread and biscuits at concentration of 10 U/g flour (Huang et al. 2014). It was reported that immobilized asparaginase was not as effective as free asparaginase to convert asparagine. The stability of the asparaginase was stable such that immobilized asparaginase was reused without any loss in the activity of asparaginase. The asparaginase was immobilized on silica-based carrier using glutaraldehyde as linking agent (Hendriksen et al. 2009).

The acrylamide mitigation in biscuits was achieved using asparaginase. The asparaginase concentration was varied from 100 to 900 ASNU in commercial asparaginase and showed that reuse of enzyme decreased the cost of the process (Anese et al. 2011). Asparaginase in gingerbread reduced the concentration of acrylamide about 55% in dough and degraded the amount of asparagine to 75%. It was reported that there were no detrimental effects on colour and taste of the food. The use of L-asparaginase was evaluated in potato-based model system and found that 90–97% of acrylamide reduction was achieved. The same L-asparaginase was used for the reduction of acrylamide in dried powdered potato products and was noted with significant reduction in acrylamide concentration. The experiments conducted on both the studies did not show any change in the organoleptic properties of the food which added the advantage of using asparaginase for acrylamide reduction (Ciesarova et al. 2006). Experiments were conducted to examine the acrylamide concentration in coffee beans. 55–74% of acrylamide reduction was reported when low dosage of enzyme (2000–6000 ASNU) is applied. The reduction of free asparagine level was achieved mainly by the treatment of asparaginase at desired conditions (Xu et al. 2014). The effect of asparaginase on acrylamide mitigation in biscuits was studied. The enzyme dosage of 100–900 ASNU was applied to determine its effect on acrylamide mitigation. The incubation conditions maintained during the study was 20–54 °C as incubation temperature and 10–30 min as incubation time. It was reported that their acrylamide development was minimum at certain concentrations of the enzyme which also did not significantly affect the final product of the food (Anese et al. 2011). The commercial enzyme known as PreventASe™ was reported for acrylamide reduction in which 500 ASNU of asparaginase solution in 10 ml of water was added to the surface of wheat/oat bread prior to baking process. The reduction of 46% was achieved in bread crust at the initial stage of enzyme treatment at 32 °C for 15 min (Ciesarová et al. 2014). The schematic representation of acrylamide mitigation using fungal asparaginase is given in Fig. 6.2.

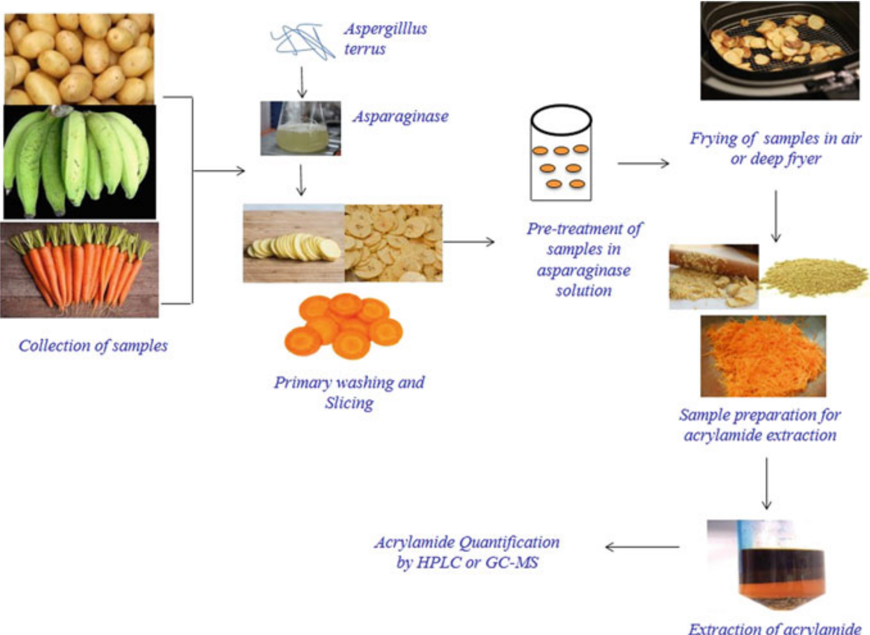


Fig. 6.2 Schematic representation of enzymatic mitigation of acrylamide in fried foods

6.8 Conclusions

The enzyme asparaginase finds its application both in pharmaceutical and food industries especially for its specificity and stability. The enzymatic mitigation using asparaginase was found to reduce the concentration of acrylamide in various fried foods. The asparaginase isolated from different sources of microorganism was proved to be effective in the mitigation of acrylamide. The use of asparaginase did not show any changes in the organoleptic properties of the food. The selection of raw materials plays a significant role during the processing of food as the presence of asparagine at lower level reduces the concentration of acrylamide. The pre-treatment of foodstuffs prior to frying process effectively helps in the reduction of acrylamide as blanching leaches the potential acrylamide precursor. The treatment of asparaginase along with additives like chitosan has potentially reduced the concentration of acrylamide in foods. The structural and computational design has helped in increasing the efficiency of the enzyme. The recombinant enzymes have brought significant improvement in increasing the efficiency of the enzyme.

References

Abboudi M, AL-Bachir M, Koudsi Y, Jouhara H (2016) Combined effects of gamma irradiation and blanching process on acrylamide content in fried potato strips. Int J Food Prop 19:1447–1454

Abha M (2006) Production of L-Asparaginase, an anticancer agent, from *Aspergillus niger* using agricultural waste in solid state fermentation. Appl Biochem Biotechnol 135:33–42

Acker MG, Auld DS (2014) Considerations for the design and reporting of enzyme assays in high throughput screening applications. Perspect Sci 1:56–73

Aiswarya R, Baskar G (2018) Microbial production of l-asparaginase and its immobilization on chitosan for the mitigation of acrylamide in heat processed carrot slices. Ind J Exp Biol 56:504–510

Aiswarya R, Baskar G (2017a) Acrylamide mitigation in fried Kochchi Kesel chips using free and immobilized fungal asparaginase Food Tech. Biotechnol 56:51–57

Aiswarya R, Baskar G (2017b) Enzymatic mitigation of acrylamide in fried potato chips using Asparaginase from *Aspergillus terreus*. Int J Food Sci Technol 53:491–498

Akoh CC, Chang SW, Lee GC, Shaw JF (2008) Biocatalysis for the production of industrial products and functional foods from rice and other agricultural produce. J Agric Food Chem 56:10445–10451

Allal O, Marwan AS, Ishita G, Yahya AF (2014) Potato chips and childhood: what does the science say? An unrecognized threat? Nutr 30:1110–1112

Anese M, Quarta B, Peloux B, Calligaris S (2011) Effect of formulation on the capacity of l-asparaginase to minimize acrylamide formation in short dough biscuits. Food Res Int 44:2837–2842

Appel IM, van Kessel-Bakvis C, Stigter R, Pieters R (2007) Influence of two different regimens of concomitant treatment with asparaginase and dexamethasone on hemostasis in childhood acute lymphoblastic leukemia. Leukemia 21(11):2377–2380

Arvanitoyannis IS, Dionisopoulou N (2014) Acrylamide: formation, occurrence in food products, detection methods, and legislation. Crit Rev Food Sci Nutr 54:708–733

Baskar G, Aiswarya R (2018) Overview in mitigation of acrylamide in starchy fried and baked foods. J Sci Food Agric 98:4385–4394

Boer AS, Priest F, Diderichsen B (1994) The industrial use of *Bacillus licheniformis*: a review. Appl Microbiol Biotechnol 40:595–598

Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K (2012) Engineering the third wave of biocatalysis. Nature 485:185–194

Bruggink A, Roos EC, de Vroom E (1998) Penicillin acylase in the industrial production of β-lactam antibiotics. Org Process Res Dev 2:128–133

Choi SL, Rha E, Lee SJ, Kim H, Kwon K, Jeong YS, Rhee YH, Song JJ, Kim HS, Lee SG (2014) Toward a generalized and high-throughput enzyme screening system based on artificial genetic circuits. ACS Synth Biol 3:163–171

Ciesarova Z, Kiss E, Boegl P (2006) Impact of L-asparaginase on acrylamide content in potato products. J Food Nutr Res 45:141–146

Ciesarová Z, Kukurová K, Mikušová L, Basil E, Polakovičová P, Duchoňová L, Vlček M, Šturdík E Nutritionally enhanced wheat-oat bread with reduced acrylamide level. Qual Assur Saf Crop 6:327–334

De Boer AS, Diderichsen B (1991) On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl Microbiol Biotechnol 36:1–4

Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ (1995) Acrylamide: a review of its genotoxicity and an assessment of heritable genetic. Mutat Res 330:71–99

Dhevendaran K, Anithakumari YK (2002) L-Asparaginase activity in growing conditions of *Streptomyces* sp. associated with *Therampon jarbua* and *Villorita cyprinoids* of Veli Lake, South India. Fish Technol 39:155–159

Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SPD, Muller DJG, Olin S, Petersen BJ, Schlatter J, Scholz G, Scimeca JA, Slimani N, Tornqvist M, Tuijtelaars S, Verger P (2005) Human exposure and internal dose assessments of acrylamide in food. Food Chem Toxicol 43:310–365

Elleuche S, Schroder C, Sahm K, Antranikian G (2014) Extremozymes-biocatalysts with unique properties from extremophilic microorganisms. Curr Opin Biotechnol 29C:116–123

Estell DA, Graycar TP, Wells JA (1985) Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. J Biol Chem 260:6518–6521

Evran S, Telefoncu A, Sterner R (2012) Directed evolution of (βα)-barrel enzymes: establish ingphosphoribosylanthranilate isomerisation activity on the scaffold of the tryptophan synthase-subunit. Protein Eng Des Sel 25:285–293

Fernandes P (2010) Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme Res 2010:862537

Flamm EL (1991) How FDA approved chymosin: a case history. Bio/Technology 9:349–351

Food Chemicals Codex (FCC) (2004) Institute of Medicine of the National Academies. The National Academies Press Washington, DC

Friedman M (2003) Chemistry, biochemistry, and safety of acrylamide—a review. J Agric Food Chem 51:4504–4526

Fu J, Yang YR, Johnson-Buck A, Liu M, Liu Y, Walter NG, Woodbury NW, Yan H (2014) Multi-enzyme complexes on DNA scaffolds capable of substrate channelling with an artificial swinging arm. Nat Nanotechnol 9:531–536

Gunasekaran S, McDonald L, Manavathu M, Manavathu E, Gunasekaran M (1995) Effect of culture media on growth and L-asparaginase production in *Nocardia asteroids*. Biomed Lett 52:197–201

Hang H, Miao M, Li Y, Jiang B, Mu W, Zhang T (2013) Difructosan anhydrides III preparation from sucrose by coupled enzyme reaction. Carbohydr Polym 92(2):1608–1611

Heinzelman P, Snow CD, Wu I, Nguyen C, Villalobos A, Govindarajan S, Minshull J, Arnold FH (2009) A family of thermostable fungal cellulases created by structure-guided recombination. Proc Natl Acad Sci 106:5610–5615

Hendriksen HV, Kornbrust BA, Østergaard PR, Stringer MA (2009) Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from *Aspergillus oryzae*. J Agric Food Chem 57:4168–4176

Huang L, Liu Y, Sun Y, Yan Q, Jiang Z (2014) Biochemical characterization of a novel L-asparaginase with low glutaminase activity from Rhizomucormiehei and its application in food safety and leukemia treatment. J Appl Environ Microbiol 80:1561–1569

Huisman GW, Liang J, Krebber A (2010) Practical chiral alcohol manufacture using ketoreductases. Curr Opin Chem Biol 14:122–129

Illanes A, Cauerhff A, Wilson L, Castro GR (2012) Recent trends in biocatalysis engineering. Biores Technol 115:48–57

Jaeger H, Janositz A, Knorr D (2010) The Maillard reaction and its control during food processing —the potential of emerging technologies. Pathol Biol 58:207–213

Jensen VJ, Rugh S (1987) Industrial-scale production and application of immobilized glucose isomerase. In: Klaus M (ed) Methods in enzymology. Academic Press, pp 356–370

Jin C, Wu X, Zhang Y (2013) Relationship between antioxidants and acrylamide formation: a review. Food Res Int 51:611–620

Jonas DA, Antignac E, Antoine JM, Classen HG, Huggett A, Knudsen I, Mahler J, Ockhuizen T, Smith M, Teuber M, Walker R, de Vogel P (1996) The safety assessment of novel foods. Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem Toxicol 34:931–940

Joo JC, Pack SP, Kim YH, Yoo YJ (2011) Thermostabilization of *Bacillus circulans* xylanase: computational optimization of unstable residues based on thermal fluctuation analysis. Biotechnol 151:56–65

Kamble VP, Srinivasa RR, Prita SB, Khobragade CN, Dawane BS (2006) Purification of L-Asparaginase from a bacteria *Erwinia carotovora* and effect of a dihydropyrimidine derivative on some of its kinetic parameters. Ind J Biochem Biophys 43:391–394

Kenari SLD, Alemzadeh I, Maghsodi V (2011) Production of L-asparaginase from *Escherichia coli* ATCC 11303: optimization by response surface methodology. Food Bioprod Process 89:315–321

Keramat J, Le Bail A, Prost C, Jafari M (2011) Acrylamide in baking products: a review article. Food Bioprocess Technol 4:530–543

Khare SD, Kipnis Y, Greisen PJ, Takeuchi R, Ashani Y, Goldsmith M et al (2012) Computational redesign of a mononuclear zinc metalloenzyme for organophosphate hydrolysis. Nat Chem Biol 8:294–300

Kim E, Lee S, Jeon A, Choi JM, Lee HS, Hohng S, Him HS (2013) A single-molecule dissection of ligand binding to a protein with intrinsic dynamics. Nat Chem Biol 9:313–318

Koushki MM, Rouhani H, Farsi M (2011) Genetic manipulation of fungal strains for the improvement of heterologous genes expression (a mini-review). Afr J Biotechnol 7939–7948

Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. Proteins 47:393–402

Krishnapura PR, Belur PD, Subramanya S (2016) A critical review on properties and applications of microbial l-asparaginases, Crit Rev Microbiol 42:720–737

Kumar A, Singh S (2013) Directed evolution: tailoring biocatalysts for industrial applications. Crit Rev Biotechnol 33:365–378

Lee SC, Kim JH, Kim HS (2010) Design and evolution of biocatalysts. Curr Org Chem 14:1894–1901

Li S, Yang X, Yang S, Zhu M, Wang X (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J 2:e201209017

Lofstedt RE (2003) Science communication and the Swedish acrylamide "alarm". J Health Commun 8:407–432

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nat 438:1157–1161

Mahajan RV, Saran S, Kameswaran K, Kumar V, Saxena RK (2012) Efficient production of L-asparaginase from *Bacillus licheniformis* with low-glutaminase activity: optimization, scale up and acrylamide degradation studies. Bioresour Technol 125:11–16

Medeiros Vinci R, Mestdagh F, De Meulenaer B (2012) Acrylamide formation in fried potato products—present and future, a critical review on mitigation strategies. Food Chem 133:1138–1154

Mohan Kumar NS, Shimray CA, Indrani D, Manonmani HK (2014) Reduction of Acrylamide Formation in Sweet Bread with L-Asparaginase Treatment. Food Bioprocess Technol 7:741–748

Momeni V, Alemzadeh I, Vosoughi M (2015) Enhancement of L-asparaginase production by *Candida utilis* in a 13l fermenter and its purification. Int J Eng-Trans B Appl 28:1134–1139

Murkonic M, Derler K (2006) Analysis of amino acids and carbohydrates in green coffee. J Biochem Biophys Methods 69:25–32

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001) Taxonomic study of aerobic *Thermophilic bacilli*: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G.*

kaustophilus, *G. thermoglucosidasius* and *G. thermodenitrificans*. Int J Syst Evol Microbiol 51:433–446
Olempska-Beer ZS, Merker RI, Ditto MD, Di Novi MJ (2006) Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharm 45:144–158
Onishi Y, Prihanto AA, Yano S, Takagi K, Umekawa M, Wakayama M (2015) Effective treatment for suppression of acrylamide formation in fried potato chips using L-asparaginase from *Bacillus subtilis*. Biotech 5:783–789
Park AR, Oh DK (2010) Galacto-oligosaccharide production using microbial beta-galactosidase: current state and perspectives. Appl Microbiol Biotechnol 85(5):1279–1286
Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century Regul Toxicol. Pharmacol 33:173–186
Paul JH, Cooksey KE (1981) Regulation of L-Asparaginase E.C.-3.5.1.1 in a *Chlamydomonas* species in response to ambient concentrations of combined nitrogen. J Bacteriol 147:9–12
Pedersen M, Von Stedingk H, Botsivali M, Agramunt S, Alexander J, Brunborg G, Chatzi L, Fleming S, Fthenou E, Granum B, Gutzkow KB, Hardie LJ, Knudsen LE, Kyrtopoulos SA, Mendez MA, Merlo DF, Nielsen JK, Rydberg P, Segerback D, Sunyer J, Wright J, Tornqvist M, Kleinjans JC, Kogevinas M (2012) Birth weight, head circumference, and prenatal exposure to acrylamide from maternal diet: the European prospective mother-child study (New Generis Consortium). Environ Health Persp 120:1739–1745
Pedersen PB, Bjørnvad ME, Rasmussen MD, Petersen JN (2002) Cytotoxic potential of industrial strains of *Bacillus* sp. Regul Toxicol Pharmacol 36:155–161
Pedreschi F, Kaach K, Granby K, Risum J (2008) The effect of asparaginase on acrylamide formation in French fries. Food Chem 109:386–392
Ramakrishnan MS, Joseph R (1996) Characterization of an extracellular asparaginase of *Rhodosporidium toruloides* CBS14 exhibiting unique physicochemical properties. Can J Microbiol 42:316–325
Rodriguez-Colinas B, de Abreu MA, Fernandez-Arrojo L, de Beer R, Poveda A, Jimenez-Barbero J, Haltrich D, Ballesteros Olmo AO, Fernandez-Lobato M, Plou FJ (2011) Production of Galacto-oligosaccharides by the β-Galactosidase from Kluyveromyces lactis: comparative analysis of permeabilized cells versus soluble enzyme. J Agric Food Chem 59(19):10477–10484
Rollin JA, Tam TK, Zhang YHP (2013) New biotechnology paradigm: cell-free biosystems for biomanufacturing. Green Chem 15:1708–1719
Rommens CM, Yan H, Swords K, Richael C, Ye J (2008) Low-acrylamide French fries and potato chips. Plant Biotechnol J 6:843–853
Sanny M, Jinap S, Bakker EJ, Van Boekel MAJS, Luning PA (2012) Possible causes of variation in acrylamide concentration in French fries prepared in food service establishments: an observational study. Food Chem 132:134–143
Savile CK, Janey JM, Mundorff EC, Moore JC, Tam S, Jarvis WR, Colbeck JC, Krebber A, Fleitz FJ, Brands J, Devine PN, Huisman GW, Hughes GJ (2010) Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. Science 329:305–309
Sedlaczek L (1988) Biotransformations of steroids. Crit Rev Biotech 7:187–236
Seo MH, Park J, Kim E, Hohng S, Kim HS (2014) Protein conformational dynamics dictate the binding affinity for a ligand. Nat Commun 5:3724
Siegel JB, Zanghellini A, Lovick HM, Kiss G, Lambert AR, St Clair JL, Gallaher JL, Hilvert D, Gelb MH, Stoddard BL, Houk KN, Michael FE, Baker D (2010) Computational design of an enzyme catalyst for a stereoselective bimolecular Diels-Alder reaction. Science 329:309–313
Silberg JJ, Endelman JB, Arnold FH (2004) SCHEMA-guided protein recombination. In: Dan ER, Joseph PN (eds) Methods in enzymology. Academic Press, pp 35–42
Sindhu R, Binod P, Sabeela Beevi U, Amith A, Anil KM, Aravind M, Sharrel R, Ashok P (2018) Applications of microbial enzymes in food industry. Food Technol Biotechnol 56:16–30
Teerayut T, Kevin DH, Saisamorn L (2009) Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxic properties. Int J Integr Biol 7:1–8

Tilson HA (1981) The neurotoxicity of acrylamide: an overview. Neurobehav Toxicol Teratol 3:445–461
Torang A, Alemzadeh I (2016) Acrylamide reduction in potato crisps using: asparaginase from *Candida utilis*, commercial asparaginase, salt immersion, and pH treatment. Int J Eng Trans A 29:879–886
Van Boekel MAJS (2006) Formation of flavour compounds in the Maillard reaction. Biotech Adv 24:230–233
Van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6: 213–218
Vera C, Guerrero C, Conejeros R, Illanes A (2012) Synthesis of galacto-oligosaccharides by β-galactosidase from Aspergillus oryzae using partially dissolved and supersaturated solution of lactose. Enzyme Microb Technol 50:188–194
Verma N, Kumar K, Kaur G, Anand S (2007) L-asparaginase: a promising chemotherapeutic agent. Crit Rev Biotechnol 27:45–62
Vleeschouwer K, Van der PI, Van LA, Hendrickx ME (2007) Kinetics of Acrylamide formation/ elimination reactions as affected by water activity. Biotechnol Progr 23:722–728
Xu Y, Cui B, Ran R, Liu Y, Chen H, Kai G, Shi J (2014) Risk assessment, formation and mitigation of dietary acrylamide: current status and future prospects. Food Chem Toxicol 69:1–12
Zhang YH (2011) Substrate channelling and enzyme complexes for biotechnological applications. Biotechnol Adv 29:715–725
Zuo S, Zhang T, Jiang B, Mu W (2015) Reduction of acrylamide level through blanching with treatment by an extremely thermostable L-asparaginase during French fries processing. Extremophiles 9:841–851
Zyzak DV, Sanders RA, Stojanovic M, Tallmadge DH, Eberhart BL, Ewald DK, Gruber DC, Eberhart DH, Ewald BL, Gruber DK (2003) Acrylamide formation mechanism in heated foods. J Agric Food Chem 51:4782–4787

Chapter 7
Xylanases for Food Applications

Kedar Sharma, Abhijeet Thakur and Arun Goyal

Abstract The development of new food products, improvement in food quality, and ease of food production process is of prime concern with the growing world population and rapidly rising demand for functional foods. These concerns make it imperative, the use of various enzymes such as glycoside hydrolases, lipases, proteases, transglutaminases, etc., in the processing of food and food ingredients. Crops and fruits used in food and brewing industry contain considerable amount of xylan. Xylan is a branched heteropolysaccharide and its main chain is composed of xylose subunits linked by β-(1 → 4) glycosidic bonds and contains different substitutions in the side chain. Xylanase cleaves β-(1 → 4) glycosidic bonds in heteroxylan randomly and converts it into xylooligosaccharides. In the last decade, xylanase has received appreciable attention owing to its applications in various food processing industries such as cereal food processing for the improvement of gluten agglomeration, baking industry for the improved texture of bread and cookies, clarification of fruit juices, production of xylooligosaccharide or arabinoxylooligosaccharides as prebiotic food supplements. This chapter presents a comprehensive overview of xylanase, its sources, production, and applications in food production and processing, with a particular focus on recent developments.

7.1 Introduction

Busy and stressful lifestyle leads to the risk of diseases related to the increase in blood cholesterol, diabetes, coronary heart disease, cardiovascular, and colon cancer. The demand of fiber-enriched, healthier, nutritious and functional meal is increasing for curing these stress-related diseases (Kendall et al. 2010). The Indian food market will reach USD 46 billion in 2020 from USD 31 billion in 2018 according to statista report. The physical and chemical methods are commonly used

K. Sharma · A. Thakur · A. Goyal (✉)
Carbohydrate Enzyme Biotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
e-mail: arungoyl@iitg.ac.in

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_7

for food processing, but these methods are not sufficient to reach the quality standards set by the food regulatory agencies (Cauvain and Young 2006). The enzymes have, however helped in overcoming the limitations of physical and chemical processes (Escobedo-Avellaneda et al. 2011). The raw material used in food preparation contains various types of plant polysaccharides, proteins, lipids, minerals, antioxidants, and vitamins, etc., in the form of intricate networks (Develaraja et al. 2016). Therefore, processing of the components such as polysaccharides, proteins and lipids require glycoside hydrolases, proteases, lipases respectively, for the enhancement of nutritional value. The major portion of plant polysaccharides is cellulose, while the second-most abundant plant polysaccharide is hemicelluloses. Hemicellulose is a group that contains various types of heteropolysaccharides, such as mannan, galactan, arabinanandxylan. These heteropolysaccharides are categorized into several types based on their composition, such as arabinoxylan, arabino-galacatan, glucomannan, glucuronoxylan, etc. The majority of these heteropolysaccharides are composed of one or more monomer units such as D-mannose, D-xylose, L-arabinose, and D-galactose linked through a glycosidic linkage (Beg et al. 2001).

The most abundant hemicellulose present in the cell wall of plants as well as in some macrophytic algae is xylan. Xylans are located between the collection of cellulose fiber and lignin present in the plant cell wall (Scheller and Ulvskov 2010). Xylans are uniform in their structure and side-chain substitutions are scattered, convoluted, and covalently linked with 'lignin sheath' at various places and forming a coat around concealed cellulose strands by hydrogen bonding (Biely 1985). Xylans are branched heteropolymers comprising D-xylose units linked by β-1,4-xylosidic bonds in the main chain. The free –OH groups of xylan main chain contains common substitutions such as glucuronic, arabinofuranosyl, and acetyl groups (Yang et al. 2006). The xylan content hardwood and softwood are different. The xylan content of hardwoods constitutes a significant amount of xylan 10–35% of the hemicelluloses, whereas, the softwoods contain 10–15% xylan of the total hemicellulose content (Chen 2014). The 4-O-methyl glucuronoxylan is present as the principal constituent in hardwood (Sjostrom 2013). The 4-O-methyl glucuronic acid is connected by α-1,2 linkage at the C-2 position of D-xylopyranosyl units of the main chain (Sjostrom 2013). The cereal plants contain arabinoxylan as a major part of the hemicellulosic component and are considered as dietary fibers. One or more L-arabinofuranosyl units are linked with β-(1 → 4) glycosidic bonds of the xylose backbone at position C2 or C3 forming substituted xylans. Arabinoxylan is present in primary cell walls of grasses and monocot plant cereals such as wheat, rye, barley, oat, rice, corn, and sorghum (Saha 2000). The presence of high content of L-arabinosyl residues as a substitution in the xylan main chain restricts its hydrolysis. Xylan hydrolysis is vital step to produce value-added nutritional products, such as xylooligosaccharides and other biochemicals. These xylooligosaccharides can be used as prebiotic which supports the growth of probiotic bacteria and therefore can be used as functional food supplement (Samanta et al. 2016). There has been growing demand for prebiotic for commercial food applications, which can be fulfilled by the production of xylooligosaccharides from

promising lignocellulosic material by rapid and efficient process. Xylooligosaccharides can be produced by auto-hydrolysis, chemical, and enzymatic methods. However, the auto-hydrolysis methods require special equipment for providing the high temperature to the biomass and the chemical method involves the use of corrosive acids. The enzymatic production method of xylooligosaccharides provides a safe alternative and does not require any special equipment for production and is preferred owing to simple downstream processing methods (Samanta et al. 2016).

The complex xylan structure requires the synergistic action of an array of enzymes such as endo-β-xylanase, α-glucuronidase, β-xylosidase, α-arabinofuranosidase, *p*-coumaric acid esterase, acetyl xylan esterase, and ferulic acid esterase for its complete hydrolysis (Sharma et al. 2018a). Among all these enzymes, the endo-β-xylanase randomly (EC.3.2.1.8) hydrolyzes the xylan and produces xylooligosaccharides, which can be used as food supplement for the improvement of probiotic bacterial growth (Goluguri et al. 2016). Xylanases belong to glycoside hydrolase family and the majority of them are classified in glycoside hydrolase (GH) families 10 and 11 and some are also found in other GH families viz. 5, 7, 8, 16, 26, 43, 52, and 62 (Liao et al. 2014).

7.2 Source of Xylanases

Xylanases are present in all life forms such as archaea (Wainø and Ingvorsen 2003), bacteria (Winterhalter and Liebl 1995), fungus (Min et al. 2002), plants (Suzuki et al. 2002), animals such as mollusc (Yamaura et al. 1997) and nematodes (Mitreva-Dautova et al. 2006). The plants and animals produce less amount of xylanase whereas, the microorganisms such as bacteria and fungi produce in large amounts. The microorganisms are preferred over plants and animals because of various advantages such as, rapid multiplication, minimal requirement of space, high biodegradation capacity towards waste biomass, controlled production in closed systems, ease of handling and utilization of cheap carbon sources which lowers the cost of production (Mitreva-Dautova et al. 2006). Therefore, the demand of various industries can be fulfilled by microbial xylanases rather than xylanases from plant and animal sources.

7.2.1 Bacterial Xylanases

Among microbes, the bacteria have proven to be abundant producers of xylanases and have considerable bioprocess interests. Bacterial genera, such as *Cellulomonas* (Laurie et al. 1997), *Bacillus* (Gupta et al. 2015; Adhyaru et al. 2014), *Microbacterium* (Kim et al. 2005) and *Paenibacillus* (Liu et al. 2018) have been reported as prolific producers of xylanase. Some prominent xylanase producers

include *Cellulomonas fimi* (Laurie et al. 1997), *Bacillus* sp. and *Bacillus halodurans* (Gupta et al. 2015), *Microbacterium xylanilyticum* (Kim et al. 2005), from *Paenibacillus barengoltzii* (Liu et al. 2018), *Cellvibrio japonicus* (Pell et al. 2004), *Bacillus subtilis* (John et al. 2006), *Streptomyces* sp. S9 (Li et al. 2008), *Bacteroides xylanisolvens XB1A* (Mirande et al. 2010), *Cellvibrio mixtus* (Wu and He 2015), *Clostridium thermocellum* (Verma and Goyal 2016) and *Pseudopedobacter saltans* (Sharma et al. 2018b). The various research groups have reported the xylanase production from marine bacteria such as *Alcaligenes* sp. XY-234 (Araki et al. 1998), *Glaciecolamesophila* KMM 241 (Guo et al. 2009), *Bacillus pumilus* GESF1 (Menon et al. 2010), and *Thermoanaerobacterium saccharolyticum* NTOU1 (Hung et al. 2011). These xylanolytic bacteria produce various types xylanases such as thermophilic (active at high temperature), mesophilic (active at low temperature), psychrophilic (active at sub-zero temperature), neutral or alkaline xylanases.

7.2.2 Fungal Xylanases

Fungal xylanases have been widely used in the food industry. Among the fungal genera, *Fusarium* (Saha 2002), *Penicillium* (Dwivedi et al. 2009), *Aspergillus* (Pal and Khanum 2010), and *Trichoderma* (Ayadi et al. 2015) are the most common producers of xylanases. The xylanases produced by fungal species are active at acidic pH and have high activity between 40 and 60 °C. Specifically, most prominent xylanases producing members include, *Fusarium proliferatum* (Saha 2002), *Penicillium oxalicum* (Dwivedi et al. 2009), *Aspergillus niger* (Pal and Khanum 2010), *Trichoderma reesei* (Ayadi et al. 2015), *Paecilomyces themophila J18* (Yang et al. 2006), *Humicola insolens* (Du et al. 2013), *Aspergillus oryzae* (Chutani and Sharma 2015), *Pichia stipitis* (Ding et al. 2018) and *Trichoderma virens* (Sadaf and Khare 2014).

7.3 Production of Xylanases

Xylanases from plant and animal sources are unable to meet industrial demands. Therefore, the microbial sources are popular for xylanases production. The biodiversity of microorganisms, the adaptability to efficient genetic manipulation and ease of growing make them a versatile source of xylanase. The production of xylanase depends on the selected microorganism, composition of growth medium, physicochemical growth parameters and the enzyme producing fermentation method. The growth medium contributed 30–40% to the total cost of enzyme production (Mitreva-Dautova et al. 2006). The selection of the efficient microorganism is essential for the production of xylanase for achieving higher yield in a short time period. The xylanase production can be affected by the type and concentration of medium components such as carbon source, nitrogen source,

micronutrient, and macronutrients. The appreciable yields of xylanase was achieved by using agro-waste such as wheat bran, rice straw, corncob, soy oil cake, sugarcane bagasse, corn stover, and banana waste (Thomas et al. 2017). Sodium nitrate, ammonium sulfate, ammonium chloride as inorganic nitrogen sources can be used for xylanase production. The peptone, tryptone, yeast extract, corn steep solid, and soybean meal are the organic nitrogen sources for xylanase production. The physiochemical growth parameters such as inoculum size, temperature, pH, the water content of substrate, aeration, and incubation period significantly influence the xylanase production (Thomas et al. 2017). Microbial xylanases are produced by solid-state and submerged fermentation processes. The efficient and cost-effective xylanase production by a fermentation process depends upon the utilization of cheaper agro-waste, productivity of microorganism, recovery and purification of xylanase by using downstream processing methods.

7.3.1 Submerged Fermentation

Submerged fermentation (SmF) method is widely adapted by the industries for xylanase production. The liquid fermentation medium containing soluble nutrients can be used for growth of a microorganism in SmF. The carbon and nitrogen source along with the nutrients is suspended or dissolved in water. The shake flask, bench scale, pilot, or industrial scale bioreactors can be used for enzyme production using SmF process. Approximately, 75–80% of commercial xylanases are produced by SmF. It is the preferred production process because of the effective control over the physicochemical factors such as, temperature, pH of the medium, the agitation speed, and aeration for microbial growth (Pandey et al. 1999). Some microbial xylanases produced by SmF are listed in Table 7.1.

7.3.2 Solid-State Fermentation

The solid-state fermentation (SSF) is a most suitable method for xylanase production by fungi. Fungi are known for their ability to grow in the presence of less water. SSF is a cost-effective technique for the production of industrially important products such as enzymes, antibiotics, organic acids, etc. The SSF method is considered as a natural type of fermentation method as compared to other fermentation methods because the growth conditions for the microorganism in SSF are almost similar to natural growth conditions. The success of fermentation and higher production of xylanases mainly depends on the selection of suitable support system for microorganism cultivation. The agro-waste residues such as wheat bran, wheat straw, rice bran, rice husk, soybean cake, corncob, and sugarcane bagasse are widely used as a support system and also utilized as a carbon source. The efficiency of xylanase production is affected by the carbon source, nitrogen source,

Table 7.1 Xylanase production from bacteria and fungi by Submerged Fermentation

Organism	Carbon source (%, w/v)	Nitrogen source (%, w/v)	Enzyme activity (U/mL)	Reference
Bacteria				
Bacillus altitudinis	Sorghum (3%)	Gelatine (0.5%)	245	Adhyaru et al. (2014)
Streptomyces violaceoruber	Wheat bran (3.5%)	Peptone (0.8%) Beef extract (0.8%)	1500	Khurana et al. (2007)
Bacillus pumulis	Corncob (2%) Cotton seed bagasse (2%) Crude wheat bran (2%) Barley (2%)	Yeast extract (0.25%)	184 183 173 134	Yasinok et al. (2010)
Bacillus mojavensis AG137	Oat bran (2%)	Yeast extract (1%) Tryptone (1%)	302	AkhavanSepahy (2011)
Streptomyces sp. RCK-2010	Wheat bran (2.5%)	Peptone (0.2%) Beef extract (1.2%)	2310	Kumar et al. (2012)
Bacillus licheniformis	Xylan (1%)	Casein (1%)	30	Bajaj and Manhas (2012)
Bacillus pumilus MTCC5015	Wheat bran (0.5%)	Yeast extract (4%)	372	Thomas et al. (2015)
Fungi				
Penicillium oxalicum SAU_E-3.510	Congress grass (0.25%)	Yeast extract (0.6%)	563	Dwivedi et al. (2009)
Aspergillus oryzae	Beechwood xylan (2.5%)	Peptone (5%) Yeast extract (5%)	7452	Chutani and Sharma (2015)
Aspergillus foetidus MTCC 4898	Birchwood xylan (1%)	Proteose-peptone (1%)	210	Shah and Madamwar (2005)
Penicillium oxalicum ZH-30	Wheat bran (10%)	Yeast extract (1%)	14.50	Li et al. (2007)
Thermomyces lanuginosus SDYKY-1	Corncob (38.7%)	Soybean meal (17.5%)	3079	Su et al. (2011)

surfactants, and metal ions present in the growth medium (Thomas et al. 2017). The xylanase production is also affected by the physicochemical growth parameters such as moisture ratio, pH, temperature, inoculum size, aeration, agitation, and

granule size of support material. The SSF method involves compact fermentation vessel that requires low water volume, simple machinery, simple medium, less space, less effluent, lower energy demand, low capital and recurring expenditure which lead to relatively easy downstream processing and higher product yield. Xylanase production from various microorganisms by SSF is listed in Table 7.2. In the last decade, only a few studies were performed on the comparative analysis of bacterial and fungal xylanase production by using submerged and solid-state fermentation methods. The organisms for xylanases production were evaluated by both methods are listed in Table 7.3.

Table 7.2 Xylanase production from bacteria and fungi by Solid-State Fermentation

Organism	Substrate	Enzyme activity (U/g)	Reference
Bacteria			
Bacillus subtilis ASH	Wheat bran	8964	Sanghi et al. (2008)
Burkholderia sp.	Anaerobically treated distillery spent wash	5200–5600	Mohana et al. (2008)
Bacillus pumilus SV-85S	Wheat bran	73,000	Nagar et al. (2011)
Bacillus pumilus	Corncob and soy meal	326	Kapilan and Arasaratnam (2011)
Bacillus sp. *PKD-9*	Wheat bran	75,000	Panwar et al. (2014)
Cellulosimicrobium cellulans CKMX1	Apple pomace	1150	Walia et al. (2015)
Fungi			
Paecilomyces themophila J18	Wheat straw	18,580	Yang et al. (2006)
Aspergillus niger DFR-5	Wheat bran and soybean cake (70:30)	4465	Pal and Khanum (2010)
Aspergillus oryzae	Wheat bran	34,442	Chutani and Sharma (2015)
Aspergillus terreus MTCC 8661	Palm fiber	115,269	Lakshmi et al. (2009)
Aspergillus foetidus MTCC 4898	Wheat bran and anaerobically treated distillery spent wash	8450	Chapla et al. (2010)
Aspergillus carneus M34	Coba husk and corn steep liquor (4.5:0.5)	1721	Fang et al. (2010)
Aspergillus sp.	Wheat bran	5059	Thomas et al. (2016)
Aspergillus tubingensis FDHN1	Sorghum straw	5177	Adhyaru et al. (2016)

Table 7.3 Comparative xylanase production by SmF and SSF method from bacteria and fungi

Organism	Carbon source	Enzyme activity		Reference
		Submerged fermentation	Solid-state fermentation	
Bacteria				
Bacillus pumilus	Rice straw	1273 U/g	11,000 U/g	Poorna and Prema (2006)
Streptomyces viridosporus T7A	*Soybean bran B complex vitamins and hydroxyethylcellulose Sugarcane bagasse, napier grass and soybean meal*	23.4 U/mL	424 U/g	Alberton et al. (2009)
Bacillus sp.	Corncob, peptone and yeast extract Corncob and wheat bran	16 U/mL	6.2 U/g	Gupta and Kar (2009)
Bacillus subtilis	Wheat bran and soybean hull Pineapple peel and soybean hull	12 U/mL	19 U/mL	Ling Ho and Heng (2015)
Bacillus subtilis	Barley husk Wheat bran	11.6 U/mL	22 U/mL	Ho (2015)
Fungi				
Sporisorium reilianum	Birch xylan	12.6 U/mL	4.2 U/mL	Álvarez-Cervantes et al. (2013)
Aspergillus oryzae	Beechwood xylan Wheat bran	7452 U/mL	34,442 U/g	Chutani and Sharma (2015)
Aspergillus niger	Wheat straw Rice husk	6658 U/g 6504 U/g	4549 U/g 5127 U/g	Membrillo Venegas et al. (2013)

7.3.3 Production of Recombinant Xylanases

The extensive application of microbial xylanases in food and wine industry necessitates its production. The sale volume of xylanase represents approximately, 10–15% of the commercial carbohydrate-active enzymes in the food and fruit juice industries. Various bioprocess applications require different types of xylanases acting under different conditions. For example, bread-making process requires the xylanases that are active and stable at a higher temperature (45–95 °C), whereas in the fruit juice extraction and clarification requires enzymes active between 25 and 40 °C. The commercial xylanase is mainly produced from filamentous fungus, *Aspergillus* genus. These xylanases are not always suitable for different industrial processes owing to their mesophilic and limited substrate specificities (Thomas et al. 2017). Various industrial processes require xylanases having capability of

performing under different conditions. Therefore, it is important to have more robust and broad-spectrum enzymes which will allow their use under most conditions for various applications. The enhanced production of xylanases is possible with the advent of recombinant DNA technology. It is now used as a replacement strategy for traditional protein production methods. The xylanase encoding genes from various cellulolytic and non-cellulolytic organisms such as *Ruminococcus flavefaciens* (Flint et al. 1991), *C. thermocellum* (Verma and Goyal 2016), *A. niger* (Elgharbi et al. 2015), and *T. reesei* (Ayadi et al. 2015) have been cloned and expressed in a heterologous host. Various recombinant xylanases from different sources expressed in the different hosts are listed in Table 7.4.

Table 7.4 Recombinant xylanase production from bacteria and fungi

Organism	Expression host	Enzyme activity (U/mg)	Reference
Bacteria			
Pseudopedobacter saltans	*Escherichia coli* BL21 (DE3)	60	Sharma et al. (2018b)
Glacier colamesophila KMM 241	*Escherichia coli* BL21 (DE3)	77	Guo et al. (2009)
Streptomyces avermitilis	*Escherichia coli* BL21 (DE3)	454	Li et al. (2008)
Paenibacillus campinasensis BL11	*E. coli HMS174 (DE3).*	2392	Ko et al. (2010)
Geobacillus thermoleovorans	*Escherichia coli* BL21 (DE3)	270	Verma and Satyanarayana (2012)
Geobacillus sp. *WSUCF1*	*Escherichia coli* BL21 (DE3)	461	Bhalla et al. (2014)
Caldicellulosiruptorkronotskyensis	*Escherichia coli* BL21 (DE3)	330	Jia et al. (2016)
Fungi			
Humicolainsolens Y1	*P. pastoris*	236	Du et al. (2013)
Lechevalieria sp.	*P. pastoris*	287	Zhou et al. (2012)
Achaetomium sp.	*P. pastoris*	767	Zhao et al. (2013)
Gloeophyllumtrabeum	*P. pastoris*	1508	Wang et al. (2016)
Corynascusthermophilus	*P. pastoris* X33	10,245	Yang and Zhang (2017)
Eupenicilliumparvum 4	*P. pastoris*	384	Long et al. (2018)

7.4 Industrial Production and Global Market

According to the Global Market Insights, Inc., the global market for food enzymes is increasing and will reach USD 3.6 Billion by 2024. Carbohydrate-active enzymes will top and would acquire approximately, 50% market with USD 1.8 Billion by 2024. The global xylanase market is anticipated to grow at a compound annual growth rate (CAGR) of 6.6%. The industrial market for xylanase sale is dominated by Alltech Inc., USA; Adisseo, France; BASF Enzymes LLC, California, USA; Novozymes, Denmark; Enzyme Development Corporation, New York, USA; Genencor, New York, USA; Danisco; Copenhagen, Denmark; DSM, Netherlands; Associated British Foods plc London, UK and Takabio, France.

7.5 Food Applications

Xylanases find applications in various processes such as food and feed, fruit juice clarification, bio-bleaching in paper industry, textile, detergent, and pharmaceutical industries (Beg et al. 2001). The industrial use of xylanases was made in the 1980s for the preparation of fodder for animal and later for biofuels, foods, paper, and textile industries.

7.5.1 Xylanases in the Baking Industry

One of the conventional staple foods consumed throughout the world is bread, which was initially manufactured by using refined flour that is deficient in dietary fibers. The consumer's awareness of nutritious and fiber-rich food encouraged manufacturers to produce whole wheat bread. The cell walls of the whole wheat grain peripheral tissues are hydrophobic and composed of cellulose, complex insoluble arabinoxylan, and lignin. The cell walls of inner tissues are hydrophilic and mostly contain arabinoxylan and β-D-glucans. The primary constituent of fibers present in wheat grain is arabinoxylan which exists in two forms as water soluble and water insoluble. The dough prepared by using whole wheat flour is an intricate and continuous network of starch embedded in gluten protein mixture. The quality of bread is profoundly influenced by the presence of fibers (Cavella et al. 2008). The utilization of xylanase has exceptionally increased in baking industry in the last decade owing to its potential of conferring beneficial properties to the bread. Xylanases transform the water-insoluble arabinoxylan into a soluble arabinoxylan form, which interacts with water in the dough, therefore increasing the volume, creating more uniform and finer crumbs and decreasing dough firmness (Butt et al. 2008). Further, it improves the dough quality in such a manner that, it does not adhere to the machine parts during dough-making (Butt et al. 2008). The bread

quality and its shelf life could be extended by the reduction in staling rate, after the xylanase treatment (Harris and Ramalingam 2010). The anti-staling action of xylanase during bread storage and softening of doughs is due to a breakdown of arabinoxylans which release water (Oliveira et al. 2014). Several reports are available on the use of xylanase alone or in combination with other enzymes used in bread manufacturing. Xylanases in combination with various other enzymes such as laccase, α-amylase, glucanase, and arabinofuranosidase have also been employed to improve the quality of baked products in the baking industry.

The efficiency of the treatment of *Trametes hirsuta* laccase and xylanase alone or in combination, on oat, wheat and mixed oat–wheat doughs and their corresponding breads were investigated by Flander et al. (2008). The combination of xylanase and laccase treatment improved the dough and fresh bread quality by increasing the proportion of water extractable arabinoxylan and water-soluble polysaccharides between medium to low molecular weight resulting in increment of the bread volume, softness of the dough, and softness of the bread. The results thus displayed that the utilization of a laccase and xylanase in combination is beneficial for the improvement of bread quality.

The cocktail of enzymes from *Thermoascus aurantiacus* (CBMAI 756) was produced using solid-state fermentation method and its potential in bread-making was tested by Oliveira et al. (2014). The water-unextractable arabinoxylan was hydrolysed by enzyme cocktail having xylanase, xylosidase, α-L-arabinofuranosidase, CMCase, β-glucosidase, protease, avicelase, α-amylase and amyloglucosidase activities displayed the beneficial effect in bread-making. The enzyme cocktail treatment reduces the amylopectin retrogradation (17%), reduced crumb firmness (25%) and increases the wheat bread specific volume (22%) during bread-making and storage. The effect of fungal xylanase, α-amylase, and a combination of these enzymes were evaluated for volume, crumb texture, cell structure, volatile compounds, and compositional analysis of the baked barely breads and wholegrain wheat bread by O'Shea et al. (2016). The baking results displayed that the xylanase alone or in combination with fungal α-amylase resulted in increase in larger loaf volume, crumb softness. The aroma and flavor of the bread was improved by the enhancement of isoamyl alcohol concentration and reduction in the concentration of pyrazine and sulfur. The utilization of enzyme combination also improves the total and soluble fiber content of the barley bread compared to the wholegrain wheat bread.

Bala and Singh (2017) displayed the production of xylanolytic and cellulolytic enzymes by *Sporotrichum thermophile* BJAMDU5 in solid-state fermentation using mixture of wheat straw and cotton oil cake as a carbon source. The application of xylanase was evaluated in bread-making to improve the nutritional value of the bread by increasing the soluble protein content, amount of amino acid and concentrations of the various metal ions (K^+, Na^+ and Ca^{2+}). The moisture content of the fresh breads was also enhanced to 32.33%. The xylanase treatment results showed improvement of bread quality that makes this enzyme a good candidate for baking industry. The *Aureobasidium pullulans* NRRL Y-2311-1 was grown on wheat bran for extremophilic xylanase production and its performance in

bread-making was evaluated by Yegin et al. (2018). Xylanase from *A. pullulans* showed enhancement in bread volume, moisture content, and reduction in crumb firmness. The slight improvement in attachment and remarkable decline in elasticity and gumminess of the bread was observed.

7.5.2 Xylanases in Papad manufacturing

Papad is a thin, crispy wafer-like texture of traditional Indian food consumed as snacks or with the meal as supplementary food. Awalgaonkar et al. (2015) demonstrated the utilization of xylanase as an aid in black gram based papad manufacturing. The black gram contains high amount of arabinoxylan which makes the papad dough very hard subsequently leading to the papad-making process cumbersome. They reported that the addition of 50 mg/kg xylanase in black gram flour and incubation at 45 °C for 30 min improved the dough rollability leading to a significant reduction in hardness of papad dough and the water requirement. They also reported the reduction in oil consumption during papad frying. The addition of xylanase did not affect the characteristics of papad, such as texture, taste or color of papad after roasting, microwaving, or frying. The sensory analysis of control papad and xylanase-treated papad also confirmed that the xylanase-treated papad was better than the control.

7.5.3 Xylanases in Fruit Juice Extraction and Clarification

The bioactive compounds present in vegetables and fruits are essential for a healthy diet. The non-alcoholic and non-addictive drinks such as fruit and vegetable juices are becoming more popular as they can be utilized as a substitute of caffeine-containing beverages such as aerated beverages, tea, and coffee (Lee et al. 2006). The cloudiness of naturally extracted fruit juices contributes significantly to provide color, aroma of juices, turbidity, and flavor. The cloudiness is due to the presence of protein, cellulose, hemicellulose, pectin, lipid, and other minor constituents (Binning and Possmann 1993). Cloudiness or turbidity in fruit juices is a physical property and considered as a quality indicator for citrus and other juices. The turbidity and haze caused by suspended substances in fruit juices scatter the light leading to visual perception. The presence of suspended pectin polysaccharide stemming from plant cell wall as well as from other disrupted cells causes immediate turbidity in freshly pressed fruit juices (Siebert 2006). During cold storage of fruit and vegetable juices, the turbidity may develop and refer to as haze formation. The haze is insoluble multi-molecular structures which were formed due to the interactions between haze-active proteins and polyphenols (Rai et al. 2004). The cloudy and turbid juices have less acceptability due to low yield and are incredibly challenging to concentrate and pasteurize (Pinelo et al. 2010). The fruit juices need

to be treated for the removal of undesired turbidity or haze and sediments by clarification process (Pal and Khanum 2011). Nowadays, in fruit juice industries in order to overcome the turbidity and haze formation, an array of plant cell wall degrading enzymes such as pectinolytic, cellulolytic, and hemicellulolytic enzymes are utilized to achieve juice clarity and high yield resulting in quality product ensuring the consumer's appeal (Siebert 2006; Rai et al. 2004; Pinelo et al. 2010; Pal and Khanum 2011).

The clarification and extraction of fruit juices by using xylanases have gained more attention in the last decade. Various research groups have demonstrated the potential of xylanase in extraction and clarification of various types of fruit juices. Xylanase from *Bacillus stearothermophilus* by batch fermentation method was produced and its potential application in clarification of citrus juicewas demonstrated by Dhiman et al. (2011). The use of xylanase in citrus fruit juice clarification increased the reducing sugar content by 2-fold, the juice yield by 53% and reduced the turbidity by 35.4%.

The apple, pineapple, and mousambi pulp were treated by xylanase from *Bacillus licheniformis* as reported by Bajaj and Manhas (2012a). The enzyme treatment resulted in enhancement in the reducing sugar content by twofold in apple juice (750–1320 mg/mL), pineapple (375–700 mg/mL), and mousambi juice (300–620 mg/mL). The reduction in percentage of turbidity of the juices was observed to be 79% for apple, 70% for pineapple, and 76% for mousambi juice.

The efficiency of purified xylanase from *B. pumilus* was investigated and the enrichment of tomato, pineapple, and apple juices was reported by Nagar et al. (2011). The xylanase treatment of pulp showed significant improvement in juice clarity and the increase in the yield of juices was 23% (apple) followed by 21% (tomato) and 11% (pineapple). The reduction in viscosity and turbidity was also observed without affecting the acid neutrality. Xylanase from *B. pumilus* was applied in tomato juice extraction and clarification (Kumar et al. 2013). The treatment of tomato pulp by xylanase (20 U/g) enhanced the yield of tomato juice by 68%, the release of reducing sugar by 198%, clarity by 7%, and filterability by 8%. The viscosity of tomato juice was also reduced, indicating that the taste, quality, and physicochemical properties of tomato juice were significantly improved after the enzyme treatment. Kumar et al. (2014) studied the effect of free and immobilized xylanase from *B. pumilus* on the orange and grape pulps. The pulps were mixed separately with the xylanase and incubated at optimum conditions. This resulted in the improvement of reducing sugar, clarity, yield, acidity, and filterability of the juices and the subsequent reduction in viscosity. The study further demonstrated that the immobilized enzyme was more efficient than the free xylanase.

7.5.4 Xylooligosaccharide Production by Xylanases

The risk of several gut-related diseases and associated dysfunctions in different age group populations can be easily determined by their diet plans. To overcome the causes of illness and higher healthcare expenditures, the demand for novel functional food products has focussed the global attention on prebiotics. The random hydrolysis of heteroxylan by xylanases produces xylooligosaccharides (XOS), which possess great potential as prebiotic and can be included as the food supplement for many food products. XOS is the only type of nutraceutical which can be produced by random hydrolysis of agro-industrial based lignocellulosic biomass. The global market for XOS was valued at USD 88.1 million in 2016. The XOS market is expected to grow at 5.3% CAGR and shall reach USD 120 million by the end of 2022. Several reports show the production of xylooligosaccharides from lignocellulosic biomass by enzymatic hydrolysis. Some of the recent reports on the production of XOS and its prebiotics application and anti-oxidant potential are discussed. The hydrolysis of corncob xylan into xylooligosaccharides by xylanase from *Aspergillus foetidus* MTCC 4898 was reported (Chapla et al. 2012). After 8 h enzymatic hydrolysis 6.8 mg mL^{-1} XOS was produced from corncob xylan and further purified by activated charcoal column chromatography. XOS was studied for their prebiotic effect using known probiotic strains and it was reported that *Bifidobacterium* sp. displayed higher growth than *Lactobacillus* sp.

Gowdhaman and Ponnusami (2015) studied the anti-oxidant properties of xylooligosaccharides (XOS) enzymatically produced from corncob xylan. The DPPH assay analysis showed concentration-dependent-free radical scavenging activity of XOS. Reddy and Krishnan (2016) studied the production of XOS and its prebiotic properties. The growth of probiotic bifidobacterial strains under anaerobic conditions was supported by XOS mixture. The bifidobacterial strains displayed the production of short chain fatty acids indicating the prebiotic function of XOS.

Endoxylanase from *Streptomyces halstedii* JM8 was immobilized on glyoxyl-agarose beads by multipoint covalent attachment (Romero-Fernández et al. 2018). The immobilized biocatalyst was used for the hydrolysis of various types of substrates such as beechwood xylan, wheat straw and corncob xylan. They reported the production of XOS to be 30 mg mL^{-1} from corncob, 21 mg mL^{-1} from beechwood xylan and 12.5 mg mL^{-1} from wheat straw.

7.6 Conclusions and Future Perspective

The demand for nutritious food and naturally processed juices is increasing with the increased awareness of health issues. To meet the current market demand for food products and fruit juices the utilization of enzymes as a processing aid has received considerable importance in last few years. The endo β-1,4-xylanases are enzymes belonging to glycoside hydrolase families and cleave the β-1,4 linkages present in

the backbone of heteroxylan. The endo β-1,4-xylanases are utilized in various food processing industries. They are used for improving bread quality by preventing staling effect in the baking industry, removal of haze and cloudiness in fruit juice extraction, and clarification process and production of substituted or non-substituted prebiotic xylooligosaccharides. It has also been extensively applied in feed technology, bioethanol, and xylitol production and paper bio-bleaching. The robustness and cost of enzyme production is the major factor for a processing industry. There is a need to develop more versatile and robust xylanase and economically viable production method for food and various other industries.

References

Adhyaru DN, Bhatt NS, Modi HA (2014) Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. Biocatal Agric Biotechnol 3(2):182–190

Adhyaru DN, Bhatt NS, Modi HA, Divecha J (2016) Insight on xylanase from *Aspergillus tubingensis* FDHN1: production, high yielding recovery optimization through statistical approach and application. Biocatal Agric Biotechnol 6:51–57

AkhavanSepahy A, Ghazi S, AkhavanSepahy, M (2011) Cost-effective production and optimization of alkaline xylanase by indigenous *Bacillus mojavensis* AG137 fermented on agricultural waste. Enzyme Res. http://dx.doi.org/10.4061/2011/593624

Alberton LR, Vandenberghe LPDS, Assmann R, Fendrich RC, Rodriguéz-León J, Soccol CR (2009) Xylanase production by *Streptomyces viridosporus* T7A in submerged and solid-state fermentation using agro-industrial residues. Braz Arch Biol Technol 52(SPE):171–180

Álvarez-Cervantes J, Hernández-Domínguez EM, Arana-Cuenca A, Díaz-Godínez G, Mercado-Flores Y (2013) Purification and characterization of xylanase SRXL1 from *Sporisorium reilianum* grown in submerged and solid-state fermentation. BioResources 8 (4):5309–5318

Araki T, Inoue N, Morishita T (1998) Purification and characterization of β-1, 3-xylanase from a marine bacterium, *Alcaligenes* sp. XY-234. J Gen Appl Microbiol 44(4):269–274

Awalgaonkar G, Sarkar S, Bankar S, Singhal RS (2015) Xylanase as a processing aid for papads, an Indian traditional food based on black gram. LWT Food Sci Technol 62(2):1148–1153

Ayadi DZ, Sayari AH, Hlima HB, Mabrouk SB, Mezghani M, Bejar S (2015) Improvement of *Trichoderma reesei* xylanase II thermal stability by serine to threonine surface mutations. Int J Biol Macromol 72:163–170

Bajaj BK, Manhas K (2012) Production and characterization of xylanase from *Bacillus licheniformis* P11 (C) with potential for fruit juice and bakery industry. Biocatal Agric Biotechnol 1(4):330–337

Bala A, Singh B (2017) Concomitant production of cellulase and xylanase by thermophilic mould Sporotrichum thermophile in solid state fermentation and their applicability in bread making. World J Microbiol Biotechnol 33(6):109

Beg Q, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56(3–4):326–338

Bhalla A, Bischoff KM, Uppugundla N, Balan V, Sani RK (2014) Novel thermostable endo-xylanase cloned and expressed from bacterium *Geobacillus* sp. WSUCF1. Biores Technol 165:314–318

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3(11):286–290

Binning R, Possmann P (1993) Apple juice. In: Fruit juice processing technology, pp 271–317. AgScience, Auburndale, FL, USA

Butt MS, Tahir-Nadeem M, Ahmad Z, Sultan MT (2008) Xylanases and their applications in baking industry. Food Technol Biotechnol 46(1):22–31

Cauvain S, Young L (2006) Ingredients and their influences. In: Baked products. Science, technology and practice. Blackwell Publishing, Oxford, pp 72–98

Cavella S, Romano A, Giancone T, Masi P (2008) The influence of dietary fibres on bubble development during bread making. In: Bubbles in food, vol 2, pp 311–321

Chapla D, Divecha J, Madamwar D, Shah A (2010) Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification. Biochem Eng J 49(3):361–369

Chapla D, Pandit P, Shah A (2012) Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Biores Technol 115:215–221

Chen H (2014) Chemical composition and structure of natural lignocellulose. In: Biotechnology of lignocellulose. Springer, Dordrecht, pp 25–71

Chutani P, Sharma KK (2015) Biochemical evaluation of xylanases from various filamentous fungi and their application for the deinking of ozone treated newspaper pulp. Carbohyd Polym 127:54–63

Develaraja S, Reddy A, Yadav M, Jain S, Yadav H (2016) Whole grains in amelioration of metabolic derangements. J Nutr Health Food Sci 4(4):1–11

Dhiman SS, Garg G, Sharma J, Mahajan R (2011) Characterization of statistically produced xylanase for enrichment of fruit juice clarification process. New Biotechnol 28(6):746–755

Ding C, Li M, Hu Y (2018) High-activity production of xylanase by *Pichia stipitis*: purification, characterization, kinetic evaluation and xylooligosaccharides production. Int J Biol Macromol 117:72–77

Du Y, Shi P, Huang H, Zhang X, Luo H, Wang Y, Yao B (2013) Characterization of three novel thermophilic xylanases from *Humicolainsolens* Y1 with application potentials in the brewing industry. Biores Technol 130:161–167

Dwivedi P, Vivekanand V, Ganguly R, Singh RP (2009) *Parthenium* sp. as a plant biomass for the production of alkalitolerant xylanase from mutant *Penicillium oxalicum* SAUE-3.510 in submerged fermentation. Biomass Bioenergy 33(4):581–588

Elgharbi F, Hmida-Sayari A, Zaafouri Y, Bejar S (2015) Expression of an *Aspergillus niger* xylanase in yeast: Application in breadmaking and in vitro digestion. Int J Biol Macromol 79:103–109

Escobedo-Avellaneda Z, Moure MP, Chotyakul N, Torres JA, Welti-Chanes J, Lamela CP (2011) Benefits and limitations of food processing by high-pressure technologies: effects on functional compounds and abiotic contaminants. CyTA J Food 9(4):351–364

Fang TJ, Liao BC, Lee SC (2010) Enhanced production of xylanase by *Aspergillus carneus* M34 in solid-state fermentation with agricultural waste using statistical approach. New Biotechnol 27(1):25–32

Flander L, Rouau X, Morel MH, Autio K, Seppänen-Laakso T, Kruus K, Buchert J (2008) Effects of laccase and xylanase on the chemical and rheological properties of oat and wheat doughs. J Agric Food Chem 56(14):5732–5742

Flint HJ, McPherson CA, Martin J (1991) Expression of two xylanase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 cloned in pUC13. Microbiology 137 (1):123–129

Goluguri BR, Thulluri C, Addepally U, Shetty PR (2016) Novel alkali-thermostable xylanase from *Thielaviopsis basicola* (MTCC 1467): purification and kinetic characterization. Int J Biol Macromol 82:823–829

Gowdhaman D, Ponnusami V (2015) Production and optimization of xylooligosaccharides from corncob by *Bacillus aerophilus* KGJ2 xylanase and its antioxidant potential. Int J Biol Macromol 79:595–600

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-β-1, 4-xylanase from marine *Glaciecola mesophila* KMM 241. Appl Microbiol Biotechnol 84(6):1107

Gupta U, Kar R (2009) Xylanase production by a thermo-tolerant *Bacillus* species under solid-state and submerged fermentation. Braz Arch Biol Technol 52(6):1363–1371
Gupta V, Garg S, Capalash N, Gupta N, Sharma P (2015) Production of thermo-alkali-stable laccase and xylanase by co-culturing of *Bacillus* sp. and *B. halodurans* for biobleaching of kraft pulp and deinking of waste paper. Bioprocess Biosyst Eng 38(5):947–956
Harris AD, Ramalingam C (2010) Xylanases and its application in food industry: a review. J Exp Sci 1(7):1–11
Ho H (2015) Xylanase production by *Bacillus subtilis* using carbon source of inexpensive agricultural wastes in two different approaches of submerged fermentation (SmF) and solid state fermentation (SsF). J Food Process Technol 6(4)
Hung KS, Liu SM, Tzou WS, Lin FP, Pan CL, Fang TY, Sun KH, Tang SJ (2011) Characterization of a novel GH10 thermostable, halophilic xylanase from the marine bacterium *Thermoanaerobacterium saccharolyticum* NTOU1. Process Biochem 46(6):1257–1263
Jia X, Qiao W, Tian W, Peng X, Mi S, Su H, Han Y (2016) Biochemical characterization of extra-and intracellular endoxylanse from thermophilic bacterium *Caldicellulosiruptor kronotskyensis*. Sci Rep 6:21672
John FJS, Rice JD, Preston JF (2006) Characterization of XynC from *Bacillus subtilis* subsp. subtilis strain 168 and analysis of its role in depolymerization of glucuronoxylan. J Bacteriol 188(24):8617–8626
Kapilan R, Arasaratnam V (2011) Paddy husk as support for solid state fermentation to produce xylanase from *Bacillus pumilus*. Rice Sci 18(1):36–45
Kendall CW, Esfahani A, Jenkins DJ (2010) The link between dietary fibre and human health. Food Hydrocolloids 24(1):42–48
Khurana S, Kapoor M, Gupta S, Kuhad RC (2007) Statistical optimization of alkaline xylanase production from *Streptomyces violaceoruber* under submerged fermentation using response surface methodology. Indian J Microbiol 47(2):144–152
Kim KK, Park HY, Park W, Kim IS, Lee ST (2005) *Microbacterium xylanilyticum* sp. nov., a xylan-degrading bacterium isolated from a biofilm. Int J Syst Evol Microbiol 55(5):2075–2079
Ko CH, Tsai CH, Tu J, Lee HY, Ku LT, Kuo PA, Lai YK (2010) Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* BL11. Process Biochem 45(10):1638–1644
Kumar A, Gupta R, Shrivastava B, Khasa YP, Kuhad RC (2012) Xylanase production from an alkalophilic actinomycete isolate *Streptomyces* sp. RCK-2010, its characterization and application in saccharification of second generation biomass. J Mol Catal B Enzymatic 74 (3–4):170–177
Kumar L, Nagar S, Kumar D, Garg N, Gupta VK (2013) Production of an alkali tolerant extracellular xylanase from *Bacillus pumilus* VLK-1 in solid state fermentation and its use in tomato juice clarification. Int J Cell Sci Biotechnol 2:1–10
Kumar L, Nagar S, Mittal A, Garg N, Gupta VK (2014) Immobilization of xylanase purified from *Bacillus pumilus* VLK-1 and its application in enrichment of orange and grape juices. J Food Sci Technol 51(9):1737–1749
Lakshmi GS, Rao CS, Rao RS, Hobbs PJ, Prakasham RS (2009) Enhanced production of xylanase by a newly isolated *Aspergillus terreus* under solid state fermentation using palm industrial waste: a statistical optimization. Biochem Eng J 48(1):51–57
Laurie JI, Clarke JH, Ciruela A, Faulds CB, Williamson G, Gilbert HJ, Rixon JE, Millward-Sadler J, Hazlewood GP (1997) The NodB domain of a multidomain xylanase from *Cellulomonas fimi* deacetylates acetylxylan. FEMS Microbiol Lett 148(2):261–264
Lee WC, Yusof SALMAH, Hamid NSA, Baharin BS (2006) Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). J Food Eng 73(1):55–63
Li Y, Liu Z, Zhao H, Xu Y, Cui F (2007) Statistical optimization of xylanase production from new isolated *Penicillium oxalicum* ZH-30 in submerged fermentation. Biochem Eng J 34(1):82–86

Li N, Meng K, Wang Y, Shi P, Luo H, Bai Y, Yang P, Yao B (2008) Cloning, expression, and characterization of a new xylanase with broad temperature adaptability from *Streptomyces sp*. S9. Appl Microbiol Biotechnol 80(2):231

Liao H, Sun S, Wang P, Bi W, Tan S, Wei Z, Mei X, Liu D, Raza W, Shen Q, Xu Y (2014) A new acidophilic endo-β-1, 4-xylanase from *Penicillium oxalicum*: cloning, purification, and insights into the influence of metal ions on xylanase activity. J Ind Microbiol Biotechnol 41(7):1071–1083

Ling Ho H, Heng KL (2015) Xylanase production by *Bacillus subtilis* in cost-effective medium using soybean hull as part of medium composition under submerged fermentation (SmF) and solid state fermentation (SsF). J Biodivers Biopros Dev 2:143

Liu X, Liu Y, Jiang Z, Liu H, Yang S, Yan Q (2018) Biochemical characterization of a novel xylanase from *Paenibacillus barengoltzii* and its application in xylooligosaccharides production from corncobs. Food Chem 264:310–318

Long L, Xu M, Shi Y, Lin Q, Wang J, Ding S (2018) Characterization of two new endo-β-1, 4-xylanases from *Eupenicillium parvum* 4–14 and their applications for production of feruloylated oligosaccharides. Appl Biochem Biotechnol 1–18. https://doi.org/10.1007/s12010-018-2775-6

Membrillo Venegas I, Fuentes-Hernández J, García-Rivero M, Martínez-Trujillo A (2013) Characteristics of *Aspergillus niger* xylanases produced on rice husk and wheat bran in submerged culture and solid-state fermentation for an applicability proposal. Int J Food Sci Technol 48(9):1798–1807

Menon G, Mody K, Keshri J, Jha B (2010) Isolation, purification, and characterization of haloalkaline xylanase from a marine *Bacillus pumilus* strain, GESF-1. Biotechnol Bioprocess Eng 15(6):998–1005

Min SY, Kim BG, Lee C, Hur HG, Ahn JH (2002) Purification, characterization, and cDNA cloning of xylanase from fungus *Trichoderma strain* SY. J Microbiol Biotechnol 12(6):1–5

Mirande C, Mosoni P, Béra-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

Mitreva-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-β-xylanase from the plant-parasitic nematode *Meloidogyne incognita*. Mol Plant Microbe Interact 19(5):521–529

Mohana S, Shah A, Divecha J, Madamwar D (2008) Xylanase production by *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent wash. Bioresour Technol 99(16):7553–7564

Nagar S, Mittal A, Kumar D, Kumar L, Kuhad RC, Gupta VK (2011) Hyper production of alkali stable xylanase in lesser duration by *Bacillus pumilus* SV-85S using wheat bran under solid state fermentation. New Biotechnol 28(6):581–587

O'Shea N, Kilcawley KN, Gallagher E (2016) Influence of α-amylase and xylanase on the chemical, physical and volatile compound properties of wheat bread supplemented with wholegrain barley flour. Eur Food Res Technol 242(9):1503–1514

Oliveira DS, Telis-Romero J, Da-Silva R, Franco CML (2014) Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread qualitiy. Food Chem 143:139–146

Pal A, Khanum F (2010) Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Biores Technol 101(19):7563–7569

Pal A, Khanum F (2011) Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: characterization of immobilized enzyme. Process Biochem 46(6):1315–1322

Pandey A, Selvakumar P, Soccol CR, Nigam P (1999) Solid state fermentation for the production of industrial enzymes. Curr Sci 149–162

Panwar D, Srivastava PK, Kapoor M (2014) Production, extraction and characterization of alkaline xylanase from *Bacillus sp*. PKD-9 with potential for poultry feed. Biocatal Agric Biotechnol 3 (2):118–125

Pell G, Szabo L, Charnock SJ, Xie H, Gloster TM, Davies GJ, Gilbert HJ (2004) Structural and biochemical analysis of *Cellvibrio japonicus* xylanase 10C how variation in substrate-binding cleft influences the catalytic profile of family GH-10 xylanases. J Biol Chem 279(12):11777–11788

Pinelo M, Zeuner B, Meyer AS (2010) Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. Food Bioprod Process 88 (2):259–265

Poorna CA, Prema P (2006) Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. Biochem Eng J 32(2):106–112

Rai P, Majumdar GC, Dasgupta SDES, De S (2004) Optimizing pectinase usage in pretreatment of mosambi juice for clarification by response surface methodology. J Food Eng 64(3):397–403

Reddy SS, Krishnan C (2016) Production of high-pure xylooligosaccharides from sugarcane bagasse using crude β-xylosidase-free xylanase of *Bacillus subtilis* KCX006 and their bifidogenic function. LWT Food Sci Technol 65:237–245

Romero-Fernández M, Moreno-Perez S, de Oliveira SM, Santamaría RI, Guisan JM, Rocha-Martin J (2018) Preparation of a robust immobilized biocatalyst of β-1,4-endoxylanase by surface coating with polymers for production of xylooligosaccharides from different xylan sources. New Biotechnol 44:50–58

Sadaf A, Khare SK (2014) Production of *Sporotrichum thermophile* xylanase by solid state fermentation utilizing deoiled *Jatropha curcas* seed cake and its application in xylooligosaccharide synthesis. Biores Technol 153:126–130

Saha BC (2000) α-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 18(5):403–423

Saha BC (2002) Production, purification and properties of xylanase from a newly isolated *Fusarium proliferatum*. Process Biochem 37(11):1279–1284

Samanta AK, Kolte AP, Elangovan AV, Dhali A, Senani S, Sridhar M, Suresh KP, Jayapal N, Jayaram C, Roy S (2016) Value addition of corn husks through enzymatic production of xylooligosaccharides. Braz Arch Biol Technol 59. http://dx.doi.org/10.1590/1678-4324-2016160078

Sanghi A, Garg N, Sharma J, Kuhar K, Kuhad RC, Gupta VK (2008) Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid-state fermentation. World J Microbiol Biotechnol 24(5):633–640

Scheller HV, Ulvskov P (2010) Hemicelluloses. Ann Rev Plant Biol 61

Shah AR, Madamwar D (2005) Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem 40(5):1763–1771

Sharma K, Antunes IL, Rajulapati V, Goyal A (2018a) Low-resolution SAXS and comparative modeling based structure analysis of endo-β-1, 4-xylanase a family 10 glycoside hydrolase from *Pseudopedobacter saltans* comb. nov. Int J Biol Macromol 112:1104–1114

Sharma K, Antunes IL, Rajulapati V, Goyal A (2018b) Molecular characterization of a first endo-acting β-1, 4-xylanase of family 10 glycoside hydrolase (*Ps*GH10A) from *Pseudopedobacter saltans* comb. nov. Process Biochem 70:79–89

Siebert KJ (2006) Haze formation in beverages. LWT-Food Sci Technol 39(9):987–994

Sjostrom E (2013) Wood chemistry: fundamentals and applications. Elsevier

Su Y, Zhang X, Hou Z, Zhu X, Guo X, Ling P (2011) Improvement of xylanase production by thermophilic fungus *Thermomyces lanuginosus* SDYKY-1 using response surface methodology. New Biotechnol 28(1):40–46

Suzuki M, Kato A, Nagata N, Komeda Y (2002) A xylanase, AtXyn1, is predominantly expressed in vascular bundles, and four putative xylanase genes were identified in the *Arabidopsis thaliana* genome. Plant Cell Physiol 43(7):759–767

Thomas L, Sindhu R, Binod P, Pandey A (2015) Production of a cellulase-free alkaline xylanase from *Bacillus pumilus* MTCC 5015 by submerged fermentation and its application in biobleaching. Indian J Exp Biol 53(6) 356–363

Thomas L, Parameswaran B, Pandey A (2016) Hydrolysis of pretreated rice straw by an enzyme cocktail comprising acidic xylanase from *Aspergillus* sp. for bioethanol production. Renew Energy 98:9–15

Thomas L, Joseph A, Singhania RR, Patel AK, Pandey A (2017) Industrial enzymes: xylanases. In: Current developments in biotechnology and bioengineering, pp 127–148

Verma AK, Goyal A (2016) A novel member of family 30 glycoside hydrolase subfamily 8 glucuronoxylan endo-β-1, 4-xylanase (*Ct*XynGH30) from *Clostridium thermocellum* orchestrates catalysis on arabinose decorated xylans. J Mol Catal B Enzym 129:6–14

Verma D, Satyanarayana T (2012) Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Biores Technol 107:333–338

Wainø M, Ingvorsen K (2003) Production of β-xylanase and β-xylosidase by the extremely halophilic archaeon *Halorhabd usutahensis*. Extremophiles 7(2):87–93

Walia A, Mehta P, Guleria S, Shirkot CK (2015) Improvement for enhanced xylanase production by *Cellulosimicrobium cellulans* CKMX1 using central composite design of response surface methodology. 3 Biotech 5(6):1053–1066

Wang X, Luo H, Yu W, Ma R, You S, Liu W, Hou L, Zheng F, Xie X, Yao B (2016) A thermostable *Gloeophyllum trabeum* xylanase with potential for the brewing industry. Food Chem 199:516–523

Winterhalter C, Liebl W (1995) Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl Environ Microbiol 61(5):1810–1815

Wu YR, He J (2015) Characterization of a xylanase-producing *Cellvibrio mixtus* strain J3-8 and its genome analysis. Sci Rep 5:10521

Yamaura I, Koga T, Matsumoto T, Kato T (1997) Purification and some properties of endo-l, 4-β-d-xylanase from a fresh-water mollusc, *Pomacea insularus* (de Ordigny). Biosci Biotechnol Biochem 61(4):615–620

Yang Z, Zhang Z (2017) Codon-optimized expression and characterization of a pH stable fungal xylanase in *Pichia pastoris*. Process Biochem 53:80–87

Yang SQ, Yan QJ, Jiang ZQ, Li LT, Tian HM, Wang YZ (2006) High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. Biores Technol 97(15):1794–1800

Yasinok AE, Biran S, Kocabas A, Bakir U (2010) Xylanase from a soil isolate, *Bacillus pumilus*: gene isolation, enzyme production, purification, characterization and one-step separation by aqueous-two-phase system. World J Microbiol Biotechnol 26(9):1641–1652

Yegin S, Altinel B, Tuluk K (2018) A novel extremophilic xylanase produced on wheat bran from *Aureobasidium pullulans* NRRL Y-2311-1: effects on dough rheology and bread quality. Food Hydrocolloids 81:389–397

Zhao L, Meng K, Shi P, Bai Y, Luo H, Huang H, Wang Y, Yang P, Yao B (2013) A novel thermophilic xylanase from *Achaetomium* sp. Xz-8 with high catalytic efficiency and application potentials in the brewing and other industries. Process Biochem 48(12):1879–1885

Zhou J, Gao Y, Dong Y, Tang X, Li J, Xu B, Mu Y, Wu Q, Huang Z (2012) A novel xylanase with tolerance to ethanol, salt, protease, SDS, heat, and alkali from actinomycete *Lechevalieria sp*. HJ3. J Ind Microbiol Biotechnol 39(7):965–975

Chapter 8
Biotechnological Avenues for Fruit Juices Debittering

Lokesh Kumar Narnoliya and Jyoti Singh Jadaun

Abstract Nowadays, the majority of the population follows the tight working schedule so it became difficult for them to maintain their balanced diet. Therefore, to obtain proper healthy nutrition, food supplements need to be included in their diet. Fruits are the best choices as a food supplement to provide nutritional constituents. The long-term storage of fruits is the major problem, therefore, the use of packed fruit juices is increasing day by day. People are using these packed materials more frequently because these can be stored for a long time, availability of juice of all fruits in all seasons and easy to carry anywhere, without any problem of leakage and spoiling. Therefore, this is the blooming phase of all fruit-based industries throughout the world. The demand and market for citrus fruit juice are worldwide and it is quite high due to their significant nutritional value. There is a huge hurdle in citrus juice market, which is related to its bitterness. There are two main components, naringin, and limonin, responsible for the "immediate" and "delayed" bitterness, respectively. During processing, this bitterness increases with time due to the conversion of limonoate A-ring-lactone (non-bitter) into limonin (bitter) and this conversion is facilitated under the acidic pH of juice. Bitterness can be reduced up to an acceptable range of the consumer by the use of certain enzymes like naringinase and limonoate dehydrogenases but production of these enzymes in such a large amount, which would be sufficient for treatment of juice at industrial scale, is a big issue of concern. Current research activities are focusing on this target to develop a technology for achieving highly efficient enzyme sources either from native or recombinant sources using synthetic biology and modern biotechnological approaches. Another aspect to enhance this technology is the immobilization of such enzymes for their reuses, which will minimize the total cost of production. Therefore, in this chapter, we will discuss various methods that were previously

L. K. Narnoliya
Department of Biotechnology (DBT), Center of Innovative and Applied Bioprocessing (CIAB), Mohali 140306, Punjab, India

J. S. Jadaun (✉)
Botany Department, Dayanand Girl's P.G. College, Kanpur 208001, Uttar Pradesh, India
e-mail: jyotisingh.jadaun@gmail.com

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_8

used for the debittering purpose and new biotechnological approaches, which can be used for debittering of juice more efficiently at large scale.

8.1 Introduction

In the current scenario, it is becoming more difficult to maintain proper health due to modern lifestyle and it is not only associated with a particular age group of people, although, it is present in all age categories from adult to old including infants. For keeping the better status of health, it is somewhat mandatory to eat food from different sources because different food materials have a variety of nutrients (Marshall et al. 2001). In addition to the regular meal of cereal, pulses, and vegetables, consumption of fruits, in any form either fresh or packaged, is becoming unavoidable to keep away several kinds of disorders and to reduce the risk of malnutrition (Slavin and Lloyd 2012). Fruits are the rich source of vitamins, minerals (electrolytes), dietary fibers, and several others phytochemicals like flavonoids and carotenoids. Recent research articles reveal that these compounds have several medicinal attributes and aid in the treatment of headache to cancer like diseases (Reiss et al. 2012; Alissa and Ferns 2017). Citrus fruits are well known for their beneficial effects on human health and these fruits are a rich source of various macronutrients, for example, vitamins mainly vitamin C (ascorbic acid), vitamin B6, dietary fibers, sugars, potassium, folate, calcium, niacin, phosphorus, copper, riboflavin, and other valuable compounds, which fall under the category of secondary metabolites such as flavanoids (flavanones, flavonols, phenolic acids, flavones, etc.), coumarins, limonoids, carotenoids, essential oils, phenolics, and alkaloids (Caristi et al. 2003; Lv et al. 2015). These secondary metabolites showed diverse biological activities such as antioxidant, anti-inflammatory, antimicrobial, and antitumor activity, as well as cardiovascular protective effects, neuroprotective effects (Yao et al. 2004; Kaur and Kapoor 2001; Kabra et al. 2012; Zou et al. 2016).

Due to health-promoting values, citrus fruits are consumed by people as a traditional herb in several Asian countries on a regular basis (Arias and Ramón-Laca 2005). Citrus fruits belong to the family Rutaceae, comprise different varieties of fruits varying in size, shape, color, and taste and most commonly used are oranges, limes, lemons, grapefruits, mandarins, and citron. Their traits of economical values play a decisive role in their organoleptic and commercial attributes (Economos and Clay 1999). Structure of citrus fruits is constituted by the pericarp and seeds, while pericarp is further subdivided into three parts epicarp, mesocarp, and endocarp. Epicarp is subdivided into epidermis and hypodermis and mesocarp is also divided into outer mesocarp and inner mesocarp. Colored epicarp and outer mesocarp are collectively called as flavedo, while white inner mesocarp is known as albedo and the both collectively form the peel of a fruit (Izquierdo and Sendra 2003). Due to its economic importance, citrus fruits are cultivated at a large scale throughout the world, approximately in 140 countries and nearly 8.7 million hectares land is devoted for cultivation (González-Molina et al. 2010). The list of major

citrus producer countries includes Brazil, China, USA, Mexico, India, and Spain, although, Brazil and USA together account for more than 90% of citrus production (Lv et al. 2015).

Citrus fruits are used by the consumers in raw form in the preparation of several kinds of drinks commonly known as lemonade or limeade by diluting the fresh juice with sugar solution, and besides this, citrus juice is used by mixing with juices of other fruits. Due to the daily consumption of citrus juice, industries are focusing on the development of technology, which can be applied for the long-term preservation of citrus juice, but the main hurdle in the processing of citrus juice is the bitterness. This bitterness of citrus fruits increases during processing hours, due to the release of some chemicals at the time of extraction process and such kind of bitterness is termed as 'delayed bitterness', which we have discussed further in detail. This bitterness is caused by the presence of chemicals belonging to the limonoid and flavonoid categories. Bitterness is acceptable up to a certain extent but the excessive bitterness is not acceptable by the consumers, although this bitterness level is fluctuates with harvesting stages, post-harvest storage, acidity level and processing stages (Raithore et al. 2016). Researchers optimized several methods for the reduction of bitterness but still, the complete solution is not in hands, which could be applied at a commercial level. Now in "Biotechnology Era", it is quite possible to search for an efficient, less time consuming and cost-effective technology, which can be used from a small-scale industry up to large-scale industry.

In this chapter, we discussed the biochemistry of bitterness, methods for quantification of bitterness level, methods developed for debittering of juice, and recent advances in debittering processes.

8.2 Biochemicals Responsible for Bitterness in Citrus Fruits

The bitterness of citrus fruits is due to the presence of certain biomolecules such as flavonoids and limonoids. Naringin and limonin are identified as major contributors for "immediate" and "delayed" bitterness, respectively. Besides these compounds, the presence of essential oil also affects the sensory threshold of juice and abundance of essential oil negatively affects the commercial value of juices.

8.2.1 Flavonoids

Flavonoids, an important group of phytochemicals, are polyphenolic compounds having C6-C3-C6 carbon skeleton. The basic structure of flavonoid includes three rings, where six carbons containing *ring A* and *ring B* are joined together by a three-carbon short chain. The carbon of short chain is linked with neighboring ring

A and B, either by directly or through oxygenation or through unsaturation process and consequently generates a five- or six-membered *ring C*.

Depending on the variation in the arrangement of ring C, flavonoids are classified into several subclasses, six major subclasses are flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins, and chalcones (Peterson et al. 1998). Among these classes of flavonoids; the flavones, flavanones, flavonols, and flavans are dominating in all the citrus species, while anthocyanins occur only in blood oranges. Flavanones in citrus fruits present in glycosidic and aglyconic forms, examples of aglyconic flavanones are naringenin and hesperetin and examples of glycosidic flavanones are neohesperidosides and rutinosides. Neohesperidosides are bitter in taste and these are synthesized by the addition of neohesperidose (rhamnosyl-a-1, 2 glucose) with flavanone ring, while rutinosides are tasteless and are formed with the addition of rutinose (rhamnosyl-a-1, 6 glucose) with the flavonone ring. Neohesperidose and rutinose are disaccharides and they differ only in carbon position at which rhamnose sugar attached to the glucose unit. Naringin, neohesperidin, and neoeriocitrin are examples of neohesperidosides and hesperidin, narirutin, and didymin are some examples of rutinosides. Neohesperidosides commonly occur in bergamot, grapefruit, and bitter orange juices and rutinosides are present in bergamot, orange, mandarin, and lemon juice. Order of bitterness of some common neohesperidosides is naringin, which is highly bitter and this is followed by poncirin, neohesperidin, and neoeriocitrin.

In grapefruit (*Citrus paradisi*) and pummelo (*Citrus maxima*, *Citrus grandis*), the predominant bittering agent is naringin while in sour orange (*Citrus sinensis*), neohesperidin is a major factor for bitterness although neoeriocitrin and poncirin are present in minor concentrations in citrus juices (Kawaii et al. 1999). Naringin is also known as 4,5,7-trihydroxyflavanone-7-rhamnoglucoside or naringenin 7-o-neohesperidoside or naringenine-7-rhamnosidoglucoside or naringenin-7-beta-neohesperidoside. Flavonoid occurs mainly in peel and seeds of citrus fruits although in peel flavonoids present in higher amount then seeds, that is why citrus fruits are peeled off before juice extraction and mixing of seeds is also avoided for making less bitter juice (Benavente-Garcia and Castillo 2008). In citrus species types, flavonoids functions as a marker for species identification, and concentration of each flavonoid varies among parts of fruits.

8.2.1.1 Biosynthesis of Flavonoids

Flavonoids are biosynthesized *via* the phenylpropanoid pathway, and in citrus fruits this pathway is operated similarly to other plants up to certain stages, the further downstream pathway is diverted for the production of specialized citrus flavonoids. A large number of enzymes are contributing to the production of flavonoids: (i) Phenylalanine ammonialyase (PAL), (ii) Cinnamate 4-hydroxylase (C4H), (iii) 4-Coumarate: CoA ligase (4CL), (iv) Chalcone synthase (CHS), (v) Chalcone isomerase (CHI), (vi) Flavone synthase I, II (FNSI, FNSII), (vii) Flavanone-3-hydroxylase (F3H), (viii) Flavonol synthase (FLS),

(ix) Dihydroflavonol 4-reductase (DFR), (x) Leucoanthocyanidin oxygenase enzyme (LDOX), and (xi) Isoflavone synthase (IFS) and Isoflavone reductase (IFR) (Fig. 8.1).

A comprehensive detail about these genes of citrus species is present in the EST database of CitEST project (USDA 2006). The first enzyme of phenylpropanoid pathway is phenylalanine ammonialyase (PAL, EC 4.3.1.5), which converts phenylalanine into *trans*-cinnamate through a *trans*-elimination of ammonia. This enzyme connects primary and secondary metabolism by connecting the shikimic acid with the flavonoid biosynthesis pathway. The concentration of *trans*-cinnamate may function as a regulatory step for this enzyme by feedback inhibition (Zhang and Liu 2015). The first time, PAL enzyme was demonstrated from flavedo of *C. paradisi* and subsequently, this enzyme was detected in different varieties of citrus such as *Citrus limon*, *C. sinensis*, *Citrus reticulata* (Lucheta et al. 2007; Sanchez-Ballesta et al. 2000; Maier and Hasegawa 1970). PAL is the most extensively studied enzyme because of its regulatory role in the production of

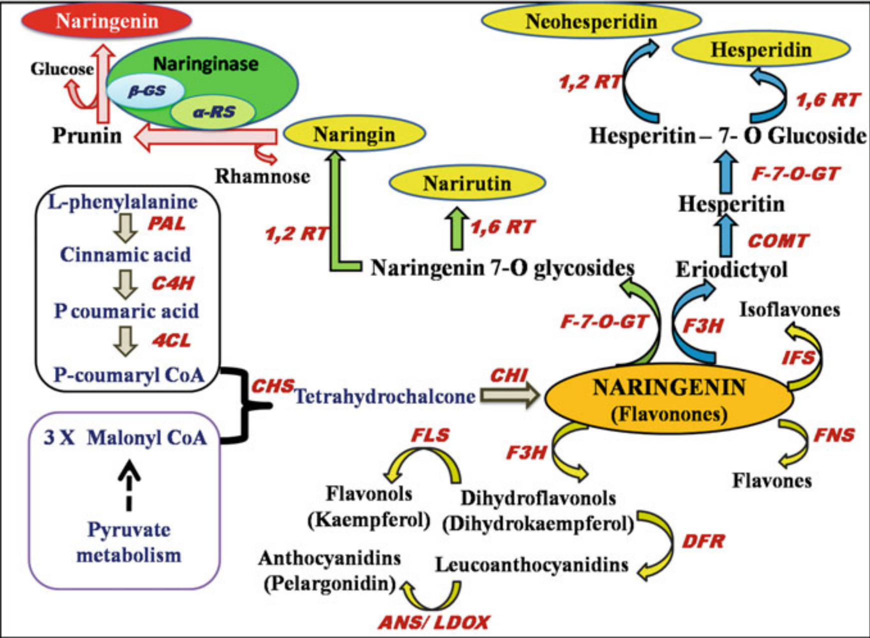


Fig. 8.1 Diagrammatic presentation of flavonoids biosynthetic pathway with the involvement of naringinase for degradation of bittering compounds. Abbreviations: PAL—Phenylalanine ammonialyase, C4H—Cinnamate 4-hydroxylase, 4CL—4-Coumarate: CoA ligase, CHS—Chalcone synthase, CHI—Chalcone isomerase, F3H—Flavanone-3-hydroxylase, FNS—Flavone synthase, FLS—Flavonol synthase, DFR—Dihydroflavonol 4-reductase, ANS—Anthocyanidin synthase/LDOX—Leucoanthocyanidin oxygenase enzyme, IFS—Isoflavone synthase, F-7-O-GT—Flavanone 7-O-glucosyltransferase, 1,2 RT—1,2 rhamnosyltransferase, 1,6 RT—1,6 rhamnosyltransferase, COMT—Caffeoylmethyltransferase, α-RS—α-L-rhamnosidase, β GS—β-D-glucosidase

flavonoids and several studies were performed by various research groups for isolation, characterization, and transcript abundance in different tissues as well as under stress conditions (Kim and Hwang 2014; Zhang and Liu 2015; Yang et al. 2016; Milan et al. 2017). PAL belongs to the multigene family and mostly it occurs in tetrameric forms having molecular weight of approximately 275–330 kDa (Appert et al. 1994).

The second enzyme of this pathway is cinnamate 4-hydroxylase (C4H; EC 1.14.13.11), which catalyzes the production of *trans*-p-coumarate from *trans*-cinnamate by hydroxylation reaction. This enzyme is also known as CYP73A and it is associated with NADPH-cytochrome P450 reductase (CPR; EC 1.6.2.4) for functioning and it requires O_2 and NADPH as a cofactor during the reaction (Gabriac et al. 1991). Two types of cDNA of cinnamate-4-hydroxylase (C4H1 and C4H2) were isolated form *C. sinensis* (sweet orange) and they differ in their expression pattern such as C4H1 is wound inducible and C4H2 is constitutively expressed in all the tissues (Betz et al. 2001). The p-coumarate is converted into 4-coumaroyl-CoA by the activity of 4-coumarate: CoA ligase (4CL; EC 6.2.1.12) enzyme and it requires Mg^{+2} and ATP as a cofactor. The EST data analysis of sweet orange (*C. sinensis* (L) Osbeck), exhibited ten putative ESTs of 4-coumarate: CoA ligase enzymes, which may function in multiple isomeric forms (Lucheta et al. 2007). This enzyme is the branching enzyme as it diverts the products from the phenylpropanoid pathway toward flavanoid biosynthesis pathway.

Another important enzyme of this pathway is chalcone synthase (CHS; 2.3.1.74), which produces naringenin chalcone (15 carbon), the first product of flavonoid pathway, by the condensation of 4-coumaroyl-CoA with three molecules of malonyl Co-A. Two cDNA (CitCHSl and CitCHS2) representing chalcone synthase genes were isolated from the cell suspension culture of *C. sinensis* and these genes were differentially expressed during embryogenesis (Moriguchi et al. 1999). Further Lucheta et al. (2007) found three transcripts of chalcone synthase genes in the EST database of *C. sinensis*. At next step, this naringenin chalcone (bicyclic) is converted into (2S)-naringenin (tricyclic flavanone) by chalcone isomerase (CHI, E.C. 5.5.1.6) or chalcone-flavanone isomerase, another committed enzyme of this pathway. Naringenin is a primary (2S)-flavanone and backbone for the synthesis of other flavanones, flavones, flavonols, and anthocyanins. Two forms of chalcone isomerase were identified, which catalyze different substrates, for one form 6-hydroxychalcones and for another, 6-hydroxy-(naringenin chalcone) and 6-deoxychalcones (isoliquirgentin) act as substrate (Joseph and Joseph 2002).

According to Moriguchi et al. (2001), there is a single copy of chalcone isomerase while EST database analysis revealed the presence of three transcripts belonging to CHI gene family (Lucheta et al. 2007). Further hydroxylation of this (2S)-naringenin is catalyzed by flavanone 3 β-hydroxylase (F3H, EC 1.14.11.9) and consequently 2R, 3R dihydroflavonols is produced. This enzyme requires different cofactors such as 2-oxoglutarate, Fe^{2+}, and ascorbate for this reaction (Pelt et al. 2003). This enzyme is present in single copy in some *Citrus* sp. (grapefruit) while in other species like mandarin it may be present in multiple copies and expression of transcripts showed tissue specificity (Pelt et al. 2003; Lucheta et al. 2007).

Another important branching enzyme is flavone synthase (FNS; EC 1.14.11.22), which catalyzes the addition of a double bond between carbon number C2 and C3 of the respective flavonone ring for producing flavone ring. Nature has evolved two different proteins of flavone synthase for synthesis of flavones compounds specified to a particular species, one is named as flavone synthase I (FNS I) and flavone synthase II (FNS II) and these two genes differ in their distribution among plant species, as FNS I is primarily found in members of Apiaceae family while FNS II is widely spread in different species. FNS I is a soluble dioxygenase and it is 2-oxoglutarate- and Fe^{2+}-dependent enzyme and FNS II is membrane-bound cytochrome P450 monooxygenase, which is dependent on NADPH and molecular oxygen for activity. Two putative transcripts of FNS II are found in the CitEST database.

Flavonol synthase (FLS; E.C.1.14.11.23) acts as another dedicated enzyme of this pathway and participate in the synthesis of flavonols from (2R, 3R)-dihydro-flavonols. It requires 2-oxoglutarate, Fe^{2+}, and ascorbate. CitEST database suggests that around four putative transcripts of the flavonol synthase gene are present in citrus. Dihydroflavonol 4-reductase (DFR; EC 1.1.1.219) catalyzes the conversion of (+)-(2R, 3R)-dihydroflavonols into the corresponding (2R, 3S, 4S) flavan-3, 4-*cis*-diols (leucoanthocyanidins) and this enzyme requires NADPH as a cofactor. CitEST database of *C. sinensis* reveals the presence of three transcripts related to the dihydroflavonol 4-reductase gene. Another branching enzyme is leucoantho-cyanidin oxygenase, which is also known as anthocyanidin synthase (ANS, LDOX; EC 1.14.11.19). It is a dioxygenase enzyme and requires 2-oxoglutarate as a cofactor, which involves the production of colored anthocyanidins from leu-coanthocyanidins. More than one transcript related to anthocyanidin synthase group are found in EST database analysis of *C. sinensis*. The occurrence of isoflavonoids is reported in some *Citrus* sp. and synthesis of isoflavonoids involves isoflavone synthase (IFS, E.C. 1.14.14) and isoflavone reductase (IFR, EC 1.3.1) enzymes (Jung et al. 2000; Lapcik et al. 2004). CitEST database analysis showed the lack of transcripts of isoflavone synthase while this database referred to the presence of five putative transcripts of isoflavone reductase gene (Lucheta et al. 2007). Further, glycosylation of these compounds is carried out by a different type of glycosyl-transferases, e.g., flavonoid 7-o-glucosyltransferase and 7-o-rhamnosyltransferase in *C. sinensis* (Fig. 8.1).

8.2.2 Limonin

Limonin belongs to limonoid group of the compound, which is generally present in Rutaceae and Meliaceae family plants. Limonin is a bitter tetracyclic triterpenoid dilactone present in citrus plants. It is the main component of citrus fruit juice, which produces bitterness (Maier et al. 1969). Generally, at initial stage, citrus fruits are nonbitter but later on, due to the presence of limonin, these fruits become bitter in taste and this phenomenon is generally known as delayed bitterness (Maier and Beverly,

1968; Guadagni et al. 1973). Previous reports state that limonin monolactone (limonoate A-ring lactone) is the non-bitter precursor in the early stage of fruit, which converts it into a bitter compound known as limonin (Maier and Beverly 1968; Maier et al. 1969).

Nomilin is an early precursor of all the limonoid groups of metabolites in citrus, which involves the biosynthesis of bitter molecule limonin. Auxins are thought to be potent inhibitors of nomilin biosynthesis, able to resolve the bitterness problem up to some extent (Hasegawa et al. 1986a, b). In practice, at commercial scale, the limonin level >6 ppm is considered as bitterness. Generally, bitterness in citrus is two types; first is flavonoids/naringin that generates immediate bitterness and second is delayed bitterness which is produced by limonoids/limonin. Non-bitter limonoids like limonin were generated from bitter limonoids like limonoate A-ring lactone (LARL) (monolactone) during juicing process, freeze damage, or physical damage of citrus fruits (Mohanpuria 2016; Wang et al. 2017). The site of limonin biosynthesis is the phloem region of citrus stem *via* the isoprenoid biosynthesis pathway. Other tissues such as leaves, fruit, peel, and seeds are not capable to synthesize limonin from its precursor acetate or mevalonate. Although, further limonin is converted into other limonoids in fruit tissues, peels, seeds, and leaves independently (Hasegawa and Hoagland 1977; Hasegawa et al. 1997).

8.2.2.1 Limonin Biosynthesis

As we know, limonin belongs to limonoids group of compounds, which are biosynthesized through the terpenoids/isoprenoids biosynthetic pathway (Wang et al. 2017). Generally, squalene is synthesized through non-mevalonate and/or mevalonate pathway and it is converted into a precursor of triterpenoid metabolites. Thereafter, terminal transferase enzymes such as CYP450s, acyltransferases, glycosyltransferases, and methyltransferases generate a diverse kind of limonoids (Thimmappa et al. 2014). Nomilin is the key precursor of all kinds of citrus limonoids. Thereafter, secondary modifications such as oxidization, isomerization, acetylation, methylation, and hydrolyzation of nomilin take place to generate a huge number of limonoids (Wang et al. 2017) (Fig. 8.2).

Generally, limonoid aglycones in citrus are responsible for its bitterness and recently discovered that limonoid glucosyltransferase enzyme is able to convert it into non-bitter limonoid glucosides. Identification of these enzymes is the key step for production of non-bitter citrus juice (Hasegawa and Hoagland 1977; Hasegawa et al. 1997). At the time of fruit maturation, natural debittering process occurs in which limonoid aglycones are endogenously converted into tasteless limonoid glucosides (Endo et al. 2002). Limonoid aglycones are present in both immature and mature fruit but test less limonoid glucosides are absent in immature fruit. Hasegawa and his coworkers identified two key enzymes, which convert bitter aglycones compounds into taste less glucosides from of that compound. These two important regulatory enzymes are limonoid glucosyltransferase (LGT) and limonoid lactone hydrolase (LLH) (Mohanpuria 2016; Hasegawa et al. 1991).

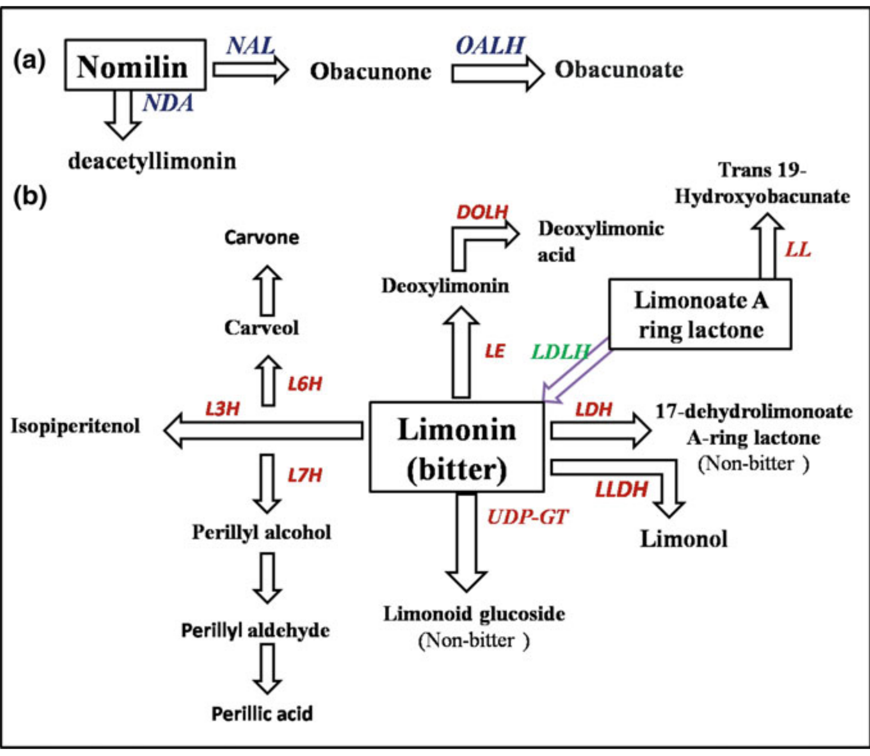


Fig. 8.2 Schematic diagram representing the conversion of bitter limonin and nomilin into non-bitter compounds through the involvement of proposed enzymes. Abbreviations: LDH—Limonoate dehydrogenase, UDP GT—UDP-D-glucose: limonoid glucosyltransferase (limonoid GTase), NDA—Nomilin deacetylase, L6H—Limonene 6-hydroxylase, L3H—Limonene 3-hydroxylase, L6H—Limonene 6-hydroxylase, L7H—Limonene 7-hydroxylase, LE—Limonin epoxidase, DOLH—Deoxylimonin hydrolase, NAL—Nomilinin Acetyl-Lyase, OALH—Obacunone A-ring lactone hydrolase, LDLH—Limonin D-ring lactone hydrolase, LL—Limonoate lyase

The glucosyltransferase enzymes transfer glycosyl moiety to the acceptor molecule and modify its properties. Several members of LGT enzymes are characterized in citrus but information about LLH enzyme is still very limited (Fig. 8.2). Till date, almost 44 aglycones and 18 glucosides limonoids are identified from citrus, which may be involved in bitterness processing and still, the number is increasing day by day (Mohanpuria 2016; Kim et al. 2012).

Predominantly, the level of limonoid glucosides in citrus fruit is increased from immature to mature fruit and they are more stable rather than other limonoids aglycones. At the time of seed germination, these limonoid glucosides are hydrolyzed into glucose and aglycones by the action of a limonoid glucoside β-glucosidase enzyme, which is only found in seeds (Ronneberg et al. 1995). Naturally, when the citrus juice is prepared, the seeds are crushed which release the

limonoid glucoside β-glucosidase enzyme which may alter the level of limonin through hydrolyzing tasteless glucosides of limonin (Hasegawa and Hoagland 1977; Hasegawa et al. 1986a, b, 1997). However, most of the enzymes of the limonoid biosynthesis pathway are yet to be identified, therefore still significant improvement can be done for citrus industries (Wang et al. 2017). Next-generation sequencing technology provided a significant amount of information about the transcriptome and genome analysis, which can solve this citrus bitterness issue. Nowadays, researchers are working on this, and hopefully, soon they will identify all the key enzymes responsible for citrus bitterness.

8.3 Analytical Quantification of Citrus Limonoids and Flavonoids

Naringin and limonin are the key flavonoid and limonoid, respectively, of citrus plant, which is responsible for its bitterness. Thus, their quantification in any food or juice materials composed of citrus fruit is very crucial. Generally, the flavonoid and limonoid compounds of the citrus plant are estimated through high-performance liquid chromatography (Magiera and Kwietniowska 2016). There are some other quantification methods used for the flavonoid and limonoid estimation such as HPLC methods, TLC methods, spectrometric method, colorimetric method [aluminum chloride method, $NaNO_2$-$Al(NO_3)_3$-NaOH method, and Davis methods] (Aturki and Sinibaldi 2003; Huang et al. 2018) but only HPLC method is most predominantly used because it takes less time, improve analytical effectiveness, and lower the cost.

Limonin and naringenin are nearly insoluble in water, therefore it is difficult to extract through the normal method and their measurement is also not easy. Thus, frequently they were extracted in an organic solvent and evaluated through HPLC or TLC methods (Ni et al. 2015). For the initial stage, TLC is the best method for the identification of limonoids. Generally, in the TLC method, Ehrlich reagent (p-dimethylaminobenzaldehyde) is used for spraying followed by HCL gas, which produces an orange spot of limonoids. Limonoids such as limonin could be detected by immunoassay methods such as RIA and ELISA, which are able to detect nanogram concentration of the compound. By using these immunoassays, limonoids could be identified without any prior extraction processer, which saves a lot of time and makes estimation processor easy (Dekker 1988). Thus, immunoassay is the best method for the identification of limonoids and HPLC is the most suitable method for the estimation of limonoids as well as flavonoids extracted from the citrus plant.

8.4 Approaches for Debittering of Fruit Juices

8.4.1 Physiochemical Methods

Earlier several physiochemical methods were investigated for the debittering process; here we presented a brief description of till date applied physiochemical process for debittering of juice from different citrus fruits.

8.4.1.1 Use of Adsorbents

Adsorption is a physiochemical process in which solute from the liquid phase is selectively transferred to the surface of the solid material (adsorbent) and this process is continued till the thermodynamic equilibrium of solute was maintained, after attaining the equilibrium there is no further absorption (Cussler 1997). In fruit juice processing, the use of adsorbents is a common practice for the separation of some targeted compounds like the removal of dark coloring agents, mycotoxins, and to reduce the acidity. For debittering of citrus juice, removal of limonin and naringin is necessary, therefore, several types of adsorptive materials have been explored since 1960 to till date. An earlier study of adsorbents showed that the use of polyamide was very successful for debittering the Washington navel orange juice.

Several other adsorptive materials like cellulose acetate and its different derivatives like cellulose acetate butyrate, cellulose triacetate, cellulose esters, and Florisil (activated magnesium silicate) have been employed for debittering of juice of different citrus fruits (Chandler et al. 1968; Chandler and Johnson 1977; Tsen and Yu 1991; Barmore et al. 1986). These adsorbents can be used separately or in combination with others but there is a large difference in the degree of bitterness removal by different adsorbents because of their selective absorption. Polystyrene divinylbenzene alone or in combination with acryl divinylbenzene resins are successfully used for debittering and removal of naringin and limonin depends on the degree of cross-linkage and surface area of adsorbents. Other polymers like polyvinylpyrrollidone, nylon polymers, synthetic neutral resins (Amberlite XAD-2, XAD-4, XAD-7, and XAD-16), diatomaceous earth, and granulated activated carbon are also attempted for the debittering process (Nisperos and Robertson 1982; Johnson and Chandler 1982; Hasegawa et al. 1989; Ribeiro et al. 2002).

8.4.1.2 Use of β-Cyclodextrin for Debittering

β-Cyclodextrin (Cycloheptaamylose) is a cyclic oligosaccharide, which is made up of seven α-1,4-linked D-glucopyranose units in a cyclic manner. It is soluble in water and sweet in taste (Chatjigakis et al. 1992) and produced by the enzymatic conversion of starch. Generally, β-cyclodextrin occurs in three forms; α, β, and γ

and among this β-cyclodextrin is relatively cheaper and used in pharmaceutical, food and nutraceutical, cosmetic, agricultural, and chemical industries (Del Valle 2004). For the debittering of juice extracted from citrus fruits (grapefruit, Iyo orange and *Citrus natsudaidai*), 0.5% β-cyclodextrin was used which reduced 58% bitter taste (Konno et al. 1982). There was the formation of an insoluble complex between k-cyclodextrin and naringin or limonin and this β-cyclodextrin polymer was regenerated by the organic solvent extraction process. After the β-cyclodextrin treatment level of flavonoids, coumarins and naringenin 7β-rutinoside were decreased while there was no adverse effect on the total acidity level and ascorbic acid content and simultaneously total soluble solids remain unchanged. Regeneration of β-cyclodextrin makes it suitable for use at pilot scale (Shaw and Wilson 1983; Shaw and Busligs 1986; Binello et al. 2008).

8.4.1.3 Use of Bitterness Suppressing Agents

Some chemicals showed effective bitterness suppressing activity such as neodiosmin, sucrose, and citric acid. Use of neodiosmin in 50–150 ppm concentration was effective for suppressing bitterness agent (Guadagni et al. 1976). Sucrose and citric acid are also used for suppression of bitterness although sucrose was weak and citric acid strongly suppressed the bitterness level (Guadagni et al. 1973). Many more compounds such as chalcone, dihydrochalcone, hesperidin dihydrochalcone, neohesperidin dihydrochalcone, and aspartyl phenylalanine methyl ester are able to act as bitterness suppressing agent due to their sweetening effect (Guadagni et al. 1974).

8.4.1.4 Debittering by Blanching

Blanching is a process of heat treatment, applied in fruit and vegetable industry for deactivating the endogenous enzymes, which affect their organoleptic and nutritional values as well as shelf life (Fellows 2009). Water and steam blanching tried to lessen the level of bittering substances of orange peel and results concluded as water blanching was able to remove ~38% and 48% of bitter flavanones when treated at 95°C and 85°C temperature, respectively, while steam blanching revealed the good retention of bitterness (Zid et al. 2015). In another study, blanching was applied on Nagpur (*C. reticulata* Blanco), Kinnow (*Citrus nobilis* × *Citrus deliciosa*), and Mandarin (*C. reticulata*) fruit and it was carried out at 65 °C which was followed by osmodehydration. By this process, naringin content was reduced up to 50% and this became lesser with the extended storage time of 6 months (Jagannath and Kumar 2016).

8.4.1.5 Using Supercritical Carbon Dioxide

Supercritical carbon dioxide behaves like fluid having gas like properties and it is used as a solvent in place of organic solvents. Treatment of citrus fruit juice with supercritical carbon dioxide showed a 25% reduction in limonin content without any adverse effect on other commercially important constituents like vitamin C, amino acids, citric acid (Kimball 1987).

8.4.1.6 Using Chemicals

Ethylene treatment reduces the limonin content in fruit juices (navel oranges, lemons, grapefruit) by accelerating the limonoate-A-ring lactone metabolism and another way to achieve ethylene effect is the spraying of 2-chloroethyl phosphonic acid in wax (Maier et al. 1973). In Pummelo (*C. grandis* (L.) Osbeck) fruit, Pichaiyongvongdee and Haruenkit (2011) reported that treatment with ethylene at 200 ppm concentration for 1.30 h leads to a decrease in limonin content by 78.38% without affecting antioxidant activity, nomilin, eriocitrin, neoeriocitrin concentration although a slight decrease in naringin occurred.

Effect of different phytohormones has been studied on nomilin biosynthesis and results revealed that auxins like indole acetic acid (IAA), indole butyric acid (IBA), naphthalene acetic acid (NAA) and 2,4,5-trichlorophenoxyacetic acid acted as a potent inhibitor of nomilin biosynthesis and abscisic acid also had an inhibitory effect but this can be revert back to treatment of cytokinin, although gibberellic acid (GA3) showed a neutral effect on nomilin biosynthesis (Hasegawa 1986a).

8.4.1.7 Limitations of Physiochemical Methods

The physiochemical process has several drawbacks so they are not accepted to be used at industrial scale. During physiochemical treatment, certain alterations in taste, turbidity, fragrance, and flavor of juice take place, which is not in favor of the consumer's acceptability. Removal of bittering compounds leads to loss of some valuable components during processing. Another limitation of these processes is the cost of chemicals and moreover, these cannot be reused. The most problematic issue with these chemicals is their hazardous impact on the environment due to lack of a proper channel for disposal of these chemicals. Consequently, these processes are not viable technologies for applying at large scale so researchers are trying to develop more sustainable and eco-friendly alternatives for the debittering of fruit juices.

8.4.2 Biotechnological Methods

Limitations of physiochemical processes can be overcome by introducing the biotechnological methods in fruit juice processing. Till date research on this topic shows the existence of various alternative techniques, which are successfully applied for the debittering process. Use of biological agents for debittering has numerous advantages over physiochemical methods as they are viable sources, have remarkable reusability, and less toxic effect on the environment. An overview of these techniques is presented below.

8.4.2.1 Use of Whole Microbial Cells

Microbes are very helpful to develop a sustainable solution to many problems whether it is related to food, pharmaceutical, chemical industry, and they are full of opportunity since the early period of research to till date. Their beneficial role in mineral cycling through degradation of several problematic compounds, which cannot be degraded by another means, makes them a powerful tool for use in biotechnology. Microbes have a good impact on the food industry as certain steps could not be completed without the aid of these microbes. These are used in the juice industry at various steps during juice processing and research reveals that certain microbes have some specific enzymes, which can be used to remove the bitterness problem of citrus juice.

Microbes Used for Limonin Debittering

Research on the screening of limonin metabolizing microbes is performed continuously from the early 1960 to till date, yet several microbes have been isolated which are capable to catalyze the bitter limonin into non-bitter compounds. These microbes such as bacteria, yeast, and some fungal species were isolated from diverse natural sources, which have remarkable limonin metabolizing activity.

Bacterial Sources

Earlier research in this area was pioneered by Dhavalikar and Bhattacharyya (1966) and they isolated a strain of *Pseudomonas* sp. (*P. putida* PL), which used d-limonene as a sole carbon source and analysis of products determined the existence of a series of compounds (carvone, dihydrocarvone, carveol, limonene1,2-*cis*-diol, 1-hydroxy-2-oxolimonene, limonene-1,2-*trans*-diol, perillic acid, 6,9-dihydroxy perillic acid, limonene-6,9-diol, β-isopropenyl pimelic acid, 2-hydroxy-8-p-menthen-7-oic acid). These large number of compounds show that limonin is attacked at several positions by the enzymes in this strain, majorly through the production of perillic acid by hydroxylation followed by oxidation.

Later several other strains of *Pseudomonas* have been reported for their limonin catalyzing activity such as *Pseudomonas incognita*, *Pseudomonas gladioli*, *P. putida* GS1, *P. putida* F1, *P. putida* MTCC 1072 (Rama Devi and Bhattacharyya 1977; Cadwallader et al. 1989; Speelmans et al. 1998; Mars et al. 2001; Chatterjee and Bhattacharyya 2001). In all the studied strains, perillic acid was found to be the main product of limonin degradation although some strain produced perillyl alcohol and limonene 6, 8 diol as a product (Chatterjee and Bhattacharyya 2001). This limonin degradation was carried out by the activity of three enzymes (cymene monooxygenase and two dehydrogenases) of cymene catalyzing pathway and function of these enzymes is confirmed by recombinant protein production in *Escherichia coli* (Mars et al. 2001). Although, Hasegawa (1976) reported that *Pseudomonas* sp. had Limonin D-ring lactone hydrolase activity.

Hasegawa et al. (1972) also screened several bacterial species by growing them on medium containing limonin or its sodium derivative. These bacterial species were able to metabolize the limonin and product analysis showed that these bacteria converted limonin into non-bitter deoxylimonin and deoxylimonic acid. Vaks and Lifshitz (1981) isolated *Acinetobacter* sp. which metabolized limonin into deoxylimonin and deoxylimonic acid. Hasegawa et al. (1972) reported a bacterium *Arthrobacter globiformis* which metabolized the limonin through the activity of limonoate dehydrogenase which was also purified and its *in vitro* activity was estimated through conversion of limonoate into 17-dehydrolimonoate in presence of NAD.

Chang and Oriel (1994) isolated *Bacillus stearothermophilus* BR388 strain which converted limonin into perillyl alcohol, α-terpineol, and perillaaldehyde, later limonene hydroxylase gene was found to be responsible for this bioconversion (Chang et al. 1995). Another limonin degrading bacterial strain *Bacillus megaterium* KU570370 was isolated from soil of kinnow orchard (Dua and Kocher 2017). *Enterobacter cowanii* 6L, isolated from citrus peel, metabolized limonin due to the presence of limonin hydroxylase activity (Yang et al. 2007). *Rhodococcus fascians* NRRL-B-15096 strain was used for debittering of kinnow juice and this strain had limonoate dehydrogenase activity for metabolizing limonin (Marwaha et al. 1994). *Rhodococcus erythropolis* A DCL14 bacterium, which was isolated from a freshwater sediment sample, followed a unique pathway for the metabolism of limonin (van der Werf et al. 1998). This pathway is assumed to be operated by the involvement of four different enzymes: 1. limonene 1, 2-monooxygenase; 2. limonene-1, 2-epoxide hydrolase; 3. limonene-1, 2-diol dehydrogenase and 4. 1-hydroxy-2-oxolimonene 1, 2-monooxygenase (van der Werf et al. 1999). Lactic acid bacteria were also screened for finding the limonin catabolizing activities. In this screening, *Streptococcus lactis* had been isolated having the maximum limonin degradation activity (66.7%) under optimum conditions (Lihua and Shaotong 2008).

Another soil bacterium, *Corynebacterium fascians* can be used for the debittering process of juice because this bacterium metabolized nomilin into obacunon by the activity of enzyme nomilin acetyl-lyase (Hasegawa et al. 1984; Herman et al. 1985).

Fungal Sources

Bowen (1975) isolated a fungal strain *Penicillium digitatum* from overripe oranges and this fungus successfully produced several products (*cis*- and *trans*-carveol, carvone, limonene-4-ol, and *cis*- and *trans*-mentha-2,8-dien-1-ol) from limonin degradation. Another *P. digitatum* DSM 62840 strain degraded limonin into α-terpineol, the earlier formation of α-terpineol from limonin was reported in another fungal strain *Cladosporium* sp. T12 but unfortunately this study was not extended for detailed research (Abraham et al. 1986; Kraidman et al. 1969). Later many other strains of *Penicillium* such as *P. digitatum* NRRL 1202, *P. digitatum* DSM 62840, *P. digitatum* ATCC 201167 were explored for the production of α-terpineol from limonin (Tan et al. 1998; Demyttenaere et al. 2001). Previously hydratase enzyme was supposed to be responsible for this conversion but recent updates show the involvement of P-450 monooxygenase in this conversion (Tan et al. 1998). Conversion of limonin into *trans*-limonene-1,2-diol, and *cis*-diol by *Cladosporium* sp. T7, *Diplodia gossypin* ATCC10936, and *Corynespora cassiicola* strains (DSM 62474 and DSM 62475) was also reported and this conversion occurred through the formation of an epoxide (Mukherjee et al. 1973; Kieslich et al. 1986).

Fungus *Armillaria mellae* produced α-terpineol and limonene-1,2-diol both from limonin while *Aspergillus cellulosae* M-77 led to the production of a mixture of compounds (α-terpineol, isopiperitenol, limonene-1,2 *trans*-diol, perillyl alcohol, isopiperitenone, and *cis*-carveol) from limonin degradation (Noma et al. 1992; Draczynska 1987). Onken and Berger (1999) reported a novel fungal strain *Pleurotus sapidus* for limonin degradation and this strain produced *cis*- and *trans*-carveol from limonin. Interestingly, the black yeast *Hormonema* sp. UOFS Y-0067 produced *trans*-isopiperitenol from limonin and this compound was not similar to the products obtained from limonin transformation by bacteria (Van Dyk et al. 1998).

Microbes Used for Naringin Debittering

A large number of microbes were screened for debittering of citrus juice. Generally, these microbes degraded naringin, the bittering substance, due to the activity of naringinase enzyme. Kishi (1955) explored 96 strains and found that *Aspergillus niger* can produce naringinase in significant quantity. Subsequently, other strains of *A. niger* were also identified for naringinase production such as *A. niger* NRRL 72-4 (Bram and Solomons 1965), *A. niger* MTCC 1344 (Puri and Kalra 2005), *A. niger* CECT 2088 (Busto et al. 2007), *A. niger* BCC 25166 (Thammawat et al. 2008). *Penicillium* sp. was also reported as a good producer of naringinase enzyme in fermentation media and subsequently, Hoechst Company filed a patent on naringinase production from *Penicillium* sp. (Fukumoto and Okada 1973; Hoechst 1994). Bread mold *Rhizopus nigricans* also produced naringinase enzyme in sucrose and rice supplemented medium (Shanmugam and Yadav 1995). Ito and

Takiguchi (1970) reported naringinase production from *Phanopsis citri*, *Rhizoctonia solani*, and *Cochiobolus miyabeanus*. *Aspergillus sojae*, isolated from a fermented soybean product, converted naringin to prunin with a negligible amount of naringenin (Chang et al. 2011). *Staphylococcus xylosus* MAK2 produced naringinase (8.45 U/mL) in a stirred tank reactor (Puri et al. 2010). Thus, these microbes, which can degrade naringin, can be used for citrus juice debittering but still this technology is not much suitable at a commercial level. Therefore, still, there is a huge scope in this area to explore debittering enzymes, which could possess remarkable applications in citrus industries.

8.4.2.2 Genetic Engineering Approach

Genetic engineering branch opens diverse paths for the development of new strategies for the production of juice without bitterness. It involves several steps and it can be performed by combining the basic knowledge with advanced techniques of molecular biology. Here, we elucidate prerequisite and modes of genetic engineering, which can be applied for debittering of citrus juice.

Targeted Enzymes for the Debittering Process

This technology needs comprehensive data about biochemical pathways and their enzymes which are involved in the production and degradation of these bittering substances. Simultaneously, we require knowledge of sources from which we can isolate these enzymes. Here we presented the enzymes, which can be targeted for manipulation of bitterness in citrus fruit juice.

Naringinase

Naringinase (α-rhamnopyranosidase) is a complex enzyme which has two activities, one is α-L-rhamnosidase (E.C. 3.2.1.40) and another is β-D-glucosidase (E.C.3.2.1.21). It uses naringin as substrate and converts it into rhamnose and prunin by its α-L-rhamnosidase activity and further this prunin is broken into glucose and naringenin by its β -D-glucosidase activity (Fig. 8.1). So, this enzyme is commercially used for debittering of citrus juice because it reduces the concentration of bitter naringin and produced naringenin, which is sweet in taste. Other than naringin, this enzyme can use a broad number of substrates, for example, α-rhamnose, and β-glucose containing other flavonoids like rutin, quercitrin, hesperidin, diosmin, and terphenyl glycosides. This enzyme has been reported from different sources such as bacteria, fungi, and plants and this enzyme is isolated and kinetically characterized for its catalytic activity (Table 8.1).

Table 8.1 List of different organisms producing naringinase enzyme

Organism	References
A. niger NRRL 72-4	Mateles et al. (1965)
Aspergillus aculeatus JMUdb058	Di et al. (2013)
Aspergillus oryzae 11250	Zhu et al. (2017a)
Aspergillus flavus	Radhakrishnan et al. (2013)
Aspergillus niger	Kishi (1955), Bram and Solomons (1965), Olson et al. (1979)
Aspergillus niger BCC 25166	Thammawat et al. (2008)
Aspergillus niger CECT 2088	Busto et al. (2007)
Aspergillus niger MTCC 1344	Puri and Karla (2005)
Aspergillus sojae	Chang et al. (2011)
Aspergillus terreus CECT 2663	Soria et al. (2004)
Bacillus amyloliquefaciensits 11568	Zhu et al. (2017b)
Cryptococcus albidus	Borzova et al. (2018)
Lasiodiplodia theobroma	Moubsher and Abdel-Aziz (2015)
Micrococcus sp.	Kumar et al. (2015)
Penicillium decumbens	Fukumoto and Okada (1973), Young et al. (1989), Magario et al. (2008), Vila-Real et al. (2010), Pedro et al. (2007), Ribeiro et al. (2010)
Penicillium decumbens PTCC 5248	Norouzian et al. (1999)
Penicillium DSM 6825	Meiwes et al. (1995)
Penicillium sp.	Hoechst (1994)
Pichia angusta	Yanai and Sato (2000)
Rhizopus nigricans	Shanmugam and Yadav 1995
Staphylococcus xylosus MAK2	Puri et al. (2011)

Limonoate Dehydrogenase

This enzyme catalyzes the dehydrogenation of bitter limonin into non-bitter 17-dehydrolimonoate A-ring lactone. This enzyme is reported from several bacterial and fungal sources (Table 8.2).

Table 8.2 Enzymes involve in limonin degradation and their sources

Enzymes	Organism	References
Limonoate dehydrogenase	*Rhodococcus fascians*	Humanes et al. (1997)
	Arthrobacter globiformis	Suhayda et al. (1995)
	Pseudomonas sp.	Hasegawa et al. (1976)
Nomilin Acetyl-lyase	*Corynebacterium fascians*	Herman et al. (1985)
Nomilin Deacetylase	*C. ichangensis*	Herman et al. (1989)
UDP-D-glucose: limonoid glucosyltransferase	*Satsuma mandarin* (*Citrus unshiu* Marc.)	Kita et al. (2000)
	Navel orange (*Citrus sinensis*)	Hasegawa et al. (1997)
	Citrus paradisi	Devaiah et al. (2016)
	C. paradisi cv. *Marsh seedless*, *C. aurantium*, *C. sinensis* cv. Shahsavar, *C. sinensis* cv. Thompson navel, *C. unshiu*, *C. limettioides*, *C. paradisi* cv. Marsh seedless	McIntosh and Mansell (1990), Zaare-Nahandi et al. (2008)
Limonin hydroxylase	*Bacillus stearothermophilus*	Cheong and Oriel (2000)
	Pseudomonas putida	Speelmans et al. (1998)
	Mentha x piperita	Lupien et al. (1999)
	Aspergillus niger ATCC 16404, *Aspergillus niger* ATCC 9642, *Aspergillus oryzae* ATCC 1003, *Penicillium notatum* ATCC 9478, *Penicillium camembertii* (CT) ATCC 4845, and *Paecilomyces variotii* ATCC 22319	Lerin et al. (2010)
	Penicillium digitatum DSM 62840	Tan and Day (1998)
	Solanum aviculare and *Dioscorea deltoidea*	Vanek et al. (1999)
	Enterobacter cowanii 6L	Yang et al. (2007)
Limonene 1,2-monooxygenase, Limonene-1, 2-epoxide hydrolase, Limonene-1,2-diol dehydrogenase, 1-Hydroxy-2-oxolimonene 1,2 monooxygenase	*Rhodococcus erythropolis* DCL14	Van der Werf et al. (1999)

UDP-D-Glucose: Limonoid Glucosyltransferase

These enzymes participate in the conversion of bitter limonoids into their glycosides which are not bitter in taste. Literature shows, the presence of these enzymes in different varieties of citrus fruits (Karim and Hashinaga 2002a, b) (Table 8.2).

Nomilin Deacetylase

It catalyzes the conversion of bitter nomilin into non-bitter deacetyllimonin. This enzyme exclusively reported from *Citrus ichangensis* and this species showed the accumulation of deacetyl limonin, other than this species this enzyme was also found in its hybrids such as Yuzu, Sudachi, and Kabosu (Herman et al. 1989) (Table 8.2).

Limonene Hydroxylase

Hydroxylation of limonene leads to lessening its bitterness and this hydroxylation can be performed on several carbon positions of limonin. In bacteria and fungus, this hydroxylation is carried out by P-450 monooxygenase enzyme and a homolog of this gene is cloned from *B. stearothermophilus* BR388 bacteria. This hydroxylase converts limonin into carveol and perillyl alcohol and simultaneously these products have undergone dehydrogenation to produce carvone and perillyl aldehyde. Similar results have been seen when the recombinant protein of this gene was produced in *E. coli* (Cheong and Oriel 2000). These P-450 systems work differentially in different bacterial and fungal system so their products also vary in their region-specificity (Duetz et al. 2000). Similarly, in plants, limonin hydroxylation is carried out by cytochrome P-450 enzymes as *Solanum aviculare* and *Dioscorea deltoidea* plant cells were able to metabolize both enantiomers of limonin with the production of a racemic mixture of *cis*- and *trans*-carveol and carvone by the hydroxylation of limonin at 6th position (Vanek et al. 1999).

Plant cell generates a large number of metabolites from limonin using a series of enzymes and these enzymes are very specific in their substrate selection and product formation (Karp et al. 1990). In plants for synthesis of diverse limonin-derived compounds hydroxylation of limonin is the first step and this hydroxylation mostly occurs at position allylic C3, C6, or C7 positions and some time at C-10 position (Duetz et al. 2003). Examples of these hydroxylases are perilla (−)-limonene-7-hydroxylase, caraway (+)- and spearmint (−)-limonene-6-hydroxylase, peppermint (−)-limonene-3-hydroxylase, putative limonene-10-hydroxylase (Karp et al. 1990). Mentha limonene C3 and C6 hydroxylases genes have been isolated and characterized for their regiospecificity (Schalk and Croteau 2000). Therefore, these enzymes have good potential to be targeted for metabolic engineering of citrus plants (Table 8.2).

Immobilization Technique for Efficient Debittering

Immobilization of the whole cell is a very common practice and this process has several advantages over the direct use of cell for product formation such as it avoids contamination of microbes in the product and reusability makes it a more preferable method to adopt at industrial scale. The immobilization of organisms producing debittering enzymes was performed such as *A. globiformis* cells in acrylamide gel was successfully immobilized with reusability of 17 cycles without a major loss in effectiveness (Hasegawa et al. 1983). Similarly, entrapment of *Acinetobacter* cells in a dialysis tube provided a platform for debittering of orange juice (Vaks and Lifshitz 1981). Treatment of citrus juice with the *C. fascians* cells, entrapped in acrylamide gel, showed the reduction in bitterness due to degradation of nomilin and limonin without any undesirable change in juice flavor (Hasegawa et al. 1985). Immobilization of *R. fascians* cells within polyurethane foam pads for batch and the continuous system was established for debittering of citrus juice (85% conversion of limonin in 200 h) under non-aerated environment and interestingly, this system was operated at different pH values (Cánovas et al. 1998). In another study immobilization of *R. fascians* in K-carrageenan is also attempted by Iborra and his coworkers (1994).

Immobilization of debittering enzymes rather than whole microbial cell provides more flexibility for exploring more combinations of supporting materials with either native or recombinant forms of enzymes. Extensive efforts have been made for immobilization of naringinase enzyme from different sources for obtaining a better platform for debittering of juice. There are several cases of immobilization of naringinase from *A. niger* such as immobilization on copolymers of styrene and maleic anhydride (Goldstein et al. 1971), on a tannin-aminohexyl cellulose (Ono et al. 1978), on chitin with glutaraldehyde and sodium borohydride (Tsen 1984), in hollow-fiber reactor (Olson et al. 1979), on controlled pore glass (Roitner et al. 1984), into poly (vinyl alcohol) (PVA) hydrogel (Busto et al. 2007). Similarly, naringinase from *Penicillium* sp. has economical importance, so immobilization of this enzyme has been performed on silicate (Turecek and Pittner 1987), on cellulose triacetate fibers (Tsen et al. 1989), on glycophase-coated controlled pore glass (Manjon et al. 1985). Naringinase enzyme of *Penicillium* sp. was stable up to 3 months at 6 °C after entrapment in sodium alginate and this enzyme was reused up to 12 cycles maintaining up to 50% activity (Puri et al. 1996).

Sometime, after immobilization certain profitable alterations occur in kinetic and thermal properties of enzymes like immobilization of *Penicillium decumbens* naringinase on mesoporous molecular sieve MCM-41 showed that K_m value of enzyme became lesser than free enzyme and simultaneously, there was excellent improvement in storage and thermal stability with recycling rate up to 6 times (Lei et al. 2011). Immobilization process proved better than physiochemical such as debittering of grapefruit (*Citrus aurantium*) juice by using Amberlite IR 400 and Amberlite IR 120 reduced naringin level up to 69.23% and 9%, respectively, while immobilized naringinase enzyme from *A. niger* successfully resulted in 83.84% reduction.

In another interesting study beyond the use of naringinase and limonin degrading enzymes the authors choose the strategy of adding glucose moiety to limonoid by using an immobilized glucosyltransferase enzyme. Pummelo limonoid glucosyltransferase (LGTase) was immobilized on different supporting systems and they found that properties of enzymes vary for different systems. LGTase was immobilized ionically on DEAE-Toyopearl and covalently on chitosan cross-linked with glutaraldehyde and on cellulose carbonate. This immobilized enzyme retained almost 80% activity and it can be reused up to 15 cycles, 9 cycles and 6 cycles on cellulose carbonate, chitosan and on DEAE-Toyopearl, respectively (Karim and Hashinaga 2002b).

8.4.2.3 Plant Metabolic Engineering Approach

The above-mentioned enzymes can be used for the generation of transgenic plants of *Citrus* sp. having the approach of silencing and overexpression of these genes. A similar case reported by Lücker et al. (2004) in which they raised the transgenic tobacco plants expressing the cDNA of (+)-limonene synthase and a (−)-limonene-3-hydroxylase from lemon and mint, respectively. These plants are able to produce limonin and its hydroxylated product, (+)-*trans*-isopiperitenol. Like other medicinal plants, plant metabolic engineering in citrus can be performed by modulating the flux of pathways by targeting the enzymes and transcription factors of the particular metabolic pathway (Jadaun et al. 2017; Sangwan et al. 2018). Use of transcriptomics and genomics approach is very supportive for analyzing the networking of these complicated pathways, by using *in silico* approach we can hypothesize the targets for further modifications which will lead to desirable alterations in metabolites (Tripathi et al. 2016; Narnoliya et al. 2017). In this direction, research work is in progress for the development of transgenic *Citrus* tree (Moriguchi et al. 2001; Pons et al. 2012; Orbović et al. 2015). There are certain chances of exploring all other possibilities for reducing the bitterness and it is possible by applying the basic knowledge about the citrus plant and pathways with modern techniques of synthetic biology.

8.5 Future Prospects

Citrus fruit juice industry has a key position in fruit juice industries and it has remarkable demand throughout worldwide consumers. The main hurdle of this industry is its bitter taste, which is mainly due to its limonoids and flavonoids, particularly limonin and naringin, respectively. Although, some technologies are available in the market, none of them is at a significant stage. Therefore, there is huge scope for the establishment of an efficient technology, which can reduce the notable level of citrus juice bittering compounds. The combination of metabolic engineering and system biology is known as synthetic biology which could be a

better approach to generate a potent technology for the problem of bitterness in fruit juice of citrus, which can solve this problem. Recently, identified genome editing CRISPR-Cas technique can play a key role in the development of such strategies. Thus, there is a need to resolve this problem through modern biotechnological strategies which will function at low cost also in less time with a reduction in bittering compounds up to consumer's acceptance level. This study provides the platform for the scientific community to develop such technologies and provides key information regarding enzyme engineering for targeting pathways to overcome the debittering issue.

References

Abraham WR, Hoffmann HMR, Kieslich K, Reng G, Stumpf B (1986) Microbial transformations of some monoterpenoids and sesquiterpenoids. In: Enzymes in organic chemistry. Ciba foundation symposium III. Pitman, London, pp 146–160

Alissa EM, Ferns GA (2017) Dietary fruits and vegetables and cardiovascular diseases risk. Crit Rev Food Sci Nutr 57(9):1950–1962

Appert C, Logemann E, Hahlbrock K, Schmid J, Amrhein N (1994) Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from Parsley (*Petroselinum crispum* Nym.). Eur J Biochem 225:491–499

Arias BA, Ramón-Laca L (2005) Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. J Ethnopharmacol 97(1):89–95

Aturki Z, Sinibaldi M (2003) Separation of diastereomers of flavanone-7-O-glycosides by capillary electrophoresis using sulfobutyl ether-β-cyclodextrin as the selector. J Sep Sci 26(9–10):844–850

Barmore CR, Fisher JF, Fellers PJ, Rouseff RL (1986) Reduction of bitterness and tartness in grapefruit juice with Florisil. J Food Sci 51:415–416

Benavente-Garcia O, Castillo J (2008) Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. J Agric Food Chem 56 (15):6185–6205

Betz C, McCollum TG, Mayer RT (2001) Differential expression of two cinnamate 4-hydroxylase genes in 'Valencia' orange (*Citrus sinensis* Osbeck). Plant Mol Biol 46(6):741–748

Binello A, Robaldo B, Barge A, Cavalli R, Cravotto G (2008) Synthesis of cyclodextrin-based polymers and their use as debittering agents. J Appl Polym Sci 107(4):2549–2557

Borzova N, Gudzenko O, Varbanets L (2018) Purification and characterization of a naringinase from *Cryptococcus albidus*. Appl Biochem Biotechnol 184(3):953–969

Bowen ER (1975) Potential by-products from microbial transformation of d-limonene. Florida State Horticultural Society, Florida, p 304

Bram B, Solomons GL (1965) Production of the enzyme naringinase by *Aspergillus niger*. Appl Microbiol 13:842–845

Busto MD, Meza V, Ortega N, Perez-Mateos M (2007) Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly (vinyl alcohol) cryogels for the debittering of juices. Food Chem 104(3):1177–1182

Cadwallader KR, Braddock RJ, Parish ME, Higgins DP (1989) Bioconversion of d-limonene by *Pseudomonas gladioli*. J Food Sci 54:1241–1245

Cánovas M, García-Cases L, Iborra J (1998) Limonin consumption at acidic pH values and absence of aeration by *Rhodococcus fascians* cells in batch and immobilized continuous systems. Enzyme Microb Technol 22(2):111–116

Caristi C, Bellocco E, Panzera V, Toscano G, Vadalà R, Leuzzi U (2003) Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from Sicilian cultivars. J Agric Food Chem 51(12):3528–3534

Chandler BV, Johnson RL (1977) Cellulose acetate as a selective sorbent for limonin in orange juice. J Sci Food Agr 28:875–884

Chandler BV, Kefford JF, Ziemelis G (1968) Removal of limonin from bitter orange juice. J Sci Food Agric 19(2):83–86

Chang HC, Oriel P (1994) Bioproduction of perillyl alcohol and related monoterpenes by isolates of *Bacillus stearothermophilus*. J Food Sci 59:660–662

Chang HC, Gage DA, Oriel PJ (1995) Cloning and expression of a limonene degradation pathway from *Bacillus stearothermophilus* in *Escherichia coli*. J Food Sci 60:551–553

Chang H-Y, Lee Y-B, Bae H-A, Huh J-Y, Nam S-H, Sohn H-S, Lee HJ, Lee S-B (2011) Purification and characterisation of *Aspergillus sojae* naringinase: the production of prunin exhibiting markedly enhanced solubility with *in vitro* inhibition of HMGCoA reductase. Food Chem 124:234–241

Chatjigakis AK, Donze C, Coleman AW, Cardot P (1992) Solubility behavior of beta-cyclodextrin in water/cosolvent mixtures. Anal Chem 64(14):1632–1634

Chatterjee T, Bhattacharyya DK (2001) Biotransformation of limonene by *Pseudomonas putida*. Appl Microbiol Biotechnol 55:541–546

Cheong TK, Oriel PJ (2000) Cloning and expression of the limonene hydroxylase of *Bacillus stearothermophilus* BR388 and utilization in two-phase limonene conversions. In: Twenty-first symposium on biotechnology for fuels and chemicals. Humana Press, Totowa, NJ, pp 903–915

Cussler EL (1997) Diffusion mass transfer in fluid systems, 2nd edn. Cambridge University Press, Cambridge, pp 308–330

Dekker RF (1988) De-bittering of citrus fruit juices: specific removal of limonin and other bitter principles. Aust J Biotechnol 2:65–76

Del Valle EM (2004) Cyclodextrins and their uses: a review. Process Biochem 39(9):1033–1046

Demyttenaere JCR, Van Belleghem K, De Kimpe N (2001) Biotransformation of (R)-(+)- and (S)-()-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry 57:199–208

Devaiah SP, Owens DK, Sibhatu MB, Sarkar TR, Strong CL, Mallampalli VK, Asiago J, Cooke J, Kiser S, Lin Z, Wamucho A (2016) Identification, recombinant expression, and biochemical analysis of putative secondary product glucosyltransferases from *Citrus paradisi*. J Agric Food Chem 64(9):1957–1969

Dhavalikar RS, Bhattacharyya PK (1966) Microbiological transformations of terpenes. VIII. Fermentation of limonene in a soil pseudomonad. Indian J Biochem 3:144–157

Di W, Ni H, Li L, Chen F, Xiao A, Cai H, Sun W (2013) Identification and characterization of *Aspergillus aculeatus* JMUdb058 for naringinase production. Wei sheng wu xue bao = Acta Microbiol Sin 53(7):691–701

Draczynska LB (1987) Oxidation of selected p-menthane derivatives by means of *Armilariella mellea* (honey fungus), a parasite of woodlands. J Basic Microbiol 27:191–196

Dua K, Kocher GS (2017) Enrichment and characterization of limonin degrading microorganisms isolated from kinnow orchard soil, Peel and Waste Site. App Biol Res 19(2):187–196

Duetz WA, Jourdat C, Witholt B (2000) Process for the preparation of trans-carveol and/or carvone, EP1205556

Duetz WA, Bouwmeester H, Van Beilen JB, Witholt B (2003) Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Appl Microbiol Biotechnol 61(4):269–277

Economos C, Clay WD (1999) Nutritional and health benefits of citrus fruits. Energy (kcal) 62 (78):37

Endo T, Kita M, Shimada T, Moriguchi T, Hidaka T, Matsumoto R, Hasegawa S, Omura M (2002) Modification of limonoid metabolism in suspension cell culture of Citrus. Plant Biotechnol 19:397–403

Fellows PJ (2009) Blanching. In: Food processing technology: principles and practice. Woodhead Publishing in Food Science, Technology and Nutrition, pp 369–380

Fukumoto J, Okada S (1973) Naringinase production by fermentation. Jpn Patent 7(306):554
Gabriac B, Werck-Reichhart D, Teutsch H, Durst F (1991) Purification and immune characterization of a plant cytochrome P450: the cinnamic acid 4-hydroxylase. Arch Biochem Biophys 288(1):302–309
Goldstein L, Lifshitz A, Skolovsky M (1971) Water insoluble derivatives of naringinase. Int J Biochem 10:448–449
González-Molina E, Domínguez-Perles R, Moreno DA, García-Viguera C (2010) Natural bioactive compounds of *Citrus limon* for food and health. J Pharm Biomed Anal 51(2):327–345
Guadagni DG, Maier VP, Turnbaugh JG (1973) Effect of some citrus juice constituents on taste thresholds for limonin and naringin bitterness. J Sci Food Agric 24(10):1277–1288
Guadagni DG, Maier VP, Turnbaugh JG (1974) Effect of subthreshold concentrations of limonin, naringin and sweeteners on bitterness perception. J Sci Food Agric 25(11):1349–1354
Guadagni DG, Maier VP, Turnbaugh JG (1976) Effect of neodiosmin on threshold and bitterness of limonin in water and orange juice. J Food Sci 41(3):681–684
Hasegawa S (1976) Metabolism of limonoids. Limonin D-ring lactone hydrolase activity in *Pseudomonas*. J Agric Food Chem 24(1):24–6
Hasegawa S, Hoagland JE (1977) Biosynthesis of limonoids in Citrus. Phytochemistry 16:469–471
Hasegawa S, Bennett RD, Maier VP, King AD Jr (1972) Limonoate dehydrogenase from *Arthrobacter globiformis*. J Agric Food Chem 20(5):1031–1034
Hasegawa S, Kim KS, Border SN, Brewster LC, Maier VP (1976) Production of limonoate dehydrogenase by *Pseudomonas*. J Food Sci 41(3):706–708
Hasegawa S, Pelton VA, Bennett RD (1983) Metabolism of limonoids by *Arthrobacter globiformis* II: basis for a practical means of reducing the limonin content of orange juice by immobilized cells. J Agric Food Chem 31(5):1002–1004
Hasegawa S, Dillberger AM, Choi GY (1984) Metabolism of limonoids: conversion of nomilin to obacunone in *Corynebacterium fascians*. J Agric Food Chem 32:457–459
Hasegawa S, Vandercook CE, Choi GY, Herman Z, Ou P (1985) Limonoid debittering of citrus juice sera by immobilized cells of *Corynebacterium fascians*. J Food Sci 50(2):330–332
Hasegawa S, Herman Z, Orme ED, Ou P (1986a) Biosynthesis of limonoids in Citrus: sites and translocation. Phytochemistry 25(12):2783–2785
Hasegawa S, Maier VP, Herman Z, Ou P (1986b) Phytohormone bioregulation of nomilin biosynthesis in *Citrus limon* seedlings. Phytochemistry 25(6):1323–1325
Hasegawa S, Bennett RD, Herman Z, Fong CH, Ou P (1989) Limonoid glucosides in Citrus. Phytochemistry 28:1717–1720
Hasegawa S, Ou P, Fong CH, Herman Z, Coggins CW Jr, Atkin DR (1991) Changes in the limonoate A-ring lactone and limonin 17-beta-D-glucopyranoside content of navel oranges during fruit growth and maturation. J Agric Food Chem 39(2):262–265
Hasegawa S, Suhayda CG, Hsu WJ, Robertson GH (1997) Purification of limonoid glucosyltransferase from navel orange albedo tissues. Phytochemistry 46:33–37
Herman Z, Hasegawa S, Ou P (1985) Nomilin acetyl-lyase, a bacterial enzyme for nomilin debittering of citrus juices. J Food Sci 50(1):118–120
Herman Z, Hasegawa S, Fong CH, Ou P (1989) Limonoids in *Citrus ichangensis*. Agric Food Chem 37:850–851
Hoechst (1994) Alpha-rhamnosidase production by *Penicillium* sp., and purification and characterization. German Patent EP 599, 159
Huang R, Wu W, Shen S, Fan J, Chang Y, Chen S, Ye X (2018) Evaluation of colorimetric methods for quantification of citrus flavonoids to avoid misuse. Anal Methods 10(22):2575–2587
Humanes L, López-Ruiz A, Merino MT, Roldan JM, Diez J (1997) Purification and characterization of limonoate dehydrogenase from *Rhodococcus fascians*. Appl Environ Microbiol 63(9):3385–3389

Iborra JL, Manjon A, Canovas M, Lozano P, Martinez C (1994) Continuous limonin degradation by immobilized *Rhodococcus fascians* cells in K-carrageenan. Appl Microbiol Biotechnol 41 (4):487–493

Ito T, Takiguchi Y (1970) Naringinase production by *Cochiobolus miyabeanus*. Jpn Patent 7 (014):875

Izquierdo L, Sendra JM (2003) Citrus fruits composition and characterization. Encycl Food Sci Nutr 2:1335–1341

Jadaun JS, Sangwan NS, Narnoliya LK, Singh N, Bansal S, Mishra B, Sangwan RS (2017) Over-expression of DXS gene enhances terpenoidal secondary metabolite accumulation in rose-scented geranium and *Withania somnifera*: active involvement of plastid isoprenogenic pathway in their biosynthesis. Physiol Plant 159(4):381–400

Jagannath A, Kumar M (2016) Monitoring blanching induced debittering and storage losses of naringin in orange subjected to osmotic dehydration. Int J Fruit Sci 16(4):410–422

Johnson RL, Chandler BV (1982) Reduction of bitterness and acidity in grapefruit juice by adsorptive processes. J Sci Food Agric 33(3):287–293

Joseph MJ, Joseph PN (2002) Reaction mechanism of chalcone isomerase. J Biol Chem 11:1361–1369

Jung W, Yu O, Lau SC, OKeefe D, Odell J, Fader G, McGonigle B (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol 18:208–212

Kabra AO, Bairagi GB, Mahamuni AS, Wanare RS (2012) *In vitro* antimicrobial activity and phytochemical analysis of the peels of *Citrus medica* L. Int J Res Pharm Biomed Sci 3(1): 34–37

Karim MR, Hashinaga F (2002a) Isolation and characterization of limonoid glucosyltransferase from pummelo albedo tissue. Food Chem 76(4):431–436

Karim MR, Hashinaga F (2002b) Preparation and properties of immobilized pummelo limonoid glucosyltransferase. Process Biochem 38(5):809–814

Karp F, Mihaliak CA, Harris JL, Croteau R (1990) Monoterpene biosynthesis: specificity of the hydroxylations of limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*) and perilla (*Perilla frutescens*) leaves. Arch Biochem Biophys 276:219–226

Kaur C, Kapoor HC (2001) Antioxidants in fruits and vegetables the millennium's health. Int J Food Sci Technol 36(7):703–725

Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M (1999) Quantitation of flavonoid constituents in citrus fruits. J Agric Food Chem 47(9):3565–3571

Kieslich K, Abraham WR, Stumpf B, Thede B, Washausen P (1986) Transformation of terpenoids. Progress in essential oil research. De Gruyter, Berlin, pp 367–394

Kim DS, Hwang BK (2014) An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J Exp Bot 65(9):2295–2306

Kim ME, Jayaprakasha GK, Vikram A, Patil BS (2012) Methyl nomilinoate from citrus can modulate cell cycle regulators to induce cytotoxicity in human colon cancer (SW480) cells in vitro. Toxicol in Vitro 26:1216–1223

Kimball DA (1987) Debittering of citrus juices using supercritical carbon dioxide. J Food Sci 52 (2):481–482

Kishi K (1955) Production of naringinase from *Aspergillus niger*. Kagaku to Kogyo (Chem Ind Jpn) 29:140

Kita M, Hirata Y, Moriguchi T, Endo-Inagaki T, Matsumoto R, Hasegawa S, Suhayda CG, Omura M (2000) Molecular cloning and characterization of a novel gene encoding limonoid UDP-glucosyltransferase in Citrus. FEBS Lett 469(2–3):173–178

Konno A, Miyawaki M, Toda J, Wada T, Yasumatsu K (1982) Bitterness reduction of naringin and limonin by β-cyclodextrin polymer. Agric Biol Chem 46:2203

Kraidman G, Mukherjee BB, Hill ID (1969) Conversion of d-limonene into an optically active isomer of a-terpineol by a *Cladosporium* species. Bacteriol Proc 63

Kumar A, Singh MK, Amena S (2015) Optimization of naringinase production and its purification from *Micrococcus* sp. Int J Pharm Pharm Sci 7(2):269–272

Lapcik O, Klejdus B, Davidová M, Kokoska L, Kuban V, Moravcová J (2004) Isoflavonoids in the Rutaceae Family: *Fortunella obovata*, *Murraya paniculata* and four *Citrus* species. Phytochem Anal 15:293–299

Lei S, Xu Y, Fan G, Xiao M, Pan S (2011) Immobilization of naringinase on mesoporous molecular sieve MCM-41 and its application to debittering of white grapefruit. Appl Surf Sci 257(9):4096–4099

Lerin L, Toniazzo G, Oliveira DD, Rottava L, Dariva C, Cansian RL, Treichel H, Padilha F, Antunes OA (2010) Microorganisms screening for limonene oxidation. J Food Sci Technol 30 (2):399–405

Lihua LS, Shaotong J (2008) Effect of *In-situ* biodegradation on limonin of *citrus* juice by lactic acid bacteria. Trans Chin Soc Agric Mach 4:022

Lucheta AR, Silva-Pinhati ACO, Basilio-Palmieri AC, Berger IJ, Freitas-Astua J, Cristofani M (2007) An *in silico* analysis of the key genes involved in flavonoid biosynthesis in *Citrus sinensis*. Genet Mol Biol 30(3):819–831

Lücker J, Schwab W, Franssen MC, Van Der Plas LH, Bouwmeester HJ, Verhoeven HA (2004) Metabolic engineering of monoterpene biosynthesis: two-step production of (+)-trans-isopiperitenol by tobacco. Plant J 39(1):135–145

Lupien S, Karp F, Wildung M, Croteau R (1999) Regiospecific cytochrome P450 limonene hydroxylases from mint (Mentha) species: cDNA isolation, characterization, and functional expression of (−)-4S-limonene-3-hydroxylase and (−)-4S-limonene-6-hydroxylase. Arch Biochem Biophys 368(1):181–192

Lv X, Zhao S, Ning Z, Zeng H, Shu Y, Tao O, Xiao C, Lu C, Liu Y (2015) *Citrus* fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chem Cent J 9(1):68

Magario I, Ma X, Neumann A, Syldatk C, Hausmann R (2008) Nonporous magnetic micro-particles: comparison to porous enzyme carriers for a diffusion rate-controlled enzymatic conversion. J Biotechnol 134:72–78

Magiera S, Kwietniowska E (2016) Fast, simple and efficient salting-out assisted liquid-liquid extraction of naringenin from fruit juice samples prior to their enantioselective determination by liquid chromatography. Food Chem 211:227–234

Maier VP, Beverly GD (1968) Limonin monolactone, the nonbitter precursor responsible for delayed bitterness in certain citrus juices. J Food Sci 33(5):488–492

Maier VP, Hasegawa S (1970) L-Phenylalanine ammonia-lyase activity and naringenin glycoside accumulation in developing grapefruit. Phytochemistry 9:139–144

Maier VP, Hasegawa S, Hera E (1969) Limonin D-ring-lactone hydrolase. A new enzyme from *Citrus* seeds. Phytochemistry 8(2):405–407

Maier VP, Brewster LC, Hsu AC (1973) Ethylene-accelerated limonoid metabolism in citrus fruits. Process for reducing juice bitterness. J Agric Food Chem 21(3):490–495

Manjon A, Bastida J, Romero C, Jimeno A, Iborra JL (1985) Immobilization of naringinase on glycophase coated porous glass beads. Biotechnol Let 7:487–492

Mars AE, Gorissen JPL, Van den Beld I, Eggink G (2001) Bioconversion of limonene to increased concentrations of perillic acid by *Pseudomonas putida* GS1 in a fed-batch reactor. Appl Microbiol Biotechnol 56:101–107

Marshall TA, Stumbo PJ, Warren JJ, Xie XJ (2001) Inadequate nutrient intakes are common and are associated with low diet variety in rural, community-dwelling elderly. J Nutr 131(8):2192–2196

Marwaha SS, Puri M, Bhullar MK, Kothari RM (1994) Optimization of parameters for hydrolysis of limonin for debittering of kinnow mandarin juice by *Rhodococcus fascians*. Enzyme Microb Technol 16(8):723–725

Mateles RI, Perlman D, Humphery AE, Deindorfer FH (1965) Fermentation review. Biotechnol Bioeng 7:54–758

McIntosh CA, Mansell RL (1990) Biosynthesis of naringin in *Citrus paradisi*: UDP-glucosyl-transferase activity in grapefruit seedlings. Phytochemistry 29(5):1533–1538
Meiwes J, Wullbrandt D, Giani C (inventors), Hoechst AG (assignee) (1995) α-L-Rhamnosidase for obtaining rhamnose, a process for its preparation and its use. United States patent US 5,468,625
Milan EB, Mandoulakani BA, Kheradmand F (2017) The effect of methyl jasmonate on the expression of phenylalanine ammonia lyase and eugenol-o-methyl transferase genes in basil. Philipp Agric Sci 100(2):163–167
Mohanpuria P (2016) Cloning and expression of delayed bitterness gene encoding limonoid glucosyltransferase from Kinnow mandarin (*Citrus reticulata* Blanco). Doctoral dissertation, Punjab Agricultural University, Ludhiana
Moriguchi T, Kita M, Tomono Y, EndoInagaki T, Omura M (1999) One type of chalcone synthase gene expressed during embryogenesis regulates the flavonoid accumulation in citrus cell cultures. Plant Cell Physiol 40(6):651–655
Moriguchi T, Kita M, Tomono Y, Endo-Inagaki T, Omura M (2001) Gene expression in flavonoid biosynthesis: correlation with flavonoid accumulation in developing citrus fruit. Physiol Plant 111(1):66–74
Moubsher H, Abdel-Aziz F (2015) Production and characterization of naringinase from new fungal isolate of *Lasiodiplodia theobromae*. J Enzyme 1(2):010
Mukherjee BB, Kraidman G, Hill ID (1973) Synthesis of glycols by microbial transformations of some monocyclic terpenes. Appl Microbiol 25:447–453
Narnoliya LK, Kaushal G, Singh SP, Sangwan RS (2017) De novo transcriptome analysis of rose-scented geranium provides insights into the metabolic specificity of terpene and tartaric acid biosynthesis. BMC Genom 18(1):74
Ni H, Zhang SF, Gao QF, Hu Y, Jiang ZD, Chen F (2015) Development and evaluation of simultaneous quantification of naringin, prunin, naringenin, and limonin in citrus juice. Food Sci Biotechnol 24(4):1239–1247
Nisperos MO, Robertson GL (1982) Removal of naringin and limonin from grapefruit juice using polyvinyl pyrrolidone. Phillip Agric 65:275
Noma Y, Yamasaki S, Asakawa Y (1992) Biotransformation of limonene and related compounds by *Aspergillus cellulosae*. Phytochemistry 31:2725–2727
Norouzian D, Hosseinzadeh A, Inanlou DN, Moazami N (1999) Various techniques used to immobilize naringinase produced by *Penicillium decumbens* PTCC 5248. World J Microbiol Biotechnol 15:501–502
Olson AC, Gray GM, Guadagni DG (1979) Naringinase bitterness of grapefruit juice debittered with naringinase immobilized in a hollow fibre. J Food Sci 44:1358–1360
Ono M, Tosa T, Chibata I (1978) Preparation and properties of immobilized naringinase using tannin-aminohexylcellulose. Agric Biol Chem 42:1847–1853
Onken J, Berger RG (1999) Effects of R-(+)-limonene on submerged cultures of the terpene transforming basidiomycete Pleurotus sapidus. J Biotechnol 69(2-3):163-8
Orbović V, Ćalović M, Dutt M, Grosser JW, Barthe G (2015) Production and characterization of transgenic *Citrus* plants carrying p35 anti-apoptotic gene. Sci Hortic 197:203–211
Pedro HAL, Alfaia AJ, Marques J, Vila-Real HJ, Calado A, Ribeiro MHL (2007) Design of an immobilized enzyme system for naringin hydrolysis at high-pressure. Enzyme Microb Technol 40:442–446
Pelt JL, Downes WA, Schoborg RW, McIntosh CA (2003) Flavanone 3-hydroxylase expression in *Citrus paradisi* and *Petunia hybrida* seedlings. Phytochemistry 64:435–444
Peterson JMS, Dwyer J, Dsc RD (1998) Flavonoids: dietary occurrence and biochemical activity. Nutr Res 18:1995–2018
Pichaiyongvongdee S, Haruenkit R (2011) Effect of ethylene treatments in limonin reduction in Thai Pummelo (*Citrus grandis* (L.) Osbeck) fruit. Kasetsart J (Nat Sci) 45:1105–1114
Pons E, Peris JE, Peña L (2012) Field performance of transgenic citrus trees: assessment of the long-term expression of uidA and nptII transgenes and its impact on relevant agronomic and phenotypic characteristics. BMC Biotechnol 12(1):41

Puri M, Kalra S (2005) Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. World J Microbiol Biotechnol 21(5):753–758

Puri M, Marwaha SS, Kothari RM (1996) Studies on the applicability of alginate-entrapped naringiase for the debittering of kinnow juice. Enzyme Microb Technol 18(4):281–285

Puri M, Kaur A, Singh RS, Singh A (2010) Response surface optimization of medium components for naringinase production from *Staphylococcus xylosus* MAK2. Appl Biochem Biotechnol 162:181–191

Puri M, Kaur A, Barrow CJ, Singh RS (2011) Citrus peel influences the production of an extracellular naringinase by *Staphylococcus xylosus* MAK2 in a stirred tank reactor. Appl Microbiol Biotechnol 89(3):715–722

Radhakrishnan I, Sampath S, Kumar S (2013) Isolation and characterization of enzyme naringinase from *Aspergillus flavus*. Int J Adv Biotechnol Res 4(2):208–212

Raithore S, Dea S, McCollum G, Manthey JA, Bai J, Leclair C, Hijaz F, Narciso JA, Baldwin EA, Plotto A (2016) Development of delayed bitterness and effect of harvest date in stored juice from two complex citrus hybrids. J Sci Food Agric 96(2):422–429

Rama Devi J, Bhattacharyya PK (1977) Microbiological transformations of terpenes. XXIII. Fermentation of geraniol, nerol and limonene by a soil pseudomonad, *Pseudomonas incognita* (linalool strain). Indian J Biochem Biophys 14:288–291

Reiss R, Johnston J, Tucker K, DeSesso JM, Keen CL (2012) Estimation of cancer risks and benefits associated with a potential increased consumption of fruits and vegetables. Food Chem Toxicol 50(12):4421–4427

Ribeiro MH, Silveira D, Ferreira-Dias S (2002) Selective adsorption of limonin and naringin from orange juice to natural and synthetic adsorbents. Eur Food Res Technol 215(6):462–471

Ribeiro MHL, Afonso C, Vila-Real HJ, Alfaia AJ, Ferreira L (2010) Contribution of response surface methodology to the modeling of naringin hydrolysis by naringinase Ca-alginate beads under high pressure. LWT Food Sci Technol 43:482–487

Roitner M, Schalkhammer T, Pittner F (1984) Preparation of prunin with the help of immobilized naringinase pretreated with alkaline buffer. Appl Biochem Biotechnol 9:483–488

Ronneberg TA, Hasegawa S, Suhayda C, Ozaki Y (1995) Limonoid glucoside β-glucosidase activity in lemon seeds. Phytochemistry 39:1305–1307

Sanchez-Ballesta MT, Lafuente MT, Zacarias L, GranellA (2000) Involvement of phenylalanine ammonia-lyase in the response of fortune mandarin fruits to cold temperature. Physiol Plant 108(4):382–389

Sangwan NS, Jadaun JS, Narnoliya LK, Mishra B, Tripathi S, Sangwan RS (2018) Plant metabolic engineering, Omics technologies and bio-engineering: towards improving quality of life. Elsevier. https://doi.org/10.1016/B978-0-12-815870-8.00009-7

Schalk M, Croteau R (2000) A single amino acid substitution (F363I) converts the regiochemistry of the spearmint (−)-limonene hydroxylase from a C6-to a C3-hydroxylase. Proc Natl Acad Sci U S A 97(22):11948–11953

Shanmugam V, Yadav KDS (1995) Extracellular production of alpha-rhamnosidase by *Rhizopus nigricans*. Ind J Exp Biol 33:705–707

Shaw PE, Buslig BS (1986) Selective removal of bitter compounds from grapefruit juice and from aqueous solution with cyclodextrin polymers and with Amberlite XAD-4. J Agric Food Chem 34(5):837–840

Shaw PE, Wilson CW (1983) Debittering *Citrus* juices with β-cyclodextrin polymer. J Food Sci 48 (2):646–647

Slavin JL, Lloyd B (2012) Health benefits of fruits and vegetables. Adv Nutr 3(4):506–516

Soria F, Ellenrieder G, Grasselli M, Navarro del Cañizo AA, Cascone O (2004) Fractionation of the naringinase complex from *Aspergillus terreus* by dye affinity chromatography. Biotechnol Lett 26:1265–1268

Speelmans G, Bijlsma A, Eggink G (1998) Limonene bioconversion to high concentrations of a single and stable product, perillic acid, by a solvent-resistant *Pseudomonas putida* strain. Appl Microbiol Biotechnol 50:538–544

Suhayda CG, Omura M, Shin H (1995) Limonoate dehydrogenase from *Arthrobacter globiformis*: the navite enzyme and its N-Terminal sequence. Phytochemistry 40(1):17–20
Tan Q, Day DF (1998) Bioconversion of limonene to alpha-terpineol by immobilized *Penicillium digitatum*. Appl Micriobiol Biotechnol 49:96–101
Tan Q, Day DF, Cadwallader KR (1998) Bioconversion of (R)-(+)-limonene by *P. digitatum* (NRRL 1202). Process Biochem 33:29–37
Thammawat K, Pongtanya P, Juntharasri V, Wongvithoonyaporn P (2008) Isolation, preliminary enzyme characterization and optimization of culture parameters for production of naringinase isolated from *Aspergillus niger* BCC 25166. Kaestsart J (Nat Sci) 42:61–72
Thimmappa R, Geisler K, Louveau T, O'Maille P, Osbourn A (2014) Triterpene biosynthesis in plants. Annu Rev Plant Biol 65:225–257
Tripathi S, Jadaun JS, Chandra M, Sangwan NS (2016) Medicinal plant transcriptomes: the new gateways for accelerated understanding of plant secondary metabolism. Plant Genet Resour 14 (4):256–269
Tsen HY (1984) Factors affecting the inactivation of naringinase immobilized on chitin during debittering from fruit juices. J Ferment Technol 62:263–267
Tsen HY, Yu GJ (1991) Limonin and naringin removal from grapefruit juice with naringinase entrapped in cellulose triacetate fibres. J Food Sci 56:31–34
Tsen HY, Tsai SY, Yu GJ (1989) Fibre entrapment of naringinase from *Penicillium* sp. and application to fruit juice debittering. J Ferment Technol 67:186–189
Turecek PL, Pittner F (1987) Application of enzyme immobilization to the analysis of naturally occurring compounds as exemplified by alpha-rhamnosides. Sci Pharm 55:275–283
Vaks B, Lifshitz A (1981) Debittering of orange juice by bacteria which degrade limonin. J Agric Food Chem 29(6):1258–1261
van der Werf MJ, Overkamp KM, de Bont JA (1998) Limonene-1, 2-epoxide hydrolase from *Rhodococcus erythropolis* DCL14 belongs to a novel class of epoxide hydrolases. J Bacteriol 180(19):5052–5057
van der Werf MJ, Swarts HJ, de Bont JA (1999) *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. Appl Environ Microbiol 65(5):2092–2102
Van Dyk MS, Van Rensburg E, Moleki N (1998) Hydroxylation of (+) limonene, (-) α-pinene and (-) β-pinene by a *Hormonema* sp. Biotechnol Lett 20:431–436
Vanek T, Valterova I, Vankova R, Vaisar T (1999) Biotransformation of (-)-limonene using *Solanum aviculare* and *Dioscorea deltoidea* immobilized plant cells. Biotechnol Lett 21:625–628
Vila-Real H, Alfaia AJ, Calado ART, Ribeiro MHL (2010) Improve-ment of activity and stability of soluble and sol–gel immobilized naringinase in co-solvent systems. J Mol Catal B Enzym 65:91–101
Wang F, Wang M, Liu X, Xu Y, Zhu S, Shen W, Zhao X (2017) Identification of putative genes involved in limonoids biosynthesis in *Citrus* by comparative transcriptomic analysis. Front Plant Sci. https://doi.org/10.3389/fpls.2017.00782
Yanai T, Sato M (2000) Purification and characterization of α-L-rhamnosidases from *Pichia angusta* X349. Biosci Biotechnol Biochem 64(10):2179–2185
Yang JE, Park YJ, Chang HC (2007) Cloning of four genes involved in limonene hydroxylation from *Enterobacter cowanii* 6L. J Microbiol Biotechnol 17(7):1169–1176
Yang HY, Dong T, Li JF, Wang MY (2016) Molecular cloning, expression, and subcellular localization of a PAL gene from *Citrus reticulata* under iron deficiency. Biol Plant 60(3):482–488
Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. Plant Foods Hum Nutr 59(3):113–122
Young NM, Johnston RAZ, Richards JC (1989) Purification of the αL-rhamnosidase of *Penicillium decumbens* and characterisation of two glycopeptide components. Carbohydr Res 191:53–62

Zaare-Nahandi F, Hosseinkhani S, Zamani Z, Asadi-Abkenar A, Omidbaigi R (2008) Delay expression of limonoid UDP-glucosyltransferase makes delayed bitterness in citrus. Biochem Biophys Res Commun 371(1):59–62

Zhang X, Liu CJ (2015) Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol Plant 8(1):17–27

Zhu Y, Jia H, Xi M, Li J, Yang L, Li X (2017a) Characterization of a naringinase from *Aspergillus oryzae* 11250 and its application in the debitterization of orange juice. Process Biochem 62:114–121

Zhu Y, Jia H, Xi M, Xu L, Wu S, Li X (2017b) Purification and characterization of a naringinase from a newly isolated strain of *Bacillus amyloliquefaciens* 11568 suitable for the transformation of flavonoids. Food Chem 214:39–46

Zid MB, Dhuique-Mayer C, Bellagha S, Sanier C, Collignan A, Servent A, Dornier M (2015) Effects of blanching on flavanones and microstructure of *Citrus aurantium* peels. Food Bioprocess Technol 8(11):2246–2255

Zou Z, Xi W, Hu Y, Nie C, Zhou Z (2016) Antioxidant activity of *Citrus* fruits. Food Chem 196:885–896

Chapter 9
Enzymes in Sweeteners Production

Filipe Carvalho and Pedro Fernandes

Abstract The eco-friendly and highly specific nature of enzymes has made these biocatalysts widely used in the production of sweeteners. Traditionally, their application is mostly associated with the production of starch-derived high-calorie sugars, and at a minor scale, to the production of invert sugar syrup. Such pattern still stands, albeit with significant developments toward improved biocatalysts for those roles. These improvements have involved several approaches such as enzyme screening/modification through genetic or chemical approaches, and enhanced enzyme formulations. Additionally, in recent years, the public perception on the impact of diet in public health has established the need for alternative low-calorie sweeteners. These abridge a diversity of compounds, from high-intensity sweeteners to oligosaccharides with low sweetening power but with a prebiotic role. The present work aims to provide an updated overview of the current enzyme-based processes in the production of sweeteners. The rationale underlying the enzymatic approaches as preferred alternative to chemical routes is addressed. Specific insight is given on the operational conditions implemented in the enzymatic processes and on biocatalyst development, while also providing the scope for the different types of sweeteners manufactured enzymatically. The key issues on industrial scale sweetener production are discussed. Finally, foreseen developments in the field are also suggested.

Keywords Sweeteners · Hydrolases · Transferases · Isomerases
Engineering

F. Carvalho · P. Fernandes (✉)
Department of Bioengineering and IBB, Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
e-mail: pedro.fernandes@tecnico.ulisboa.com

P. Fernandes
DREAMS and Faculty of Engineering, Universidade Lusófona de Humanidades e Tecnologias, Av. Campo Grande 376, 1749-024 Lisbon, Portugal

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_9

9.1 Caloric Sweeteners

9.1.1 Starch-Based Sweeteners

At industrial scale the production of starch-derived sweeteners is conventionally of multienzyme nature and involves the use of: (1) amylases (α and β-amylases, isoamylases); (2) gluconases (pullulanase); and (3) glucose isomerases. The process flow includes several processing steps (Fig. 9.1) where under the scope of this publication, the major ones are liquefaction of starch though the action of amylases resulting into oligosaccharides and short-chain dextrins; saccharification of these oligosaccharides into monomeric units of glucose, through the action of amyloglucosidase, highly active in the hydrolysis of α-1,4 glycosyl bonds, eventually superseded with pullulanase, active in the hydrolysis of α-1,6 glycosyl bonds, should these be in significant number; and isomerization of glucose to fructose with glucose (xylose) isomerase (Parker et al. 2010; Hii et al. 2012). The equilibrium of the isomerization of glucose to fructose depends on the temperature as it increases, and equilibrium shifts to fructose. Commercially available immobilized glucose isomerase formulations currently can operate at 60 °C, at which temperature roughly 50% fructose is formed (DiCosimo et al. 2013).

Liquefaction of starch is performed under high temperatures, e.g., 105 °C, therefore many efforts have been made to put into market highly active thermostable amylases. These will be particularly addressed in this work.

α-Amylases

Starch can be found in roots, seeds, and tubers. Industrially, the most widely used starch source is corn; nonetheless, there are others such as potato, wheat, tapioca, and rice that are also broadly exploited. The first step toward the production of starch-derived sweeteners is the liquefaction and partial hydrolysis of starch. In the case of corn, the starch obtained from the wet milling of kernels undergoes enzymatic hydrolysis through the action of thermostable α-amylases (EC 3.2.1.1). α-amylases are one type of endoamylases that catalyze the hydrolysis of internal α-1,4-glycosidic linkages of starch into low molecular weight products such as dextrins and progressively smaller polymers composed of glucose units. As a result, α-amylases can rapidly reduce the viscosity of starch solutions (Windish and Mhatre 1965; Gupta et al. 2003). Accordingly, α-amylases are among the most important and widely used enzymes in industry, holding roughly 25% of the world enzyme market (de Souza and Magalhães 2010).

α-Amylases can be obtained from several sources such as plants, animals, and microorganisms; yet microbial α-amylases more often fulfill the industrial demands regarding catalytic performance and window of operation. During the liquefaction of starch, pH is traditionally maintained on the 5.8–6.5 range to match the optimal pH values of the α-amylases and because higher pH values increase by-product formation. However, starch native pH is around 3.2–4.5 which gave the incompatibility with the biocatalyst operational range results in the need to introduce

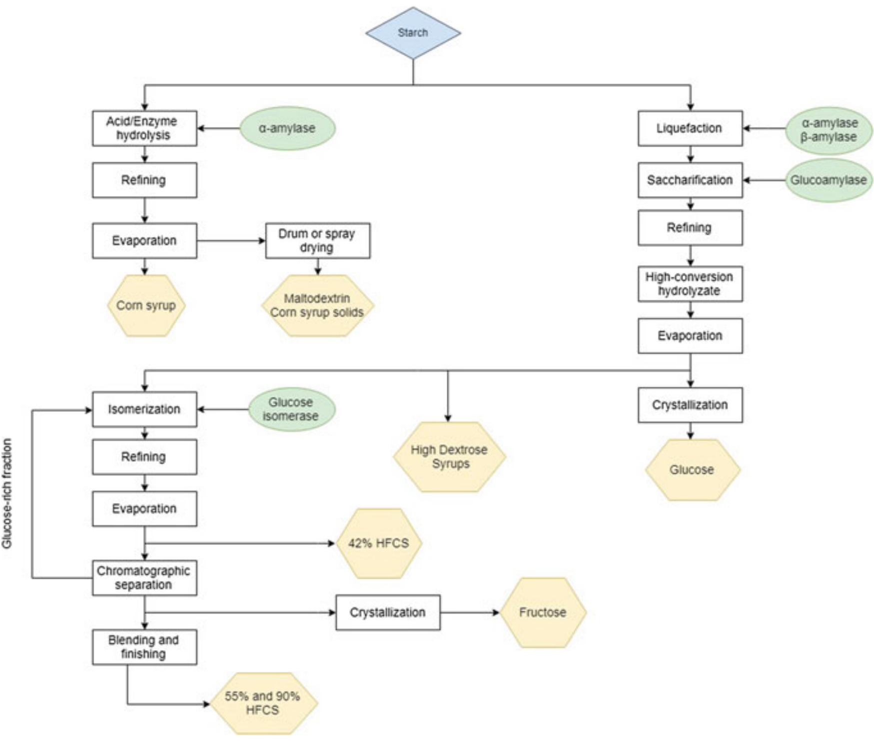


Fig. 9.1 Flowsheet for the production of sweeteners from starch. HFCS stands for high-fructose corn syrup (Lloyd and Nelson 1984). Percent refer to the relative content of fructose, the remaining being essentially non-isomerized glucose

additional steps of pH correction prior to and after the liquefaction step. Solid levels are usually kept around 30–35% to achieve full starch gelatinization. Typically, Ca^{2+} is an important α-amylase cofactor that enhances activity and thermostability, and it is added in calcium oxide or calcium chloride form at concentrations ranging from 50 to 300 ppm depending on the source of the amylase. One of the drawbacks of the Ca^{2+} salt addition is the need of its removal downstream with ion-exchange methodologies. Reaction times and temperatures are carefully tuned to provide optimal operational conditions for enzyme performance and full starch gelatinization (Sharma and Satyanarayana 2013; Singh et al. 2017).

The identification of extremozymes from extremophiles able to withstand the demanding industrial conditions of starch liquefaction together with the protein engineering of the novel and previously used α-amylases have raised considerable attention among industrial players (Sharma and Satyanarayana 2013; Dey et al. 2016). Furthermore, process engineering options (e.g., reactor design, mode of operation, biocatalyst immobilization methodologies, among others) also contribute for process optimization, cost reduction, and overall increase in productivities (Tufvesson et al. 2013). It is required to maintain activity at high temperatures,

usually 105 °C for few minutes followed by 1–2 h at 90–100 °C, and low pHs, understanding, and increasing α-amylases operational stability has been the focus of considerable amount of research (Dey et al. 2016; Olempska-Beer et al. 2006).

The structural stability of proteins is largely dependent on the refined balance among noncovalent forces or interactions such as ion pairs, van der Waals interactions and H bonds, among others. Weakening of these interactions will result in stability disturbance and consequently in protein unfolding and/or denaturation (Dill 1990; Fitter 2005). Biocatalyst inactivation occurs when protein unfolds becomes irreversible, which is generally attributed to aggregation, misfolding and chemical modifications (Feller 2010). Currently, analytical technics such as circular dichroism (CD) spectroscopy, X-ray crystallography, Fourier transform infrared (FTIR), and nuclear magnetic resonance (NMR) spectroscopies among others are widely applied to unveil protein structure and conformation and the mechanisms that govern it (Moorthy et al. 2015). Comparison of extremophiles proteins with their mesophilic homologues allows the establishment of a link between sequence and function, and therefore, the mechanisms underlying protein rigidity and stabilization are disclosed. It is now acknowledged the contribution of several factors such as: the number of disulphide bonds, a high core hydrophobicity (Gromiha et al. 2013), salt bridge formation (Chakravarty and Varadarajan 2002), ionic interaction (Vogt et al. 1997), metal-binding activity, and ionic interactions, to the overall thermal stability of proteins (Chen and Stites 2004; Yin et al. 2017).

Highly stable α-amylases have been obtained by three preferential routes: (a) bioprospection of microbial extremophiles (thermophiles, hyperthermophiles, halophiles, acidophiles, etc.), (b) genetically engineering of microbial extremophiles and mesophiles, i.e., application of site-directed mutagenesis and directed evolution methodologies, and more recently by (c) metagenomics approaches. α-amylases, and enzymes in general, produced by extremophiles present high stability at extreme temperatures. Thus, these enzymes are particularly relevant for industrial processes that include steps performed at high temperatures such as the case of the production of sweeteners from starch. Among the several microbial species producing enzymes for industrial processing, the *Bacillus* species are particularly relevant because they produce enzymes with remarkable thermostability, are regarded as nonpathogenic and also because efficient expression systems are available (Olempska-Beer et al. 2006; Fitter 2005). Some examples of microbial thermophiles-producing α-amylases with industrial relevant thermal characteristics are presented in Table 9.1. More examples may be found elsewhere (de Souza and Magalhães 2010; Dey et al. 2016; Ajita and Thirupathihalli 2014). On the other hand, fungal α-amylases usually have limited application due to lack of thermal stability; nonetheless, several examples of fungal sources of α-amylases are found in the literature. These are usually confined to terrestrial isolates and mostly belong to *Aspergillus* species and a few to *Penicillium* spp. (Ajita and Thirupathihalli 2014). The following have been more widely applied: *Aspergillus niger* (Wang et al. 2016a), *Aspergillus oryzae* (Porfirif et al. 2016), *Aspergillus awamori* (Karam et al. 2017), *Aspergillus fumigatus* (Pervez et al. 2014), and *Aspergillus terreus*

Table 9.1 Some examples of microbial thermophiles-producing α-amylases with industrial potential

Microorganism	Source	Temperature optimum (°C)	Observations	Kinetic parameters	Reference
B. stearothermophilus	–	70–80	– Enzyme successfully produced in *B. subtilis* and *E. coli* – Purified enzyme presented similar properties irrespective of the production host – Ca^{2+}, Na^{+}, and bovine sérum albumin used for stabilization	K_m = 14 mg/mL	Vihinen and Mäntsälä (1990)
B. stearothermophilus	Soil	50	– Presented a relative activity of 82% at 100 °C – Maintained 90% of initial activity after 1 h incubation at 100 °C – Metal ions did not enhance enzyme activity	–	Chakraborty et al. (2000)
B. stearothermophilus	Potato processing facility	70	– Low starch concentrations and low growth temperatures stimulated enzyme production – Dissolved O_2 concentration in the growth media played a critical role in enzyme production	$t_{1/2}$ = 5.1 h at 80 °C; 2.4 h at 90 °C	Wind et al. (1994)
B. subtilis JS-2004	–	70	– Enzyme is stable when incubated for 1 h at 60 °C – 1 h incubation at 80 and 90 °C resulted in loss of 12 and 48% of initial activity, respectively – Enzyme activity was increased upon incubation with Ca^{2+}	–	Asgher et al. (2007)
Bacillus sp. strain *SMIA-2*	Soil sample	90	– Stable for 1 h at temperatures in the range of 40–50 °C – Loss of 66% of activity upon 1 h incubation at 90 °C – Presence of Ca^{2+} granted stabilization at 90 °C for 30 min	–	Carvalho et al. (2008)

(continued)

Table 9.1 (continued)

Microorganism	Source	Temperature optimum (°C)	Observations	Kinetic parameters	Reference
Geobacillus thermoleovorans	Hot springs	80	– Thermal stability increases in the presence of substrate. That is, 50% activity retained after: (1) 6 h incubation at 70 °C; (2) 2 h incubation at 80 °C – The presence of Ca^{2+} did not impact activity	–	Sudan et al. (2018)
B. amyloliquefaciens BH072	Honey	60	– High hydrolysis rate toward starch from corn, wheat and potato – Enzyme retained roughly 86, 79, 75, and 71% of the initial activity after 42 h incubation at 65, 70, 75, and 80 °C, respectively – Ca^{2+}-independent activity	K_m = 4.27 ± 0.21 mg/mL	Du et al. (2018)
B. licheniformis B4-423	Hot springs	100	– More than 90% of maximum activity in a broad range of temperatures (20–80 °C) – Ca^{2+} independent activity – Organic solvent tolerance and excellent stability in detergents	–	Wu et al. (2018)
Streptomyces fragilis DA7-7	Desert soil	50	– Physical, chemical, and nutrients properties influenced significantly the enzyme production – Presented high stability (above 75%) on the range of 40–80 °C	K_m = 0.624 mU/mg V_{max} = 0.836 mg/mL	Nithya et al. (2017)
Pyrococcus furiosus	Volcanic marine sediments	80–100	– Preserves more than 80% of its initial activity upon incubation for 4 h at 90, 100, and 110 °C – Ca^{2+}-independent activity – Authors reported the expression of the thermostable α-amylases in tobacco plant and in *E. coli*	–	Zhu et al. (2017)

(continued)

Table 9.1 (continued)

Microorganism	Source	Temperature optimum (°C)	Observations	Kinetic parameters	Reference
Anoxybacillus sp. AH1	Hot springs	60	– Enzyme presented increased activity in the presence of Ca^{2+} and Mg^{2+} – Enzyme retained roughly 85% of its initial activity upon incubation at 60 °C	K_m = 0.102 µmol V_{max} = 0.929 µmol/min	Acer et al. (2016)
Geobacillus sp. 4j	Sub-seafloor sediments of "warm pool"	60–65	– Enzyme was successfully overexpressed in *E. coli* BL21 – Production yield increased 22-fold when compared to the native strain	$t_{1/2}$ = 4.25 h at 80 °C	

(Sethi et al. 2016) are some of the sources used for production of α-amylases, employing submerged (SmF), or solid-state fermentation (SSF) methodologies.

Site-directed mutagenesis methodologies are widely used for the construction of de novo enzymes with improved catalytic features. Furthermore, it allows researchers to link enzyme structure with function. In these methodologies, a targeted amino acid located specifically on a known enzyme is modified and the catalytic performance, i.e., kinetic parameters, stability, promiscuity, etc., and production related properties, i.e., solubility, expression, correct folding, etc., of the new enzyme are evaluated (Sindhu et al. 2017). On this process, amino acid selection and 3D structure analysis are crucial criteria to obtain functional catalysts. A recent example of the use of site-directed mutagenesis methodologies on the development of α-amylases with enhanced catalytic performance was presented by Ranjani and coworkers. On this work, α-amylases from *Anoxybacillus* species (ASKA and ADTA) were proposed as part of a novel group of the α-amylases family GH13. Given the high yields obtained in starch hydrolysis, ASKA was chosen as a model system to study the residues that govern the catalytic features. Four residues from the conserved sequence regions were selected and the mutants F113V (CSR-I), Y187F and L189I (CSR-II), and A161D (CSR-V) were characterized. No major changes on optimum reaction temperatures and pHs were found. The most remarkable change was observed for the A161D mutant that exhibits a specific activity, turnover rate (K_{cat}) and catalytic efficiency (K_{cat}/K_m) higher (1.23, 1.17, and 2.88 times, respectively) than the values determined for the wild type (Ranjani et al. 2015). In another recent study, Gai et al. improved the properties of α-amylase from *Bacillus stearothermophilus* (AmyS) by deleting specific arginine and glycine residues. With this approach, the authors were able to increase the thermostability of the produced α-amylase. Additionally, the constructed mutant presented greater acid stability and lower calcium requirements (Gai et al. 2018). Liu et al., managed to improve pH stability of a native α-amylase from *Bacillus licheniformis* by site-directed mutagenesis of two amino acids (Leu134 and Ser320). In order to favor purification, high levels of expression, secretion and stability of the targeted enzyme, protease-deficient strain *Bacillus subtilis* WB600 was used as host. The obtained α-amylase presented an optimum pH of 4.5 and pH stable range of 4.0–6.5, compared to pH 6.5 and 5.5–7.0 of the wild type (Liu et al. 2008).

Directed evolution mimics natural evolution but at a considerable faster pace. The starting point is known enzymes (wild type or engineered) in which, contrary to rational design, random genome mutations are introduced. Following, screening of the mutants for improved or pursued catalytic features is performed. Several cycles of mutation and screening may take place until the targeted traits or level of performance is achieved (Arnold 2018). The use of direct evolution is widely disseminated and, as a result, several examples of enzymes stabilized by these methodologies can be found in the literature (Wintrode and Arnold 2000; Wintrode et al. 2000; Feng et al. 2017; Yuan et al. 2005; Kelly et al. 2009). Regarding the use of directed evolution on the scope of α-amylase feature improvement, Liu and coworkers successfully enhanced the stability of *B. licheniformis* α-amylase under

acid conditions through direct evolution. A favorable mutation site, H281I, was obtained in the targeted enzyme. Considerable increase in specific activity was reported, furthermore, the pH optimum for the mutant decreased roughly 1 unit when compared with the wild type, pH optimum 5.5 and 6.5, respectively (Yuan et al. 2005). Wang et al. proposed a new direct evolution methodology, named combinatorial coevolving-site saturation mutagenesis, in which focused mutant libraries are constructed by choosing the functionally correlated variation sites of proteins as the hotspot sites. Employing this methodology Wang et al. managed to successfully improve the thermal stability of the α-amylase from *B. subtilis* CN7 (AMY7C) by roughly 10 °C (Kelly et al. 2009). Directed evolution coupled with high-throughput automated platforms was used to improve the properties of an α-amylase from *Bacillus* sp. TS-25, now available under the tradename Novamyl. Two error-prone PCR libraries were generated and expressed recombinantly in *B. subtilis*. Screening for mutants with enhanced thermal stability and activity at low pH was performed. When compared to the wild type, several mutants presented an increase in thermal stability superior to 10 °C at pH 4.5 (Jones et al. 2008).

Microorganisms have successfully colonized almost every corner of the earth and represent the largest proportion of biomass on earth, with a total number of bacterial prokaryotic cells estimated to be around 4 to 6 × 10^{30} (Bunge et al. 2014). They have evolved and adapted to a myriad of environments, including some of the most extreme places on earth, i.e., hot springs, acid mine water at pH near zero, deep-sea hydrothermal vents, Antarctic ices (Berini et al. 2017; Mirete et al. 2016). Nonetheless, it is estimated that using standard laboratory techniques less than 1% of the prokaryotic biodiversity is successfully cultured (Culligan et al. 2014). Metagenomics allows to easily bypass the complexities of isolation and cultivation of microorganism, in order to access their exceptional diversity in terms of biocatalysts and bioactive molecules. Metagenomic techniques rely on the direct isolation of genomic DNA from environmental samples often collected at locations where microorganism communities had to adapt to specific conditions of complex ecosystems (Tringe 2005). The sites of collection often correlate with the final application of the biocatalyst. This is because microorganism will carry enzymes that are active and stable under conditions similar to those in the ecosystems where they were collected (Steele et al. 2009). While screening a metagenomic library constructed from marine sediments, Nair et al., discovered an amylolytic clone BTM109. The discovered α-amylase presented moderate tolerance to NaCl, maintaining more than 51% of its initial activity in 2.5 M NaCl. At higher concentration of NaCl, it was observed that Ca^{2+} improved enzyme stability (Nair et al. 2017). A cold-adapted α-amylase, Amyl3C6, was discovered by functional metagenomics methodologies from a metagenomics library of a cold and alkaline ecosystem. Sequence analysis showed similarities with α-amylase from the class *Clostridia*. Optimum temperature was on the range of 10–15 °C, while more than 70% of activity was maintained at 1 °C (Vester et al. 2015). Pooja and coworkers constructed a metagenomics library by cloning cow dung metagenomic DNA fragments into a vector containing a GFP. The library was screened for clones expressing GFP on maltose induction. A periplasmatic α-amylase was discovered

and its expression enhanced by cloning its sequence in *Escherichia coli* Rosetta using T7 expression system (Pooja et al. 2015). On another work, Xu et al. constructed a fecal microbial metagenomic library of the pygmy loris. The screening for amylolytic activity resulted in the discovery of eight recombinant clones. Subcloning and sequence analysis led to the discovery of a novel α-amylase gene that was successfully cloned in *E. coli* BL21 (DE3) (Xu et al. 2014). An extensive list of examples of industrially relevant enzymes recently discovered employing metagenomics methodologies are presented elsewhere (Berini et al. 2017).

Enzyme production for industrial application is a challenging task, and some of the difficulties often met include: (a) native producer strains are difficult to culture under laboratory conditions, (b) production yields are low (c) presence of undesirable enzymes (i.e., proteases that degrade the heterologous protein and/or enzymes that catalyze undesirable reactions) (d) enzyme of interest is produced by pathogenic or toxin-producing microorganism (c) produced enzymes do not present enough stability, activity, or specificity (Olempska-Beer et al. 2006; Adrio and Demain 2010). The advent of recombinant DNA methodologies has revolutionized the application of enzymes for industrial processing by introducing the tools that allow to tackle the aforementioned difficulties. Accordingly, nowadays, over 60% of the enzymes used in detergent, food, and starch processing are of recombinant origin (Adrio and Demain 2010). GRAS labeled microorganisms strains used as hosts for the production of heterologous α-amylases include: *B. subtilis* (Li et al. 2018; Yan and Wu 2017), *B. licheniformis* (Niu et al. 2009), *Bacillus amyloliquefaciens* (Wang et al. 2016b), *Pseudomonas fluorescens* (Landry et al. 2003), *A. niger* (Juge et al. 1998) and *A. oryzae* (He et al. 2017) among others (Olempska-Beer et al. 2006). Usually, the construction of a recombinant production strain involves an expression vector which is a DNA plasmid that contains the expression cassette. The latter is the gene that codes for the enzyme of interest under the control of regulatory sequences. Further details regarding the construction of recombinant production strains can be found elsewhere (Olempska-Beer et al. 2006; Xu et al. 2014). Taking advantage of genetic engineering methodologies, enzyme manufacturers can bring to market biocatalysts with tailored properties. These enzymes are designed to fit the demanding and harsh conditions of industrial processes. Through the application of recombinant DNA methodologies, it is possible to engineer α-amylases with increase thermal and pH stability. Accordingly, Li et al. constructed a recombinant plasmid containing the gene of a thermostable maltogenic amylase (SMMA) and a constitutive promoter, and then transformed into a GRAS *B. subtilis* strain. The obtained recombinant SMMA from *B. subtilis* presented a specific activity two times higher than that obtained from *E. coli* (Adrio and Demain 2010). A hyperthermostable and Ca^{2+}-independent α-amylase from *Pyrococcus furiosus* was expressed successfully in *Nicotiana tabacum*. This plant expression system allowed to overcome the production difficulties overserved when using *E. coli* as host, i.e., the formation of insoluble inclusion bodies (Zhu et al. 2017). In another study, Ghasemi et al. amplified and cloned the hyperthermostable α-amylase gene from *Pyrococcus woesei* into a pTYB2 vector, and *E. coli* BL21 was used as expression host. Activity values of

roughly 185,000 U/L of bacterial culture were obtained (Ghasemi et al. 2015). A putative highly thermostable α-amylase gene of *Thermotoga petrophila* was successfully cloned and expressed in *E. coli* BL21 (DE3) using the expression vector pET-21a(+). The authors managed to optimize the production conditions regarding temperature, pH and induction protocol. The produced enzyme presented remarkable stability at 100 °C and its activity was increase in the presence of Ca^{2+} (Zafar et al. 2016).

9.1.2 Other Common Caloric Sweeteners

Production of pure fructose from starch requires thus a chromatographic step. A more straightforward manner to obtain fructose involves the hydrolysis of inulin, a polyfructan widely available in Jerusalem artichoke tubers, dahlia tubers, and chicory tubers. Hydrolysis of inulin is performed by the action of exoinulinase (EC 3.2.1.80) that sequentially releases fructose units form the terminal endoinulin and of endoinulinase (EC 3.2.1.7) that randomly hydrolyzes internal bonds, thus releasing fructo-oligosaccharides. Free and immobilized enzyme formulations have been used for the hydrolysis of inulin, in solutions with concentrations within 50–150 g/L (Singh et al. 2018).

Invert syrup, resulting from the hydrolysis of sucrose to yield an equimolar mixture of fructose and glucose that is slightly sweeter than sucrose. It is used as sweetener in common beverages. Moreover, due to its hygroscopic nature, invert syrup is used in the manufacture of candies and fondants and. Invertase, in both free and immobilized form, is a sound alternative to acid hydrolysis as it prevents the formation of color compounds and unwanted flavors (Kotwal and Shankar 2009).

9.2 Low-Caloric Sweeteners

9.2.1 Sugar Alcohols

Sugar alcohols (or polyols) are noncyclic hydrogenated carbohydrates, where either an aldehyde or a ketone group is reduced to a hydroxyl group. They convey a varied array of sweetness, albeit not exceeding that of sucrose, impart a cooling effect, have a low-caloric and non-cariogenic nature and a low glycemic index, as they are typically poorly absorbed into the bloodstream from the small intestine. Additionally, sugar alcohols are nonhygroscopic and display high solubility and thermal stability (Park et al. 2016a; Chattopadhyay et al. 2014). Sugar alcohols are thus widely used within the scope of the food industry as additives, namely as sweeteners, but also with humectant, stabilizing, and thickening roles (Chattopadhyay et al. 2014; Grembecka 2015). Catalytic hydrogenation is used for

the industrial production of most of the sugar alcohols currently in the market, namely hydrogenated starch hydrolysates (HSH), lactitol, maltitol, and sorbitol. Mannitol and xylitol are produced either by catalytic hydrogenation or by fermentation, although the former can be also obtained by extraction from seaweeds. Erythritol is produced exclusively by fermentation, mostly due to the high cost of erythrose and only in the production of isomalt, a mixture of gluco-mannitol and gluco-sorbitol, is an enzymatic step involved (Grembecka 2015; Godswill 2017). Thus, α-glucosyltransferase (EC 5.4.99.11), commonly known as sucrose isomerase, promotes the transglycosylation of sucrose into isomaltulose, in a process where trehalulose, glucose, and fructose are also formed as by-products (Fig. 9.2).

The ratio between isomaltulose and trehalulose formed depend on enzyme source and concomitant features of the active site but also on environmental conditions (Ravaud et al. 2009; Xu et al. 2013; Wu and Birch 2005). Isomerization typically occurs in mildly acidic and mesophilic environment ($5.0 < pH < 6.0$ and $20\ °C < T < 40\ °C$) and substrate concentrations within 10–65% (w/w) (Hellmers et al. 2018; Mu et al. 2017; Orsi and Sato 2016; Li et al. 2017; Wu et al. 2017). Food grade sucrose is used as substrate, yet the use of crude raw materials, e.g., cane molasses has been reported (Orsi and Sato 2016; Wu et al. 2017). Isomaltulose can then be converted into sugar alcohol isomalt, again by catalytic hydrogenation (Hellmers et al. 2018; Varzakas and Labropoulos 2012). When implemented on industrial scale, fermentative production of sugar alcohols is preferred over cell-free enzymatic pathways, as these require costly cofactors, e.g., NADH or NADPH (Park et al. 2016a; Godswill 2017). Notwithstanding its intermediate role in the production of isomalt, isomaltulose (Palatinose) has also been increasingly used as food additive, mostly as an alternative to sucrose to yield healthier goods. Thus, albeit with a sweetening power 30–40% that of sucrose, when compared to the later,

Fig. 9.2 Synthesis of isomaltulose from sucrose catalyzed by α-glycosyltransferase. Trehalulose, glucose, and fructose by-products are also formed

isomaltulose has higher bacterial and chemical stability, lower cariogenicity and glycemic index, and slower digestibility, hence making it widely used as sweetener but also as a bulking agent in a wide variety of beverages, confectionary and cereal products (Sawale et al. 2017; Irwin and Sträter 2001). Like isomaltulose, trehalulose also displays non-cariogenic, high solubility, and slow digestion in the small intestine features and it is about 70% as sweet as sucrose. Accordingly, it is used as additive in sweetened foods, such as jams and jellies (Daudé et al. 2012; Desai et al. 2017; Wach et al. 2017).

Sucrose isomerase can be obtained from several microorganisms, yet the most efficient currently is likely that obtained from *Protaminobacter rubrum* CBS 574.77, namely if catalyst efficiency (k_{cat}/K_m) ratio is considered (over one order of magnitude above that of other enzymes), in addition to isomaltulose-to-trehalulose ratio above 22, one of the highest identified (Mu et al. 2017). Accordingly, this sucrose isomerase is used in commercial processes for the preparation of isomaltulose, where conversion close to 100% and yield and selectivity of 85% are reported (Orsi and Sato 2016; Liese et al. 2006). Still, some sucrose isomerases have exhibited virtually no trehalulose production, e.g., sucrose isomerase from *Pantoea dispersa* displayed on the cell surface of *Yarrowia lipolytica* (Li et al. 2017). Oppositely, the sucrose isomerase from *Pseudomonas mesoacidophila* MX-45 produces virtually no isomaltulose (Mu et al. 2017; Nagai et al. 1994). Irrespectively of the enzyme source, due to the relatively high cost/complexity of recovery and purification of the intracellular enzyme, isomerization of sucrose for isomaltulose/trehalulose production is typically carried out using whole cells, either free or immobilized, in both bench and industrial scale (Hellmers et al. 2018; Mu et al. 2017; Orsi and Sato 2016; Li et al. 2017; Wu et al. 2017; Liese et al. 2006).

9.2.2 Rare Sugars

Rare sugars abridge monosaccharides that are seldom found in nature (Mooradian et al. 2017; Manthey and Xu 2010). Their use as sweeteners, bulking and browning agents in food products has become particularly appealing in recent years due to their low, if any, caloric value and the lack of (or at least slow) metabolization in humans and a sweetness that is 70–92% that of sucrose. In addition to these features, rare sugars present no questionable aftertaste (Chattopadhyay et al. 2014; Mooradian et al. 2017; Manthey and Xu 2010). Rare sugars such as D-allose, D-allulose, D-tagatose, and D-talose have been incorporated into health foods and beverages. Moreover, D-allulose and D-tagatose have generally regarded as safe (GRAS) status (Mooradian et al. 2017; Manthey and Xu 2010; Mu et al. 2015).

D-allose

In addition to common features of rare sugars, anticancer, antihypertensive, antioxidative, and immunosuppressive roles have been associated with this *cis*-aldohexose,

which is 80% as sweet as sucrose (Chattopadhyay et al. 2014; Mooradian et al. 2017). D-allose is produced by the reversible isomerization of D-allulose, a reaction that can be catalyzed by several enzymes, namely L-rhamnose isomerase (EC 5.3.1.14), D-ribose-5-phosphateisomerase (EC 5.3.1.6), D-galactose-6-phosphate isomerase (EC 5.3.1.26), and glucose-6-phosphate isomerase (EC 5.3.1.9), from diverse microbial sources (Mu et al. 2015; Zhang et al. 2017; Chen et al. 2018). The isomerization is typically performed in mild acidic environment ($7.0 < pH < 9.0$) and at temperatures within 60°–85°, with conversion yields around 30% (Chen et al. 2018). L-rhamnose isomerase is the preferred enzyme for the isomerization of D-allulose to D-allose, due to the high thermal stability and detailed information gathered (Chen et al. 2018). Accordingly, large-scale biological production of D-allose has been reported to be performed with recombinant rhamnose isomerase from *Pseudomonas stutzeri* expressed in *E. coli*, and immobilized either as a cross-linked aggregate or preferably on Chitopearl beads (Chen et al. 2018; Menavuvu et al. 2006; Morimoto et al. 2006). In the latter case, a continuous operation set-up allowed 30% conversion in D-allose out of a feed D-allulose solution (500 g/L) at 42 °C and pH 9.0 (Morimoto et al. 2006). Recently, D-ribose-5-phosphate isomerase from *Thermotoga maritima* ATCC 43589D-5 and glucose-6-phosphate isomerase from *P. furiosus* have been suggested as potential candidates for large-scale production of D-allose, given their high thermal stability and high specific activity (Chen et al. 2018). As D-allulose is an expensive substrate, efforts have been made to produce D-allose from a cheap substrate such as fructose. The implementation of this approach combines the use of either D-tagatose 3-epimerase or D-allulose 3-epimerase for the first step, where fructose is converted to D-allulose; and either L-rhamnose isomerase or ribose 5-phosphate isomerase to produce D-allose (Lee et al. 2018). Based on this approach, a one-pot, two-reaction system involving D-allulose 3-epimerase and ribose 5-phosphate isomerase has been recently suggested, that allowed the production of D-allose (79 g/L) from 600 g/L fructose solution.

D-Allulose (D-psicose)

This hexose has a sweetness that is 70% that of sucrose. Blood glucose suppressive and neuroprotective effects, as well as anti-obesity and reactive oxygen species scavenging feature have been reported for this sugar (Mooradian et al. 2017; Mu et al. 2015). D-Allulose is obtained from the epimerization of D-fructose using D-tagatose 3-epimerase family members from different microbial sources. Among these enzymes, one of the most effective is from *A. tumefaciens*, although other strains such as *Clostridium bolteae*, *Clostridium cellulolyticum*, *Clostridium scindens*, *Desmospora* sp., *Dorea* sp., *Flavonifractor plautii*, *Rhodobacter sphaeroides*, or *Ruminococcus* sp. have also displayed high catalytic efficiency toward D-allulose production. Hence, these enzymes are usually named D-allulose (psicose) 3-epimerase (EC 5.1.3.30) (Parker et al. 2010). The epimerization typically occurs in mild alkaline environment, temperatures from 40 to 65 °C and substrate concentration up to 750 g/L, with conversion yields that can reach 35%. The use of

(partially) purified enzymes and whole cells, often permeabilized to ease mass transfer, both in free form, is the most often reported biocatalyst formulation (Mu et al. 2015; Park et al. 2016b, c; Mu et al. 2012). *E. coli* cells are often used for heterologous 3-epimerase expression but since generally recognized as safe (GRAS) host, thus limiting the application as food additive of d-allulose produced by such recombinant strains. To overcome this, expression of D-allulose 3-epimerase from *F. plautii* in *Corynebacterium glutamicum* has been assayed and implemented successfully. Thus, 235 g/L D-allulose were produced from 750 g/L D-fructose by the recombinant permeabilized whole cells, resulting in a volumetric productivity of 353 g/L h (Park et al. 2016b). Immobilized D-allulose 3-epimerases have been also used for this isomerization such as those immobilized on Duolite A586 beads (Lim et al. 2009) and on graphene oxide (Dedania et al. 2017). In the former case, continuous operation was performed for 384 h, with a productivity of 606 g/L h. In the latter, the immobilized enzyme formulation was reused over 11 cycles. Throughout this process, the relative activity decreased noticeably, to about 20% of the initial activity at the final cycle, a feature that may condition its wider application.

The production of D-allulose from D-glucose has also been described, involving the combination of xylose isomerase and D-allulose 3-epimerase. This has been implemented either by immobilization of both enzymes in *Saccharomyces cerevisiae* spores (Li et al. 2015) or their co-expression in *E. coli* (Men et al. 2014; Chen et al. 2017). In the former case, a two-step approach, enabling glucose isomerization at 85 °C and fructose isomerization at 60 °C, allowed the conversion of 3 g/L glucose with a final yield of 12%. The strategy enabled each enzyme to operate under the most adequate temperature. The product was obtained at a much lower concentration as compared to a traditional single reaction, fructose-based approach. Still, both immobilized enzymes could be reused in eight cycles with minor loss of activity (Li et al. 2015). The co-expression approach developed by Men and coworkers allowed the production of 135 g/L D-allulose from a mixture of D-glucose (520 g/L) and D-fructose (380 g/L) from high-fructose corn syrup (HFCS), at 65 °C (Men et al. 2014). The approach developed by Chen and coworkers was evaluated over either cellulose hydrolysate, containing 24.5 g/L D-glucose, or microalgae hydrolysate, containing 26.4 g/L D-glucose. Final D-psicose concentrations of 1.42 and 1.69 g/L, respectively, were reported, at 40 °C (Chen et al. 2017). As far as currently known industrial production of D-allulose is still limited to Japan and Korea (Mu et al. 2012).

D-Tagatose

D-tagatose is 92% as sweet as sucrose, has a caloric content half of that of sucrose and low glycemic effect. It is hence used in the prevention of diabetes and tooth care (Mooradian et al. 2017; Beerens et al. 2012). Detailed information on the properties of D-tagatose can be found in a recent review (Jayamuthunagai et al. 2017). Several approaches have been used for the biocatalytic production of D-tagatose, as summarized in Table 9.2 (Jayamuthunagai et al. 2017; Li et al. 2013).

Table 9.2 Biocatalytic production of D-tagatose using a single enzyme

Substrate	Enzyme	Comments
D-altritol D, L-galactitol	Oxidoreductases	Cofactors, typically expensive, required
D-fructose D-sorbose	Epimerases	Substrate specificity required
D-galactose D-talose	Isomerases	By-product formation, purification difficult due to substrate/ product similarities

The most widely used approach, which will be detailed, involves the isomerization of D-galactose to D-tagatose promoted by L-arabinose isomerase (EC 5.3.1.4), from diverse microbial sources (Jayamuthunagai et al. 2017; Li et al. 2013). Thermodynamic equilibrium is shifted to the product side with temperature increase, thus enzymes with high thermal stability are favored, such as those from *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus*, *T. maritima*, and *Thermotoga neapolitana*. Enzymes from those sources enabled operation at 60–95 °C, and conversion yields from 48 to 68% (Jayamuthunagai et al. 2017; Li et al. 2013). However, browning reaction becomes significant when the temperature exceeds 70 °C, with a negative impact in the final product, hence commercial production favors isomerization at temperatures around 60 °C (Mei et al. 2016). The isomerization is typically performed in mild alkaline environment (Jayamuthunagai et al. 2017; Li et al. 2013). The higher D-tagatose production reported so far involved: (a) the use of L-arabinose isomerase from *E. coli* immobilized in calcium alginate and packed into a reactor operating in continuous recycling model, yielding 230 g/L tagatose from 500 g/L galactose, with a productivity of 9.6 g/(L h); (b) the use of L-arabinose isomerase mutant enzyme from *Geobacillus thermodenitrifican*s in the presence of boric acid, that has a higher affinity to D-tagatose than to the substrate. The isomerase produced 370 g/L D-tagatose from 500 g/L D-galactose, corresponding to a productivity of 15.4 g/(L h) (Li et al. 2013; Kim et al. 2003; Lim et al. 2007). Boric acid removal, required to enable the use of tagatose as food additive, was performed with Amberlite IRA-743 and Dowex X50X8 resins (Lim et al. 2007). Most arabinose isomerases require metal cofactors such as Mn^{2+} and Co^{2+} at concentrations of 1–5 mM for improvement of thermal stability and optimal activity. This requirement increases the cost and complexity of the downstream process, to remove (or significantly reduce) the titer of metal ions, as to comply with requirements for food applications. Efforts are hence being made toward the identification/engineering of enzymes that display high stability and activity under negligible metal ion concentration (Jayamuthunagai et al. 2017). A pioneering work involved cloning and overexpression of mutant L-arabinose isomerases from *B. stearothermophilus* US100 in *E. coli* (Rhimi et al. 2009). The addition of metal ions had no effect on

activity and stability of the mutant at temperatures below 65 °C. Moreover, a double mutant displayed optimal activity at pH 6.0–6.5 and at temperatures from 50 to 65 °C, operational conditions that minimize browning reaction. The mutant enzymes displayed optimal conversion rates within 41–47% (Rhimi et al. 2009). A more recent example is the aspartate isomerase of *Bacillus coagulans* NL01 overexpressed in *E. coli*. The recombinant *E. coli* cells displayed optimal pH and temperature of 7.5 and 60 °C, respectively. High activity was observed in the absence of metal ions, and the thermal stability trend at temperatures from 70 to 90 °C remained roughly unchanged irrespective of either the absence or the presence (0.5 mM) of Mn^{2+}. Under pH 7.5 and 60 °C, 70 g/L of D-tagatose were produced from 250 g/L D-galactose corresponding to a productivity of roughly 1.5 g/(L h) (Mei et al. 2016). The L-arabinose isomerase from *Clostridium hylemonae* cloned and expressed in *E. coli* also displayed features that also suggest its potential for large-scale application (Nguyen et al. 2018). Thus, the activity peaked at 50 °C and pH 7.0–7.5, only requiring 1 mM of Mg^{2+} as cofactor, conversion yield was highest at 60 °C. Moreover, the catalytic efficiency 3.691/(mM s) on galactose exceeded that of several other enzymes (Mei et al. 2016; Nguyen et al. 2018).

Recently, a one-pot three-step enzyme cascade was used to produce D-tagatose (144 g/L) from D-fructose (180 g/L). In the first step, hexokinase was used for fructose phosphorylation with equimolar amount of ATP yielding fructose-6-phosphate; fructose-1,6-biphosphate aldolase epimerized fructose-6-phosphate to tagatose-6-phosphate; and finally, phytase fully dephosphorylated the latter, to yield tagatose. Although high-yield conversion was achieved, the process is likely to be economically unfeasible for large-scale application unless external ATP requirements are overcome. One possibility, as suggested by the authors of the work, relies on expression of the pathway in genetically engineered cells expressing the pathway and fulfilling endogenously ATP requirements (Lee et al. 2017).

D-Sorbose

D-sorbose is 70% as sweet as sucrose and besides its role as low-calorie sweetener, it may be used as insect control agent and as a building block for commercially relevant compounds (Mooradian et al. 2017; Li et al. 2013). This hexose was produced from galactitol using resting whole cells of *Pseudomonas* sp. ST 24 under pH 7.5 and temperature 30 °C. A product yield of 70% from 10 g/L galactitol was obtained after 48 h of incubation (Khan et al. 1992). A two-enzyme cascade system was involved in the biotransformation, combining a dehydrogenase, that converts galactitol to D-tagatose, with an epimerase, that transforms D-tagatose into D-sorbose (Khan et al. 1992). The D-tagatose 3-epimerase from *Pseudomonas* ST-24 was later partially purified, immobilized on BCW 2503 Chitopearl beads. This formulation was used for the direct epimerization of 100 g/L D-tagatose to D-sorbose, at pH 7.5 and temperature 30 °C, to form 70 g/L of product after 24 h incubation. This yield

was kept constant for five consecutive batch runs (Itoh et al. 1995). Glucitol, prepared from D-gulono-1,4-1actone, a relatively cheap starting material, by borohydride reduction, was used as a substrate for the synthesis of D-sorbose by oxidation at C-5 position, with resting *Pseudomonas* sp. Ac whole cells with dehydrogenase activity at pH 6.6 and 30 °C. A product yield of 97% out of an initial substrate concentration of 22 g/L was obtained after 35 h incubation. The cells were effectively reused in four consecutive batch runs of 22 h in average, with initial substrate concentration of 10 g/L in average, as product yields exceeding 95% were obtained (Huwig et al. 1996). More recently, a recombinant L-glucitol oxidizing dehydrogenase from *Bradyrhizobium japonicum* USDA 110 was shown to oxidize 18 g/L L-glucitol to 16 g/L D-sorbose in 25 h of incubation, when coupled to a cofactor regeneration system (Gauer et al. 2014).

9.2.3 High-Intensity Sweeteners

High-intensity sweeteners designation abridge a set of compounds that display significantly higher sweetness that sucrose, yet they are noncaloric and non-cariogenic. Overall, these features make high-intensity sweeteners appealing to be used as sweeteners and occasionally as flavor enhancers, so that healthy foods are produced (Mooradian et al. 2017). The most widely disseminated and approved for most foods with GRAS status high-intensity sweeteners are: acesulfame potassium (200 fold sweeter than sucrose); advantame (20.000 fold sweeter than sucrose); aspartame (200 fold sweeter than sucrose); neotame (7.000–13.000 fold sweeter than sucrose); saccharin (200–700 sweeter than sucrose); *Siraitia grosvenorii* (Luo han guo) fruit extracts (100–250 fold sweeter than sucrose); steviol glycosides, that include dulcoside A, rebaudiosides A to E, steviolbioside, and stevioside (200–400 fold sweeter than sucrose); and sucralose (600 fold sweeter than sucrose) (Chattopadhyay et al. 2014; Mooradian et al. 2017; Li et al. 2014; Carocho et al. 2014). Most of these are artificial and chemically synthesized, while stevia glycosides and Luo han guo fruit extracts are natural products.

Aspartame

Besides chemical synthesis, aspartame, N-(L-α-Aspartyl)-L-phenylalanine methyl ester or Asp-Phe-OMe, is produced enzymatically, an approach that is well documented (Liese et al. 2006; Yagasaki and Hashimoto 2008; Pitzer and Steiner 2016). Industrial production relies on the use of thermolysin (EC 3.4.24.27), a heat stable, enantioselective Zn^{2+} protease from *Bacillus thermoproteolyticus*, that catalyzes the condensation of carbobenzoxy-L-aspartic acid (protected amino acid) and L-phenyl-alanine methyl ester to yield carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, a protected precursor of aspartame that is subsequently deprotected by catalytic hydrogenation over Pd catalyst to obtain aspartame. The process is carried out at neutral pH and 50 °C, in aqueous or biphasic media, and precipitation of the

product in salt form has been suggested to shift equilibrium to product formation (Liese et al. 2006; Birrane et al. 2014). Recently, the use of a mutant of organic solvent stable protease PT121 from *Pseudomonas aeruginosa* expressed in *E. coli* was suggested for this process. As the mutant protease retains significant activity at pH 6.0, at which the product significantly precipitates, the condensation reaction can be performed at such pH, to shift the thermodynamic equilibrium to product formation with no need for further action. Condensation in aqueous medium at pH 6.0 and 37 °C led to product yields close to 90% for carbobenzoxy-L-aspartic acid and L-phenyl-alanine methyl ester concentrations of 13.4 and 89.5 g/L, respectively, in 12 h runs. Product yield slightly decreased to 82.2% when the concentration of carbobenzoxy-L-aspartic acid doubled, thus molecular docking studies were developed to highlight the inhibition mechanism of this substrate in product synthesis (Liu et al. 2015). The simplicity of the process and high yields suggest potential application of this protease for large-scale synthesis of aspartame.

Sucralose

Sucralose, 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranosid, is a chlorinated derivative of sucrose, obtained by the replacement replacing the hydroxyl groups in positions 4, 1′ and 6′ with chlorine. Large-scale production relies on chemical synthesis, but efforts have been made involving an enzymatic contribution toward the production of this sweetener (Chattopadhyay et al. 2014). Recent efforts have mostly focused on the synthesis of sucrose 6-acetate from sucrose and, at a minor scale, on the deacetylation of sucralose-6-acetate to sucralose. The synthesis of sucrose-6-acetate from sucrose typically relies on the use of glucose 6-acetate and sucrose as substrates. Fructosyl transferases have been often used to catalyze this reaction, but product yields have been far from satisfactory, in both aqueous and organic environment. The use of ionic liquids as cosolvents has however been shown to improve product yield and limiting substrate conversion of 3.18-fold and 2.90-fold, respectively, as compared to pure aqueous environment. Thus, in the presence of a mixture of 1-decatyl-3-methylimidazolium hexafluorophosphate and Tris–HCl buffer pH 8.0 (20:80, v/v ratio) and a temperature of 55 °C, glucose 6-acetate conversion of 88.2% and sucrose 6-acetate yield of 77.2% were observed after 8 h of reaction, for initial concentrations of sucrose and glucose 6-acetate of 10 and 2.5 g/L, respectively (Wei et al. 2016). Still, the use of ionic liquid in the reaction medium for the production of goods to the food industry may cause some concern (Martins et al. 2017).

Cross-linked aggregates of Lipozyme TL 100 L, based on glutaraldehyde reticulation, were also used for the synthesis of sucrose 6-acetate, with sucrose and vinyl acetate as substrates. A product yield of 87.46% and a product concentration of 49.8 g/L were obtained, for initial sucrose concentrations of 20 and 60 g/L, respectively, and vinyl acetate-to-sucrose mole ratio 8:1. Moreover, the immobilized enzyme could be reused up to eight times (Yang et al. 2012).

Sucralose 6-acetate was effectively converted to sucralose with alginate entrapped *B. subtilis* cells. This green approach which allowed full conversion of

10–300 g/L substrate was observed for incubation periods within 48–250 h. Moreover, the immobilized cells could be used for three consecutive batch bioconversions (Chaubey et al. 2013).

Recently, *B. amyloliquefaciens* WZS01 was shown to allow for the regioselective acylation and deacetylation within the scope of sucralose synthesis. Thus, whole cells immobilized on polyurethane foam were used to synthesize sucrose 6-acetate, with product yield in excess of 95% for initial sucrose and vinyl acetate concentrations of concentration of 20.5 and 60.3 g/L, respectively, after 22 h of reaction. Free cells hydrolyzed 33 g/L sucralose 6-acetate to produce sucralose, with product yield in excess of 99% yield after 24 h of reaction. These results suggest the potential application of these microorganisms in the chemical enzymatic production of sucralose (Sun et al. 2017).

Steviol glycosides

Steviol glycosides are mostly obtained from *Stevia rebaudiana* Bertoni by extraction (Mathur et al. 2017) but as a major component, namely stevioside, display an unpleasant aftertaste, enzyme-catalyzed changes have been developed to overcome this, without altering the remaining properties. These modifications mostly consider C-13 and C-19 positions of stevia glycosides, as these compounds share the same backbone, only differing in the carbohydrate residues attached to those positions (Kochikyan et al. 2006; Adari et al. 2016). For instance, rebaudioside A, the second major steviol glycoside from stevia leaves, displays an additional glucose unit at C-13, that improves its sweetness and taste, as compared to stevioside (Adari et al. 2016; Chranioti et al. 2016). There are several published works from the late 1980s henceforth, where enzymatic transglycosylation is used as a tool to increase the organoleptic properties of steviol glycosides. Most of those works involve enzymes with transglycosylation activity used for related applications, such as α-amylase, β-cyclodextrin glycosyltransferase, dextranase, isomaltase, and pullulanase, coupled with different glycosyl donors, namely cyclodextrins, glucose, lactose, maltose, and partially hydrolyzed starch. These works often aim at increasing the rebaudioside A, but other steviol glycoside derivatives with improved organoleptic properties as compared to stevioside were obtained. Several drawbacks have been associated with these enzymatic processes such as low yields, lack of selectivity of the product, and relatively poor thermal stability of the enzymes (Mathur et al. 2017; Adari et al. 2016). Moreover, it can be established that the glycosyl donor and its ratio to stevioside is casuistic.

Recent examples of the enzymatic modification of stevioside include:

(a) The use of α-amylase from *B. amyloliquefaciens* with starch as donor (Ye et al. 2013). Under optimized conditions, namely pH 6.5, temperature 70 °C, and stevioside and starch concentrations of 20 and 80 g/L, respectively, 38.3% conversion yield was achieved after 12 h. Two major products, accounting for 96% of the final product were identified as 13-{[2-O-(β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy}ent-kaur-16-en-19-oic acid 2-O-(α-D-glucopyranosyl)-β-D-glucopyranosyl ester and 13-{[2-O-(β-D-glucopyranosyl)-β-D-glucopyranosyl]

oxy} ent-kaur-16-en-19-oic acid 2-O-[(4-O-α-D-glucopyranosyl)-α-D-glucopyranosyl]-β-D-glucopyranosyl ester. Challenged for sensory evaluation, the products displayed improved sweetness and decreased aftertaste, as compared to the substrate.

(b) The use of alternansucrase (EC 2.4.1.140) from *Leuconostoc citreum* SK24.002 with sucrose as donor (Musa et al. 2014; Hii et al. 2012). Under optimized reaction conditions of pH 5.4, temperature 20 °C, donor, and stevioside concentrations of 10 g/L, the authors reported a maximum transglycosylation yield of 43.7%. The products were a mixture of mono-, di-, and tri-glycosylated steviosides, yet no sensory evaluation was performed in the study. On the other hand, operation at 20 °C suggests significant energy saving, a positive feature for large-scale operation.

(c) Dextransucrase from *L. citreum* KM20 was used for transglycosylation of stevioside to produce an α-D-glucosyl stevioside (Ko et al. 2016). More specifically, the stevioside molecule was glycosylated at the free hydroxyl group of the glycosyl moiety at the 13-carboxyl group. Under optimized reaction conditions, pH 5.5, temperature 30 °C, 200 g/L stevioside, and a total of 600 g/L sucrose as glycosyl donor were incubated in a 30 L reactor for 5 days to achieve a production yield of 94%. As sucrose hampered transglycosylation and favored the formation of by-products above 171 g/L, the donor was periodically fed to the reaction medium so that the concentration of the donor never exceeded 60 g/L. The organoleptic properties of the glycosyl stevioside were assessed to establish that flavor and sweetness were twofold improved as compared with stevioside but did not match rebaudioside A, being instead similar to those of rebaudioside E. Nevertheless, the stability of the glycosyl stevioside in soft drinks either matched or exceeded that of stevioside and rebaudioside A. Overall, it was suggested that the glycosyl stevioside formed is a potential candidate for use as sweetener in soft drinks.

(d) The use of cellulase for the pretreatment of stevia leaves that releases intracellular glycosyl-transferases present in the leaves. These enzymes catalyze the transglycosylation of stevioside into rebaudioside A (Adari et al. 2016). The pretreatment of the leaves was performed at pH 4.6, 50 °C, at a buffer-to-leaf ratio of 15:1, 10 g/L soluble starch and 10 g/L cellulase. The outcome led to an increase in the rebaudioside A:stevioside ratio to 5.3, as compared to a ratio of 0.11 when untreated leaves were considered. This approach is quite original, as it relies on the use of stevia leaves endogenous transglycosylation enzymes.

(e) A mechanistic approach was developed by Singla and Jaitak to establish the debittering features of rebaudioside A as compared to stevioside (Singla and Jaitak 2016). Through molecular simulation, these authors established that the sugar moiety in the C-3″ position in rebaudioside A limits the access to the bitter taste receptor. The authors then synthesized rebaudioside A from stevioside with β-1,3-glucanase from *Irpex lacteus* and curdlan as glycosyl

donor. Under optimized reaction conditions, pH 4.5, temperature 55 °C, donor-to-stevioside ratio 2:1, and a production yield of 62.5% from 200 mg stevioside was observed after 3 h of incubation. Unfortunately, no data for concentration was given.

(f) The combined application of a recombinant UDP-glucosyltransferase from *S. rebaudiana* and a sucrose synthase from *Arabidopsis thaliana* to produce rebaudioside A from stevioside (Wang et al. 2016c). Under optimal reaction conditions, pH 7.2, temperature 30 °C, 1.9 g/L stevioside, 2.5 g/L sucrose, and 2.4 mg/L UDP, and a product yield of 78% was obtained after 30 h. In this approach, that involves the combined activity of the two enzymes, UDP-glucosyltransferase transfers a glycosyl unit from UDP-glucose to stevioside so that rebaudioside A is formed and UDP is formed. Using sucrose as substrate, sucrose synthase regenerates UDP-glucose with the release of a fructosyl unit, so that minimal amounts of expensive ADP are required. This approach was also used by Li et al. (2016), but using as biocatalyst permeabilized whole cells of *S. cerevisiae*, engineered to express *S. rebaudiana* UDP-glucosyltransferase. In this work, glucose, rather than sucrose, was used for the in vivo regeneration UDP-glucose, through the endogenous UDP-glucose synthesis pathway, where sodium citrate was added to inhibit the action of phosphofructokinase during glycolysis, hence preventing diversion of glucose-1-phosphate into fructose 2,6-bisphosphate. Under optimized conditions, pH 7.2, temperature 30 °C, 40 g/L glucose, 15 g/L sodium citrate and 6 g/L $MgCl_2$, and 1.2 g/L rebaudioside A were produced from 2 g/L stevioside after 48 h, again with no need for exogenous UDP-glucose.

9.2.4 Oligosaccharides

Oligosaccharides are low-calorie sweeteners which have mostly a prebiotic role (Chen 2018). The most common are galacto-, fructo-, and xylo-oligosaccharides, that can be produced either by partial hydrolysis of a polysaccharide, e.g., hydrolysis of inulin with endoinulinases that yield long-chain (2–9 fructosyl residues) fructo-oligosaccharide (Singh et al. 2016); hydrolysis of corncob with xylanases (Boonchuay et al. 2014). Short-chain (2–4 units) fructo-oligosaccharides and galactooligosacharides, on the other hand, can be synthesized using fructosyltransferases or β-D-fructofuranosidase, and sucrose as substrate (Singh et al. 2016; Wang 2015) or β-galactosidase and lactose as substrate (Chen and Gänzle 2017).

References

Acer Ö, Bekler FM, Pirinççioğlu H, Güven RG, Güven K (2016) Purification and characterization of thermostable and detergent-stable α-amylase from *Anoxybacillus* sp. AH1. Food Technol Biotechnol 54:70–77

Adari BR, Alavala S, George SA, Meshram HM, Tiwari AK, Sarma AV (2016) Synthesis of rebaudioside-A by enzymatic transglycosylation of stevioside present in the leaves of *Stevia rebaudiana* Bertoni. Food Chem 200:154–158

Adrio J-L, Demain AL (2010) Recombinant organisms for production of industrial products. Bioeng Bugs 1:116–131

Ajita S, Thirupathihalli M (2014) α-amylase production and applications: a review. J Appl Environ Microbiol 2:166–175

Arnold FH (2018) Directed evolution: bringing new chemistry to life. Angew Chem Int Ed 57:4143–4148

Asgher M, Asad MJ, Rahman SU, Legge RL (2007) A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. J Food Eng 79:950–955

Beerens K, Desmet T, Soetaert W (2012) Enzymes for the biocatalytic production of rare sugars. J Ind Microbiol Biotechnol 39:823–834

Berini F, Casciello C, Marcone GL, Marinelli F (2017) Metagenomics: novel enzymes from non-culturable microbes. FEMS Microbiol Lett 364, fnx211

Birrane G, Bhyravbhatla B, Navia MA (2014) Synthesis of aspartame by thermolysin: a X-ray structural study. ACS Med Chem Lett 5:706–710

Boonchuay P, Techapun C, Seesuriyachan P, Chaiyaso T (2014) Production of xylooligosaccharides from corncob using a crude thermostable endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 and prebiotic properties. Food Sci Biotechnol 23:1515–1523

Bunge J, Willis A, Walsh F (2014) Estimating the number of species in microbial diversity studies. Ann Rev Stat Appl 1:427–445

Carocho M, Morales P, Ferreira ICFR (2014) Sweeteners as food additives in the XXI century: a review of what is known, and what is to come. Food Chem Toxicol 107(Pt A):302–317

Carvalho RV, Côrrea TLR, Silva JCM, de Oliveira Mansur LR, Martins ML (2008) Properties of an amylase from thermophilic *Bacillus* sp. Braz J Microbiol 39:102–107

Chakravarty S, Varadarajan R (2002) Elucidation of factors responsible for enhanced thermal stability of proteins: a structural genomics based study. Biochemistry 41:8152–8161

Chakraborty K, Bhattacharyya BK, Sem SK (2000) Purification and characterization of a thermostable alpha-amylase from *Bacillus stearothermophilus*. Folia Microbiol (Praha) 45:207–210

Chattopadhyay S, Raychaudhuri U, Chakraborty R (2014) Artificial sweeteners—a review. J Food Sci Technol 51:611–621

Chaubey A, Raina C, Parshad R, Rouf A, Gupta P, Taneja SC (2013) Bioconversion of sucralose-6-acetate to sucralose using immobilized microbial cells. J Mol Catal B Enzym 91:81–86

Chen R (2018) Enzyme and microbial technology for synthesis of bioactive oligosaccharides: an update. Appl Microbiol Biotechnol 102:3017–3026

Chen XY, Gänzle MG (2017) Lactose and lactose-derived oligosaccharides: more than prebiotics? Int Dairy J 67:61–72

Chen J, Stites WE (2004) Replacement of staphylococcal nuclease hydrophobic core residues with those from thermophilic homologues indicates packing is improved in some thermostable proteins. J Mol Biol 344:271–280

Chen X, Wang W, Xu J, Yuan Z, Yuan T, Zhang Y, Liang C, He M, Guo Y (2017) Production of D-psicose from D-glucose by co-expression of D-psicose 3-epimerase and xylose isomerase. Enzyme Microb Technol 105:18–23

Chen Z, Chen J, Zhang W, Zhang T, Guang C, Um W (2018) Recent research on the physiological functions, applications, and biotechnological production of D-allose. Appl Microbiol Biotechnol 102:4269–4278

Chranioti C, Chanioti S, Tzia C (2016) Comparison of spray, freeze and oven drying as a means of reducing bitter after taste of steviol glycosides (derived from *Stevia rebaudian* Bertoni plant)-evaluation of the final products. Food Chem 190:1151–1158

Culligan EP, Sleator RD, Marchesi JR, Hill C (2014) Metagenomics and novel gene discovery: promise and potential for novel therapeutics. Virulence 5:399–412

Daudé D, Remaud-Siméon M, André I (2012) Sucrose analogs: an attractive (bio)source for glycol diversification. Nat Prod Rep 29(9):945–960

de Souza PM, Magalhães PO (2010) Application of microbial α-amylase in industry—a review. Braz J Microbiol 41:850–861

Dedania SR, Patel MJ, Patel DM, Akhani RC, Patel DH (2017) Immobilization on graphene oxide improves the thermal stability and bioconversion efficiency of D-psicose 3-epimerase for rare sugar production. Enzyme Microb Technol 107:49–56

Desai SS, Gachhi DB, Hungund BS (2017) Glucose isomerizing enzymes. In: Ray RC, Rosell CM (eds) Microbial enzyme technology in food applications. CRC Press, Boca Raton, pp 69–84

Dey TB, Kumar A, Banerjee R, Chandna P, Kuhad RC (2016) Improvement of microbial α-amylase stability: strategic approaches. Process Biochem 51:1380–1390

DiCosimo R, McAuliffe J, Poulose AJ, Bohlmann G (2013) Industrial use of immobilized enzymes. Chem Soc Rev 42(15):6437–6474

Dill KA (1990) Dominant forces in protein folding. Biochemistry 29:7133–7155

Du R, Song Q, Zhang Q, Zhao F, Kim RC, Zhou Z, Han Y (2018) Purification and characterization of novel thermostable and Ca-independent α-amylase produced by *Bacillus amyloliquefaciens* BH072. Int J Biol Macromol 115:1151–1156

Feller G (2010) Protein stability and enzyme activity at extreme biological temperatures. J Phys Condens Matter 22:323101

Feng H, Wang H-Y, Zhao H-Y (2017) Novel random mutagenesis method for directed evolution. Methods Mol Biol 1498:483–490

Fitter J (2005) Structural and dynamical features contributing to thermostability in α-amylases. Cell Mol Life Sci 62:1925–1937

Gai Y, Chen J, Zhang S, Zhu B, Zhang D (2018) Property improvement of α-amylase from *Bacillus stearothermophilus* by deletion of amino acid residues arginine 179-glycine 180. Food Technol Biotechnol 56:58–64

Gauer S, Wang Z, Otten H, Etienne M, Bjerrum MJ, Lo Leggio L, Walcarius A, Giffhorn F, Kohring GW (2014) An L-glucitol oxidizing dehydrogenase from *Bradyrhizobium japonicum* USDA 110 for production of D-sorbose with enzymatic or electrochemical cofactor regeneration. Appl Microbiol Biotechnol 98:3023–3032

Ghasemi A, Ghafourian S, Vafaei S, Mohebi R, Farzi M, Taherikalani M, Sadeghifard N (2015) Cloning, expression, and purification of hyperthermophile α-amylase from *Pyrococcus woesei*. Osong Public Heal Res Perspect 6:336–340

Godswill AC (2017) Sugar alcohols: chemistry, production, health concerns and nutritional importance of mannitol, sorbitol, xylitol, and erythritol. Int J Adv Res 3:31–66

Grembecka M (2015) Sugar alcohols—their role in the modern world of sweeteners: a review. Eur Food Res Technol 241:1–14

Gromiha MM, Pathak MC, Saraboji K, Ortlund EA, Gaucher EA (2013) Hydrophobic environment is a key factor for the stability of thermophilic proteins. Proteins Struct Funct Bioinf 81:715–721

Gupta R, Gigras P, Mohapatra H, Kumar V, Chauhan GB (2003) Microbial α-amylases: a biotechnological perspective. Process Biochem 38:1599–1616

He L, Mao Y, Zhang L, Wang H, Alias SA, Gao B (2017) Functional expression of a novel α-amylase from Antarctic psychrotolerant fungus for baking industry and its magnetic immobilization. BMC Biotechnol 17:22

Hellmers F, Takors R, Thum O (2018) Robust enzyme immobilizates for industrial isomalt production. Mol Catal 445:293–298

Hii SL, Tan JS, Ling TC, Ariff AB (2012) Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res 2012:921362

Huwig A, Emmel S, Giffhorn F (1996) Preparation of d-sorbose from L-glucitol by bioconversion with *Pseudomonas* sp. Ac. Carbohydr Res 281:183–186

Irwin WE, Sträter PJ (2001) Isomaltulose. In: Nabors LB (ed) Alternative sweeteners, 3rd edn. Marcel Dekker, New York, pp 413–421

Itoh H, Sato T, Takeuchi T, Khan AR, Izumori K (1995) Preparation of D-sorbose from D-tagatose by immobilized D-tagatose 3-epimerase. J Ferment Bioeng 79:184–185

Jayamuthunagai J, Gautam P, Srisowmeya G, Chakravarthy M (2017) Biocatalytic production of D-tagatose: a potential rare sugar with versatile applications. Crit Rev Food Sci Nutr 57:3430–3437

Jones A, Lamsa M, Frandsen T, Spendler T, Harris P, Sloma A, Xu F, Nielsen JB, Cherry JR (2008) Directed evolution of a maltogenic α-amylase from Bacillus sp. TS-25. J Biotechnol 134:325–333

Juge N, Svensson B, Williamson G (1998) Secretion, purification, and characterisation of barley alpha-amylase produced by heterologous gene expression in *Aspergillus niger*. Appl Microbiol Biotechnol 49:385–392

Karam EA, Abdel Wahab WA, Saleh SAA, Hassan ME, Kansoh AL, Esawy MA (2017) Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase. Int J Biol Macromol 102:694–703

Kelly RM, Dijkhuizen L, Leemhuis H (2009) Starch and α-glucan acting enzymes, modulating their properties by directed evolution. J Biotechnol 140:184–193

Khan AR, Takahata S, Okaya H, Tsumura T, Izumori K (1992) "D-sorbose fermentation" from galactitol by *Pseudomonas* sp. ST 24. J Ferment Bioeng 74:149–152

Kim HJ, Ryu SA, Kim P, Oh DK (2003) A feasible enzymatic process for D-tagatose production by an immobilized thermostable L-arabinose isomerase in a packed-bed bioreactor. Biotechnol Prog 19:400–404

Ko JA, Nam SH, Park JY, Wee Y, Kim D, Lee WS, Ryu YB, Kim YM (2016) Synthesis and characterization of glucosyl stevioside using *Leuconostoc dextransucrase*. Food Chem 211:577–582

Kochikyan VT, Markosyan AA, Abelyan LA, Balayan AM, Abelyan VA (2006) Combined enzymatic modification of stevioside and rebaudioside A. Appl Biochem Microbiol 42:31–37

Kotwal SM, Shankar V (2009) Immobilized invertase. Biotechnol Adv 27:311–322

Landry TD, Chew L, Davis JW, Frawley N, Foley HH, Stelman SJ, Thomas J, Wolt J, Hanselman DS (2003) Safety evaluation of an alpha-amylase enzyme preparation derived from the archaeal order *Thermococcales* as expressed in *Pseudomonas fluorescens* biovar I. Regul Toxicol Pharmacol 37:149–168

Lee SH, Hong SH, Kim KR, Oh DK (2017) High-yield production of pure tagatose from fructose by a three-step enzymatic cascade reaction. Biotechnol Lett 39:1141–1148

Lee TE, Shin KC, Oh DK (2018) Biotransformation of fructose to allose by a one-pot reaction using *Flavonifractor plautii* D-allulose 3-epimerase and *Clostridium thermocellum* ribose 5-phosphate isomerase. J Microbiol Biotechnol 28:418–424

Li Z, Gao Y, Nakanishi H, Gao X, Cai L (2013) Biosynthesis of rare hexoses using microorganisms and related enzymes. Beilstein J Org Chem 9:2434–2445

Li C, Lin LM, Sui F, Wang ZM, Huo HR, Dai L, Jiang TL (2014) Chemistry and pharmacology of *Siraitia grosvenorii*: a review. Chin J Nat Med 12(2):89–102

Li Z, Li Y, Duan S, Liu J, Yuan P, Nakanishi H, Gao XD (2015) Bioconversion of D-glucose to D-psicose with immobilized D-xylose isomerase and D-psicose 3-epimerase on *Saccharomyces cerevisiae* spores. J Ind Microbiol Biotechnol 42:1117–1128

Li Y, Li Y, Wang Y, Chen L, Yan M, Chen K, Xu L, Ouyang P (2016) Production of rebaudioside A from stevioside catalyzed by the engineered *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 178:1586–1598

Li L, Wang H, Cheng H, Deng Z (2017) Isomaltulose production by yeast surface display of sucrose isomerase from *Pantoea dispersa* on *Yarrowia lipolytica*. J Funct Foods 32:208–217
Li X, Wang Y, Park J-T, Gu L, Li D (2018) An extremely thermostable maltogenic amylase from *Staphylothermus marinus*: *Bacillus* expression of the gene and its application in genistin glycosylation. Int J Biol Macromol 107:413–417
Liese A, Seelbach K, Buchholz A, Haberland J (2006) Processes. In: Liese A, Seelbach K, Wandrey C (eds) Industrial biotransformations, 2nd edn. Wiley-VCH, Weinheim, pp 147–513
Lim BC, Kim HJ, Oh DK (2007) High production of D-tagatose by the addition of boric acid. Biotechnol Prog 23:824–828
Lim BC, Kim HJ, Oh DK (2009) A stable immobilized D-psicose 3-epimerase for the production of D-psicose in the presence of borate. Process Biochem 44:822–828
Liu Y, Lu F, Li Y, Yin XB, Wang Y, Gao C (2008) Characterisation of mutagenised acid-resistant alpha-amylase expressed in *Bacillus subtilis* WB600. Appl Microbiol Biotechnol 78:85–94
Liu K, Zhu F, Zhu L, Chen G, He B (2015) Highly efficient enzymatic synthesis of Z-aspartame in aqueous medium via in situ product removal. Biochem Eng J 98:63–67
Lloyd NE, Nelson WJ (1984) Glucose- and fructose-containing sweeteners from starch. In: Whistler RL, BeMiller JN, Paschall EF (eds) Starch: chemistry and technology, 2nd edn. Academic Press, Orlando, Florida, pp 611–660
Manthey FA, Xu Y (2010) Glycobiology of foods: food carbohydrates—occurrence, production, food uses, and healthful properties. In: Yildiz F (ed) Advances in food biochemistry. CRC Press Taylor & Francis Group, Boca Raton, pp 25–49
Martins PLG, Braga AR, de Rosso VV (2017) Can ionic liquid solvents be applied in the food industry? Trends Food Sci Technol 66:117–124
Mathur S, Bulchandani N, Parihar S, Shekhawat GS (2017) Critical review on steviol glycosides: pharmacological, toxicological and therapeutic aspects of high potency zero caloric sweetener. Int J Pharmacol 13:916–928
Mei W, Wang L, Zang Y, Zheng Z, Ouyang J (2016) Characterization of an L-arabinose isomerase from *Bacillus coagulans* NL01 and its application for D-tagatose production. BMC Biotechnol 16:55
Men Y, Zhu Y, Zeng Y, Izumori K, Sun Y, Ma Y (2014) Co-expression of D-glucose isomerase and D-psicose 3-epimerase: development of an efficient one-step production of D-psicose. Enzyme Microb Technol 64–65:1–5
Menavuvu BT, Poonperm W, Leang K, Noguchi N, Okada H, Morimoto K, Granstrom TB, Takada G, Izumori K (2006) Efficient biosynthesis of D-allose from D-psicose by cross linked recombinant L-rhamnose Isomerase: separation of product by ethanol crystallization. J Biosci Bioeng 101:340–345
Mirete S, Morgante V, González-Pastor JE (2016) Functional metagenomics of extreme environments. Curr Opin Biotechnol 38:143–149
Mooradian AD, Smith M, Tokuda M (2017) The role of artificial and natural sweeteners in reducing the consumption of table sugar: a narrative review. Clin Nutr ESPEN 18:1–8
Moorthy BS, Iyer LK, Topp EM (2015) Characterizing protein structure, dynamics and conformation in lyophilized solids. Curr Pharm Des 21:5845–5853
Morimoto K, Park CS, Ozaki M, Takeshita K, Shimonishi T, Granström TB, Takata G, Tokuda M, Izumori K (2006) Large scale production of D-allose from D-psicose using continuous bioreactor and separation system. Enzyme Microb Technol 38:855–859
Mu W, Zhang W, Feng Y, Jiang B, Zhou L (2012) Recent advances on applications and biotechnological production of D-psicose. Appl Microbiol Biotechnol 94:1461–1467
Mu W, Yu L, Zhang W, Zhang T, Jiang B (2015) Isomerases for biotransformation of D-hexoses. Appl Microbiol Biotechnol 99(16):6571–6584
Mu W, Li W, Wang X, Zhang T, Jiang B (2017) Current studies on sucrose isomerase and biological isomaltulose production using sucrose isomerase. Food Chem 229:761–768
Musa A, Miao M, Zhang T, Jiang B (2014) Biotransformation of stevioside by *Leuconostoc citreum* SK24.002 alternansucrase acceptor reaction. Food Chem 146:23–29

Nagai Y, Sugitani T, Tsuyuki K (1994) Characterization of alpha glucosyltransferase from *Pseudomonas mesoacidophila* MX-45. Biosci Biotechnol Biochem 58:1789–1793
Nair HP, Vincent H, Puthusseri RM, Bhat SG (2017) Molecular cloning and characterization of a halotolerant α-amylase from marine metagenomic library derived from Arabian Sea sediments. 3 Biotech 7:65
Nguyen TK, Hong MG, Chang PS, Lee BH, Yoo SH (2018) Biochemical properties of L-arabinose isomerase from *Clostridium hylemonae* to produce D-tagatose as a functional sweetener. PLoS ONE 13(4):e0196099
Nithya K, Muthukumar C, Kadaikunnan S, Alharbi NS, Khaled JM, Dhanasekaran D (2017) Purification, characterization, and statistical optimization of a thermostable α-amylase from desert actinobacterium *Streptomyces fragilis* DA7-7. 3 Biotech 7:350
Niu D, Zuo Z, Shi G-Y, Wang Z-X (2009) High yield recombinant thermostable α-amylase production using an improved *Bacillus licheniformis* system. Microb Cell Fact 8:58
Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharmacol 45:144–158
Orsi DC, Sato HH (2016) Isomaltulose production using free and immobilized *Serratia plymuthica* cells. African J Biotechnol 15(20):835–842
Park YC, Oh EJ, Jo JH, Jin YS, Seo JH (2016a) Recent advances in biological production of sugar alcohols. Curr Opin Biotechnol 37:105–113
Park CS, Kim T, Hong SH, Shin KC, Kim KR, Oh DK (2016b) D-allulose production from D-fructose by permeabilized recombinant cells of *Corynebacterium glutamicum* cells expressing D-allulose 3-epimerase *Flavonifractor plautii*. PLoS One 11(7):e0160044
Park CS, Park CS, Shin KC, Oh DK (2016c) Production of D-psicose from D-fructose by whole recombinant cells with high-level expression of D-psicose 3-epimerase from *Agrobacterium tumefaciens*. J Biosci Bioeng 121:186–190
Parker K, Salas M, Nwosu VC (2010) High fructose corn syrup: production, uses and public health concerns. Biotechnol Mol Biol Rev 5(5):71–78
Pervez S, Aman A, Iqbal S, Siddiqui NN, Ul Qader SA (2014) Saccharification and liquefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. BMC Biotechnol 14:49
Pitzer J, Steiner K (2016) Amides in nature and biocatalysis. J Biotechnol 10(235):32–46
Pooja S, Pushpanathan M, Jayashree S, Gunasekaran P, Rajendhran J (2015) Identification of periplasmic α-amlyase from cow dung metagenome by product induced gene expression profiling (Pigex). Indian J Microbiol 55:57–65
Porfirif MC, Milatich EJ, Farruggia BM, Romanini D (2016) Production of alpha-amylase from *Aspergillus oryzae* for several industrial applications in a single step. J Chromatogr B 1022: 87–92
Ranjani V, Janeček Š, Chai KP, Shahir S, Abdul Rahman RN, Chan KG, Goh KM (2015) Protein engineering of selected residues from conserved sequence regions of a novel *Anoxybacillus* α-amylase. Sci Rep 4:5850
Ravaud S, Robert X, Watzlawick H, Haser R, Mattes R, Aghajari N (2009) Structural determinants of product specificity of sucrose isomerases. FEBS Lett 583:1964–1968
Rhimi M, Aghajari N, Juy M, Chouayekh H, Maguin E, Haser R, Bejar S (2009) Rational design of *Bacillus stearothermophilus* US100 L-arabinose isomerase: potential applications for D-tagatose production. Biochimie 91:650–653
Sawale PD, Shendurse AM, Mohan MS, Patil GR (2017) Isomaltulose (Palatinose)—an emerging carbohydrate. Food Biosci 18:46–52
Sethi BK, Jana A, Nanda PK, DasMohapatra PK, Sahoo SL, Patra JK (2016) Production of α-amylase by *Aspergillus terreus* NCFT 4269.10 using pearl millet and its structural characterization. Front Plant Sci 7:639
Sharma A, Satyanarayana T (2013) Microbial acid-stable α-amylases: characteristics, genetic, engineering and applications. Process Biochem 48:201–211

Sindhu R, Binod P, Madhavan A, Beevi US, Mathew AK, Abraham A, Pandey A, Kumar V (2017) Molecular improvements in microbial α-amylases for enhanced stability and catalytic efficiency. Bioresour Technol 245:1740–1748
Singh RS, Singh RP, Kennedy JF (2016) Recent insights in enzymatic synthesis of fructooligosaccharides from inulin. Int J Biol Macromol 85:565–572
Singh RS, Chauhan K, Singh RP (2017) Enzymatic approaches for the synthesis of high-fructose syrup. In: Gahlawat SK, Salar RK, Siwach P, Duhan JS, Kumar S, Kaur P (eds) Plant biotechnology: recent advancements and developments. Springer, Singapore, pp 189–211
Singh RS, Chauhan K, Pandey A, Larroche C (2018) Biocatalytic strategies for the production of high fructose syrup from inulin. Bioresour Technol 260:395–403
Singla R, Jaitak V (2016) Synthesis of rebaudioside A from stevioside and their interaction model with hTAS2R4 bitter taste receptor. Phytochemistry 125:106–111
Steele HL, Jaeger K-E, Daniel R, Streit WR (2009) Advances in recovery of novel biocatalysts from metagenomes. J Mol Microbiol Biotechnol 16:25–37
Sudan SK, Kumar N, Kaur I, Sahni G (2018) Production, purification and characterization of raw starch hydrolyzing thermostable acidic α-amylase from hot springs. India Int J Biol Macromol 117:831–839
Sun J, Chen L, Lou B, Bai Y, Yu X, Zhao M, Wang Z (2017) Acetylation and deacetylation for sucralose preparation by a newly isolated *Bacillus amyloliquefaciens* WZS01. J Biosci Bioeng 123:576–580
Tringe SG (2005) Comparative metagenomics of microbial communities. Science (80-) 308: 554–557
Tufvesson P, Lima-Ramos J, Al Haque N, Gernaey KV, Woodley JM (2013) Advances in the process development of biocatalytic processes. Org Process Res Dev 17:1233–1238
Varzakas T, Labropoulos A (2012) Other sweeteners. In: Varzakas T, Labropoulos A, Anestis S (eds) Sweeteners: nutritional aspects, applications and production technology. CRC Press, Taylor and Francis Group, Boca Raton, USA, pp 175–208
Vester JK, Glaring MA, Stougaard P (2015) An exceptionally cold-adapted alpha-amylase from a metagenomic library of a cold and alkaline environment. Appl Microbiol Biotechnol 99: 717–727
Vihinen M, Mäntsälä P (1990) Characterization of a thermostable *Bacillus stearothermophilus* alpha-amylase. Biotechnol Appl Biochem 12:427–435
Vogt G, Woell S, Argos P (1997) Protein thermal stability, hydrogen bonds, and ion pairs. J Mol Biol 269:631–643
Wach W, Rose T, Klingeberg M, Peters S, Dörr T, Theis S, Kowalczyk J, Hausmann S (2017) Trehalulose-containing composition, its preparation and use. Patent US9744184
Wang T-H (2015) Synthesis of neofructooligosaccharides. Org Chem Insights 5:1–6
Wang S, Lin C, Liu Y, Shen Z, Jeyaseelan J, Qin W (2016a) Characterization of a starch-hydrolyzing α-amylase produced by *Aspergillus niger* WLB42 mutated by ethyl methanesulfonate treatment. Int J Biochem Mol Biol 7:1–10
Wang P, Wang P, Tian J et al (2016b) A new strategy to express the extracellular α-amylase from *Pyrococcus furiosus* in *Bacillus amyloliquefaciens*. Sci Rep 6:22229
Wang Y, Chen L, Li Y, Li Y, Yan M, Chen K, Hao N, Xu L (2016c) Efficient enzymatic production of rebaudioside A from stevioside. Biosci Biotechnol Biochem 80(1):67–73
Wei T, Jia W, Yu X, Mao D (2016) Enzymatic synthesis of sucrose-6-acetate by a novel immobilized fructosyltransferase from *Aspergillus* sp. GX-001. J Mol Catal B: Enzym 123:100–106
Wind RD, Buitelaar RM, Eggink G, Huizing HJ, Dijkhuizen L (1994) Characterization of a new *Bacillus stearothermophilus* isolate: a highly thermostable α-amylase-producing strain. Appl Microbiol Biotechnol 41:155–162
Windish WW, Mhatre NS (1965) Microbial amylases. Adv Appl Microbiol 7:273–304
Wintrode PL, Arnold FH (2000) Temperature adaptation of enzymes: lessons from laboratory evolution. Adv Protein Chem 55:161–225

Wintrode PL, Miyazaki K, Arnold FH (2000) Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. J Biol Chem 275:31635–31640

Wu L, Birch RG (2005) Characterization of the highly efficient sucrose isomerase from *Pantoea dispersa* UQ68J and cloning of the sucrose isomerase gene. Appl Environ Microbiol 71:1581–1590

Wu L, Wu S, Qiu J, Xu C, Li S, Xu H (2017) Green synthesis of isomaltulose from cane molasses by *Bacillus subtilis* WB800-pHA01-palI in a biologic membrane reactor. Food Chem 229: 761–768

Wu X, Wang Y, Tong B, Zhao F, Kim RC, Zhou Z, Han Y (2018) Purification and biochemical characterization of a thermostable and acid-stable alpha-amylase from *Bacillus licheniformis* B4-423. Int J Biol Macromol 109:329–337

Xu Z, Li S, Li J, Li Y, Feng X, Wang R, Xu H, Zhou J (2013) The structural basis of *Erwinia rhapontici* isomaltulose synthase. PLoS ONE 8(9):e74788

Xu B, Yang F, Xiong C, Li J, Tang X, Zhou J, Xie Z, Ding J, Yang Y, Huang Z (2014) Cloning and characterization of a novel α-amylase from a fecal microbial metagenome. J Microbiol Biotechnol 24:447–452

Yagasaki M, Hashimoto S-i (2008) Synthesis and application of dipeptides; current status and perspectives. Appl Microbiol Biotechnol 81:13–22

Yan S, Wu G (2017) Bottleneck in secretion of α-amylase in *Bacillus subtilis*. Microb Cell Fact 16:124

Yang X, Zheng P, Ni Y, Sun Z (2012) Highly efficient biosynthesis of sucrose-6-acetate with cross-linked aggregates of Lipozyme TL 100 L. J Biotechnol 161:27–33

Ye F, Yang R, Hua X, Shen Q, Zhao W, Zhang W (2013) Modification of stevioside using transglucosylation activity of *Bacillus amyloliquefaciens* α-amylase to reduce its bitter aftertaste. LWT–Food Sci Technol 51:524–530

Yin H, Yang Z, Nie X, Li S, Sun X, Gao C, Wang Z, Zhou G, Xu P, Yang C (2017) Functional and cooperative stabilization of a two-metal (Ca, Zn) center in α-amylase derived from *Flavobact eriaceae* species. Sci Rep 7:1–8

Yuan L, Kurek I, English J, Keenan R (2005) Laboratory-directed protein evolution. Microbiol Mol Biol Rev 69:373–392

Zafar A, Aftab MN, ud Din Z, Aftab S, Iqbal I, ul Haq I (2016) Cloning, purification and characterization of a highly thermostable amylase gene of *Thermotoga petrophila* into *Escherichia coli*. Appl Biochem Biotechnol 178:831–848

Zhang W, Zhang T, Jiang B, Um W (2017) Enzymatic approaches to rare sugar production. Biotechnol Adv 35:267–274

Zhu H, Reynolds LB, Menassa R (2017) A hyper-thermostable α-amylase from *Pyrococcus furiosus* accumulates in *Nicotiana tabacum* as functional aggregates. BMC Biotechnol 17:53

Chapter 10
Lipases: A Promising Tool for Food Industry

Sangeeta Negi

Abstract Lipases (triacylglycerol acylhydrolases EC: 3.1.1.3) are universal enzymes, present in all the living creatures, i.e. plants, animals, fungi and bacteria. Their basic function is to catalyze the hydrolysis of lipid into free fatty acid and glycerol at the interface of aqueous and organic solvent, which broadens its applications in various industries. Lipases catalyze a wide range of industrially important reactions: transesterifications, esterifications, interesterifications, etc. and also shows enantio-selectivity due to which they are considered as indispensable tools in food, pharmaceuticals, biofuel, diagnostics, chiral chemistry, drug, detergent, oleochemicals, cosmetics, leather, biosensor industry, etc. The present chapter deals with the production of lipases and their various applications in the food industry such as dairy, bakery, egg processing, oil and fat, flavouring and aroma, meat and fish processing, etc. Various advanced technologies such as metagenomics, directed evolution, genetic engineering, protein engineering, etc. have been discussed to add desired trades in enzymes and to achieve high yield. Light has also been thrown on the key players in global lipase industry and commercially available lipases in the ending notes.

Keywords Lipases · Esterification · Production · Food industry Commercial lipase

10.1 Introduction

Lipolytic enzymes, which encompass lipases (EC 3.1.1.1, triacylglycerol hydrolases) and esterases (EC 3.1.1.3, carboxyl ester hydrolases) belong to one of the industrially important hydrolases family. Lipases are one of the most versatile biocatalysts explored in a wide range of industrially important bioconversions

---

S. Negi (✉)
Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad 211004, India
e-mail: sn5@mnnit.ac.in

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_10

reactions such as hydrolysis, esterification, transesterification, interesterification, acidolysis, alcoholysis and aminolysis, etc. (Fig. 10.1) (Villeneuve et al. 2000). A very important aspect of lipases is that they can act on a wide range of substrates such as triacylglycerides, esters of fatty acids, lipids, synthetic and natural oils, etc. Lipase activity do not require water as sole solvent, therefore, they can catalyze reactions at the interface of organic solvent and water as well as in solvent only, which makes it a versatile tool for chemical industry, fat and oil industry, dairy industry, pharmaceuticals and bakery. Apart from this, its applications are expanding in detergent, leather, organic chemistry, pharmaceutical, oleochemical, cosmetics, perfumes, biosensor, food industries, etc. However, the food industry is widely using lipases making it more profitable and attractive.

The global business of lipase is expected to reach $590.5 Million by 2020, at a CAGR of 6.5% between 2015 and 2020. Asia-Pacific was the biggest and fastest

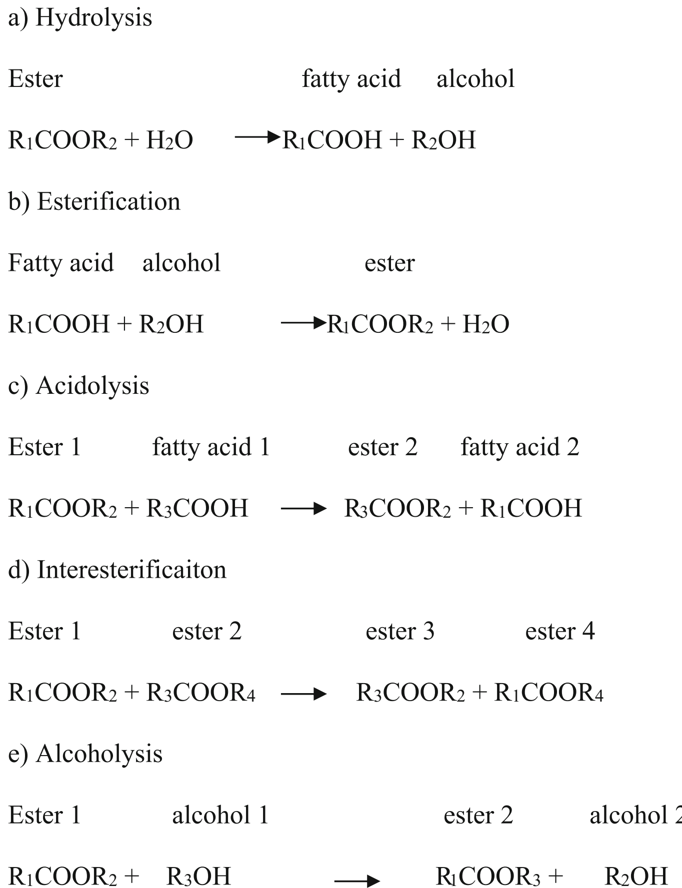


Fig. 10.1 Types of reactions catalyzed by lipases (Villeneuve et al. 2000)

growing market in 2014 for lipases. After the Asia-Pacific, U.S. is the next biggest and fastest growing market for lipase. In future, the market of lipase is expected to grow much faster globally, due to increasing awareness of people about their health, dietary habits, upcoming hi-tech food and beverage industries, etc.

Lipases are omnipresent enzymes and present in bacteria, fungi, animals, plants, yeast, etc. Lipases and esterases catalyze both the hydrolysis and synthesis of ester bonds. They are very pliant in nature, can catalyze reactions and process at wide ranges of pH and temperatures, in presence of low water content or organic solvents (Gupta et al. 2004), which makes it a versatile tool for industries.

Lipases and esterases possess characteristic α/β-hydrolase fold and specific α-helices and β-sheets patterns in their three-dimensional structure (Ollis et al. 1992). Based on amino acid sequences and biological properties, bacterial lipases are classified into eight families (families I–VIII) (Arpigny and Jaeger 1999). Out of eight different families, family I correspond to the 'true' lipases, which is the largest one also and are further divided into six subfamilies. Family I of lipases possesses lid at active site of the enzyme and undergoes interfacial activation. This lid is a hydrophobic domain and gets activated in the presence of small amount of substrate. In active form, it is presented in non-covalent homodimer form and has multiple functional domains of catalytic domain, cofactor, heparin and lipid-binding domains. Their hydrolysis mechanism involves a catalytic triad, which mostly includes a serine as nucleophile, a histidine as base and one acid residue (usually aspartic acid).

10.1.1 Action Mechanism

Lipase-mediated catalysis starts from the formation of enzyme-substrate complex. It is accomplished in three steps: step one is acylation, in which enzyme-substrate complex is formed through covalent bonding performed by proton transfer from aspartate and histidine to OH of serine, which on activation attacks carbonyl group resulting in negative charge on oxygen of carbonyl as an intermediate 'oxyanion'. This oxyanion is stabilized with hydrogen bonding with histidine where serine acts as a nucleophile and aspartate or glutamate as a catalytic acid residue that forms hydrogen bonds with amino acids present at active sites. In the second step, deacylation is initiated by nucleophilic attack of water molecule on enzyme-substrate complex at oxyanion site and fatty acid releases on leaving active enzyme freed for next reaction (Casas-Godoy et al. 2012). They also possess additional catalytic properties like cholesterol esterase, amidase, cutinase, isophospholipase, phospholipase and many more activities (Svendsen 2000).

10.1.2 Sources of Lipases

Lipases are hydrolytic enzyme and remained part of the digestive system of all living beings. Therefore, animal, plant and microorganism all are good sources of lipases. For industrial applications, lipases are sourced mostly from microorganism. The characteristics and specificity of lipases vary from microorganism to microorganism, therefore it has wide applications. In the current era, green and clean chemical processes are in demand and have become the need of the day, therefore, enzymes are in full demand. The demand of microbial, both fungal and bacterial lipases, has multifariously broadened in most of the industries and processes, because of their capability to catalyze processes/reactions in non-aqueous and organic solvent system as well. In the food industry, lipases have become indispensible tool for processing, flavour and taste enhancement, cheese maturation and flavouring, fermented food, fruit processing, dairy products processing, bread baking, confectionaries, etc. Microbial lipases are more advantageous over animal- and plant-originated lipases because they can feed market with high yield at low cost and can act on a broad range of substrates. Microbial lipases can easily be modified into lipases with more applicability and stability through genetic engineering tools. Therefore, mainly, microbial sources have been discussed in this chapter.

Bacterial Sources

Bacterial lipases are not very much explored in the food industry in comparison to fungal lipases because of their lesser specificity towards their substrate and high-temperature sensitivity (thermolabile). However, the bacterial system is more appropriate for lipase production in order to achieve high yield with desirable characteristics. Therefore, bacterial species such as *Pseudomonas, Staphylococcus, Alcaligenes, Achromobacter, Arthrobacter, Chromobacterium* and several others have been explored for the lipase production. However, Gram-negative bacteria, *Pseudomonas* species have been explored more than Gram-positive bacteria (Fatima et al. 2014). In extracellular lipases, translocation mechanism (TAT pathway) is required to reach the location where reaction has to catalyze, for which they have to cross bacterial membranes (Fekkes and Driessen 1999).

Bacterial lipases mostly work at alkaline pH (7–9), however, few bacterial lipases from *Pseudomonas fluorescens* SIK W1, *Bacillus stearothermophilus* SB-1, *Bacillus atrophaeus* SB-2 and *Bacillus licheniformis* SB-3 have shown a broad catalytic range of pH 3–11. However, thermostable *B. licheniformis* MTCC-10498 lipase can work up to pH 14. Few reports are there about thermostable lipases and some bacterial species such as *Bacillus, Chromobacterium, Pseudomonas* and *Staphylococcus* produce lipases which can withstand temperature up to 75 °C in presence of some stabilizers like ethylene glycol, sorbitol, glycerol, etc. (Verma et al. 2012).

Bacterial lipases are lipoprotein or glycoprotein in nature, requires Ca++ as cofactor, therefore have limited application in the food industry. The molecular

weight of bacterial lipase lies in between 30 and 50 kDa and are constitutive in nature (Godfredson 1990).

Fungal Sources

Fungi are more advantageous for the production of lipases because fungal lipases are extracellular in nature, therefore up and downstream processing is easy and cost-effective. Common fungal genera known for good yield of lipases are *Aspergillus, Penicillium, Rhizopus, Geotrichum*, *Humicola lanuginosa*, *Mucor miehei, Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae*, etc. *Aspergillus* species found more potent among others and one is isolated from terrestrial source have been reported with high specificity and thermal, pH stability for wide biotechnological applications (Sharma et al. 2016).

Yeast Sources

Various genera of yeast such as *Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida* and *Torulaspora*, etc. have been used for lipase production (Romo-Sánchez et al. 2010). Some of the natural sources of lipases are listed in Table 10.1.

Table 10.1 Sources of lipases

Microorganism	Source	Application	Reference
Bacillus coagulans	Bacterial	Food processing	Thakur (2012)
Staphylococcus epidermidis	Bacterial	Food industry	Xie et al. (2012)
Staphylococcus haemolyticus	Bacterial	Food industry	Jo et al. (2014)
B. licheniformis	Bacterial	Oil and fat industry	Rashid et al. (2013)
P. fluorescens	Bacterial	Food industry	Patil et al. (2011)
Aspergillus niger	Fungal	Cheese	Custry et al. (2000)
Pecillium chrysogenum	Fungal	Waste cooking oil transformation	Kumar et al. (2012)
Mucor meihei	Fungal	Chess processing	Rathi et al. (2002)
A. nidulans	Fungal	Food industry	Shukla (2014)
A. oryzae	Fungal	Production of mizo, bakery, Cheese	Custry et al. (2000), Abe et al. (2006), Sanchez et al. (2002)
R. miehei	Fungal	Flavour and fragrance	Chang et al. (2003)
R. chinensis	Fungal	Flavoured milk products	Xiao et al. (2015)

(continued)

Table 10.1 (continued)

Microorganism	Source	Application	Reference
Williopsis californica	Yeast	Food	Thakur (2012)
C. rugosa	Yeast	Food processing	Dhake et al.(2013)
T. lanuginosus	Yeast	Food	Cavalcanti-Oliveira et al. (2011)
C. antarctica	Yeast	Food	Kapoor and Gupta (2012), Primozic et al. (2016), Baldessari and Iglesias (2012), Senanayake and Shahidi (2002)
Castor beans	Plant	Vegetable oils	Salaberria et al. (2017)
Almond	Plant	oil	Huang et al. (2017)
Sorghum	Plant	Alcoholic beverages	Moreau et al. (2016)
Coconut	Plant	Coconut oil	Zin et al. (2017)
Oats	Plant	Oats processing	Lampi et al. (2015)

10.2 Production of Lipases

To meet the current and expected market demand of lipases, many approaches have been undertaken for better yield and lipases of wide specificity and stability. All sources of lipase have their own significance with respect to their application. For industrial purposes, mostly, extracellular lipases are in demand.

Yield and properties of extracellular lipases are highly subjective to the physico-chemical parameters such as media/substrate composition (carbon and nitrogen source, inducers, cofactors), incubation temperature, media pH, agitation, K_La, etc. Various physico-chemical conditions for bacterial lipase production have been listed by many scientists.

Production of lipases is inducible, therefore, triacylglycerols, fatty acids, hydrolyzable esters, tweens, bile salts and glycerol are usually added in the media to induce and enhance its production (Bradoo et al. 1999). Other carbon sources such as different forms of carbohydrates, alcohols, polysaccharides, amino acids, whey and many more complex sources impart substantial impact on the yield (Rathi et al. 2001; Ghanem et al. 2000; Rashid et al. 2001). However, presence of compounds similar to the products of lipase-mediated reactions such as olic acid may hinder the yield of lipase due to the effect of product inhibition (Gilbert et al. 1991; Mahler et al. 2000) and in contrast to this, long-chain fatty acids such as oleic, linoleic and linolenic acids enhanced lipase production from *P. mephitica* (Ghosh et al. 1996). Some reports such as Kanwar et al. (2002) support the fact that hydrocarbon such as n-alkane enhanced the production of lipase up to 25 units/ml and with 0.05%

tributyrin in the production medium, production was enhanced about 2.4 fold. In another study, Liu and Tsai (2003) have reported about the production of alkaline lipase from *A. radioresistens* taking *n*-hexadecane and olive oil as the carbon source. However, nitrogen sources also impart great impact on the yield of lipase and many researchers have worked on it (Ghosh et al. 1996). Kumar et al. (2011) reported optimization of lipase production by *Penicillium chrysogenum* using grease waste as substrate through EVOP-factorial design technique (Kumar et al. 2011). Kumar et al. (2014) carried out a comparative study of immobilized lipase produced from *Penicillium chrysogenum* on two different anionic carriers for its pH and thermostability (Kumar et al. 2014).

10.2.1 Heterogeneous Production

Day by day, the demand for enzymes is increasing due to high demand to replace the costly and hazardous chemical processes with eco-friendly and safe enzymatic processes. Due to enhanced awareness among people about the slowly diminishing ecosystems, more efforts have been made to bring it back in its original state and enzymes has been proven one of the best suitable tools to accomplish this. To achieve the diversified roles of enzymes, new technologies such as metagenomics, directed evolution, genetic engineering, protein engineering, etc. are required to be adopted.

(a) **Metagenomics**
In metagenomics, genes are collected from the natural habitat/environmental samples (microorganism), known as metagenome and cloned, then analysed for the presence of desired genome of novel catalyst. The advantage of this technique is that there is no need to culture the microorganism away from natural environment due to which uncultivable microorganism can also be explored.

(b) **Directed Evolution and Advance Protein Engineering**
Directed evolution has emerged as a novel approach in which enzymes are acclimatized and transformed into one that can act in desirable fashion in industry. Directed evolution mimics the processes of Darwinian evolution in a test tube, combining random mutagenesis and/or recombination with screening or selection for enzyme variants that have the desired properties (Kuchner and Arnold 1997). It is very tedious and difficult to screen enzymes of all desired properties from natural diversity and technique like rational protein design also does not work satisfactorily because of being very tedious and time taking. Nature has evolved enzymes according to the demand of environment for survival, therefore, the answer lies in natural evolution and can be implemented in the laboratory. On the other ways, mutation can be carried out at specific regions to improve some specific property.

Bacillus subtilis and *Saccharomyces cerevisiae* have been proved successful host organisms for producing and expressing large functional enzyme mutant libraries. Directed evolution is particularly well suited to 'tuning' enzyme function, i.e. improving an activity that already exists at some, although, at low level.

After manipulating the metagenome and enzymes with desired characteristics its large-scale production/overexpression requires an efficient host system. *Pseudomonas* spp. is known for extracellular enzyme production and is found as a suitable alternative for expression and secretion of enzymes (Jaeger and Rosenau 2004).

To express lipases in *Pseudomonas* species for heterologous production about 30 different cellular proteins are required to get functional enzyme, as folding and secretion process is specific in each organism (Rosenau et al. 2004; Rosenau and Jaeger 2000).

Gram-negative and Gram-positive bacteria use the TAT pathway for translocation of lipase for production (Shruthi et al. 2010). Proteins of this pathway possess characteristic twin-arginine translocation motif in their signal sequence (Tjalsma et al. 2000). Therefore, to express functional enzymes, host like *Pseudomonas* and *Burkholderia* system requires the assistance of a chaperone protein, i.e. lipase-specific foldase (Lif), for accurate folding of the lipase (Quyen et al. 2012; Wu et al. 2012).

Other microbial systems such as *Saccharomyces cerevisiae* and *Pichia pastoris* is also well explored for the production of heterologous lipase of *Rhizopus oryzae* successfully (Valero 2012).

Tremendous work has been done in the field of production of heterologous lipases from the genus *Rhizopus*, which is promising catalysts in lipid modification processes, as they are specific for esters of primary alcohols and acts only at the sn1 and sn3 locations. Lipase gene from Rhizopus oryzae was successfully expressed in Escherichia coli and yielded a correctly folded product, present only in the cytoplasm fraction (Lorenzo et al. 2005).

Sun et al. (2016) have found Thermophilic *Neosartorya fischeri* P1 as an excellent lipase producer and isolated seven lipase genes. All genes were found to be functional after heterologous expression in *E. coli*. Out of all, yield achieved of LIP09 in *Pichia pastoris* was 2.0 g/L in a 3.7-L fermenter, which has shown high activity at 60°C and had broad pH adaptability (Sun et al. 2016).

10.3 Application of Lipases in Food Industry

Enzymes have many industrial applications for day-to-day products used by human being as well as animals. Lipases are one of the important enzymes used for a variety of applications in the food industry. Although, enzymes are used in numerous applications to enhance food quality or to improve the processes from environment angle or to increase the yield, but lipase in food products are mostly

used for fat removal or flavouring. Applications of lipases in the food industry are well established in dairy, bakery, oil, meat and fish processing, beverages, etc.

10.3.1 Dairy

Lipases produced from microbial origins such as fungi (*Rhizomucor meihei*) and lipases produced from animals such as lamb and calf are utilized for flavouring applications in dairy industry. It is used for hydrolyzing the milk fats and give desired flavours to cheeses (Jooyandeh et al. 2009). It is used in dairy industry to alter fatty acid chain lengths to improve the flavours of cheeses. Selection of type of lipases, i.e. animal or microbial lipases, depends on the type of flavours desired. Lipases are also used to modify the structure by interesterification or transesterification to enhance nutritional value of the products. Hydrolysis of the shorter fats by lipases is used for desirable taste in cheeses (Verma et al. 2012).

Enzymatic processes with lipase are used for the production of fat from different edible oils, which can be used as cocoa butter substitute (David 2017). Cocoa butter is formed by triacylglycerides with palmitic, stearic and oleic acids. It melts around 37 °C, thus, has distinctive melting behaviour and mouthfeel because it melts in the mouth (Aravindan et al. 2007). However, its fluctuating supply and prices have led to the development of enzymatic processes to produce fats having similar properties, known as cocoa butter substitutes (CBS). Cocoa butter substitutes are synthesized from various edible oils through interesterification reaction catalyzed by lipases (David 2017).

Lipases are also used for producing human milk fat substitutes. Human milk fat includes—oleic acid (30–35%), palmitic acid (20–30%), linoleic acid (7–14%) and stearic acids (5.7–8%). Lipases have been used to produce human milk fat substitute by sn-1,3 lipase-catalyzed acidolysis of tripalmitin, butterfat, palm oil, palm stearin or lard (rich in palmitic acid in sn-2 position) with free fatty acids (FFA) from different sources (David 2017).

Nowadays, lipases are being commonly used for enhancing cheese and milk flavour and shortening the maturity period of cheese (Aravindan et al. 2007; Maugard et al. 2002). Lipase catalyzes the production of free fatty acids and flavour substances like iso-valeraldehyde, diacetyl, 3-hydroxy butanone, which enhances cheese flavour (Xiao et al. 2017; Ling and Huiping 2005).

10.3.2 Bakery and Confectioneries

Lipases are also used for manufacturing of cocoa butter-equivalent from palm oil (Bloomer et al. 1992), which is used as an ingredient in the production of chocolates and confectionery items. Similar cocoa butter-like fat has been produced from the reaction between cottonseed and olive oils catalyzed by Lipozyme (immobilized

preparation of the *M. miehei* lipase) (Chang 1990). Substrate specificity of lipases from *M. miehei, P. fluorescens* and *C. viscosum* make such triglyceride property modification feasible (Haelck and Spener 1989; Macrae 1984).

Lipases are used in bread dough to hydrolyze triglycerides into diglycerides, monoglycerides and fatty acids. Hydrolysis of triglycerides enhances softness, increases size of bread, its tolerance to over-fermentation and increases shelf life of bread. Lipases are also used along with other enzymes like glucose oxidase to further improve the quality of bakery products and enhance bread texture (Xiao et al. 2017). Monoglyceride content is increased using lipases for partial hydrolysis of triglycerides, which helps in slowing down the staling of bread dough.

Lipase is also used in confectionary to produce emulsifying lipids as an alternative or additive to traditional emulsifiers. This is done by catalyzing the degradation of wheat lipids to produce emulsifying lipids. Like in other food processes, lipase is also used to improve the flavour of confectionary items by liberating short-chain fatty acids through esterification. It is also used to increase the loaf volume and enhance crumb firmness, delay staling, improve softness and texture of bakery items in synergy with other enzymes (i.e. amylases, xylanases) used in bakery (David 2017; Robert 2015).

Lipases are widely used in the chocolate industry to enhance the flavour of toffees, milk chocolate, caramels and buttercreams and also decrease the excess sweetness and improve the buttery character of caramels and toffees.

LipopanF®, a commercial phospholipase from *Fusarium oxysporum* is used in the baking industry. This is marketed by Novozymes A/S (Denmark) and it has phospholipase as well as lipase activities (Raveendran et al. 2018; De Maria et al. 2007).

10.3.3 Food Enrichment

Lipases are used for the production of fat-free meats, monoglycerides such as monolaurin, sugar esters, fatty acyl amino esters (Gandhi 1997; Seino et al. 1984; David et al. 1989; Ota 1990). Sugar acyl esters such as 3-stearoyl D-glucose, are useful as bread-softening agents. Monoglycerides synthesized from octanoic and decanoic acids are used as base material for edible films or edible lubricants. Lipase such as from *P. fluorescens,* are used for the production of glycerides by transesterification (Gandhi 1997; Zaks and Gross 1990). Lipases are also used for synthesis of biosurfactants used for emulsion stabilization in salad dressings (Gandhi 1997; Ota 1990; Nagao 1990). The demand for synthetic fat is increasing as it is a good alternative to a number of high-caloric fats and oils. Synthetic fats have high thermal stability, hence, can be used for high-temperature applications (Gandhi 1997).

10.3.4 Egg Processing

Egg yolk is made of 16% protein, 32% lipids and 50% water. About one-third of the lipids are phospholipids, which is mainly phoshatidylcholine (about 80%). Conversion of egg yolk phospholipids into lyso-phospholipids through enzymes increases the emulsion stability. Phospholipases are used in enhancing the emulsifying power of egg yolk, thus, not only improving the performance of egg lipids but also reducing the requirement of egg yolk rate in food processing such as dressings and mayonnaise-like products. Emulsifying properties of eggs comes from its lipids. Phospholipase hydrolyzes egg lecithin and iso-lecithin. Processing of egg yolk increases the emulsifying capacity as well as temperature stability of the processed egg yolk. So processed egg yolk is used for producing mayonnaises, custards, baby foods, sauces, etc. (Aravindan et al. 2007). As per report published in OCL 24(4), approximately 150,000 metric tons of egg yolk is used globally in emulsified dressings. Annual global production of emulsified dressings is estimated about 3,000,000 metric tons. Eastern European countries and Russia are the main markets with approximately one-third of emulsified dressings being consumed there. Big global brands such as Nestlé, Kraft and Unilever are a major player in this industry (David 2017).

10.3.5 Edible Oil Processing

In the oil industry, lipases are mainly used for hydrolysis, esterification and transesterification. 1,3-oriented lipase is used for transesterification of oils and fats that converts ordinary fats and oils into high-value oils and fats. Refining process of vegetable oils is very crucial which removes impurities, improves the taste, smell, visual appearance and storage stability of the oil. Main impurities are phospholipids. The oil industry has been using thermochemical processes to remove phospholipids impurities, which is also termed as degumming. Phospholipases have provided the oil industry with enzymatic alternative to the thermochemical processes, which are more environment-friendly and energy efficient compared to the thermochemical processes. Triacylglycerol lipase obtained from genetically modified *A. oryzae* has been effectively used for oil degumming (Aravindan et al. 2007). Phospholipases hydrolyses the phopholipids at the water–oil interface, which inhibits the emulsion and releases the oil trapped in the gum phase. Phopholipase assisted processes for removing the impurities is used in oil refining from soyabean, ricebran, palm, sunflower seeds, corn and canola. Enzymatic degumming is reported to increase yield up to 2% and energy efficient as enzyme assisted processes normally operates around 50–60 °C, whereas thermochemical processes operate at about 85 °C. Other advantages of enzyme assisted processes are easier pumping and separation operations, very low oil losses, limited formation of soaps and lower water consumption (David 2017).

Phospholipases differ from each other not only by their cleavage patterns but also by their pH and temperature profiles. Their properties depend on their origin and mode of production. Mostly, the phospholipases from microbial origin are used in the oil industry (David 2017).

Lipophilic antioxidants used in sunflower oil have been synthesized by esterification of functionalized phenols using immobilized lipases from *C. antarctica* (CAL-B), *C. cylindracea* Ay30, *H. lanuginosa*, *Pseudomonas* sp. and *G. candidum* (Aravindan et al. 2007; Buisman et al. 1998). Lipase hydrolyzed soybean oil is used in making Koji, a traditional Asian food (Ang et al. 1999) and lipase from *R. oligosporous* are used to prepare soybean fermented food Tempeh (Gerhartz 1990). With more and more use of enzyme assisted degumming by the oil industry, suppliers are continuously working on research and innovation with an aim to improve the performance such as improved yield, increased stability at higher temperature and lower pH, etc.

Lipases can be used to change the location of fatty acid chains in the glyceride and replacing one or more of these with new ones. By this process properties of lipids can be altered to convert an ordinary lipid to a higher value fat. Lipases are used in the pure form as well as in the immobilized form or even in the cell-bound form.

10.3.6 Flavouring and Aroma

Most of the aromatic and flavouring compounds used in the food industry are synthesized by microbial enzymes. Use of ingredients obtained by natural processes has a significant impact in the food industry. Lipases are used for flavouring and aroma in bakery and confectionary products, dairy products, oils, salad dressing, etc. Lipases are also used in the tea industry to reduce the lipid composition in tea and increase the concentration of polyunsaturated fatty acids, which enhances tea flavour. It is also used in alcohol brewing to give specific aroma.

Lipases are used for the production of esters from short-chain fatty acids, which are used for giving flavour to food products. Level of flavour and fragrance depends on the process parameters such as temperature, pH, amount of lipase and emulsion content (Aravindan et al. 2007).

Metabolism of lactose, fat and protein in dairy products such as cheese, margarine and butter are responsible for the aroma and texture (Gerhartz 1990). Lipase application in these product processing releases free fatty acids, soluble peptides and amino acids, which enhance flavours (Gandhi 1997).

Selection of enzymes is important for developing a particular flavour. For example, cheese flavour development is due to enzymes from *Pencillium roqueforti* (Farahat et al. 1990). Milk fats modified by lipase are used for developing butter/ margarine flavours. Thus, lipase application in flavouring is huge, as it is used right from bakeries for customizing the flavour of cake, cookie, dough, cheese to candy,

chocolate, toffee, creams, coffee whiteners and products like margarines, popcorn oils, salad dressings, sauces, soups, etc.

10.3.7 Meat and Fish Industry

Lipases are used in the meat industry to remove excess fat and produce lean meat. Similarly, in fish industry lipases are used to remove fat. It is also used for fermentation of meat products to enhance its flavour (Xiaolong et al. 2010) and to improve the quality of fermented sausages. Microbial lipases are also used for hydrolysis of fish oil and increasing unsaturated fatty acid (n-3 PUFA) (Xiao 2017).

10.4 Industries and Key Players

The enzymes used in industries are producing from microbes, plants or animals. However, about 90% of the global enzyme is obtained from microbial sources, i.e. bacteria, yeast or fungi. Major producers of the enzymes are Novozymes, DuPont and DSM, which capture more than 75% of the global enzyme market. Novozymes is the biggest player with approximately 45–50% share. Other companies dealing with enzymes are AB Enzymes, BASF, Chr. Hansen, Kerry and Souffle Biotechnologies, all of them are mainly based in Europe. Other than Europe, Japan has also developed enzyme industry with companies like Ajinomoto, Amano, Nagase and Shin Nihon making name for them. Off late enzymes industries in China and India are also picking up. Some of the commercial lipases used in various applications in food processing industries are listed in Table 10.2.

Table 10.2 List of commercial lipases and its applications

Product	Company	Application
LipopanF	Novozymes	Baking
Lipopan®	Nova Nordisk	Baking
Lipozyme®	Nova Nordisk	Oil and fat
Palatase A	Nova Nordisk	Dairy, cheese processing
Lipase G ‘Amano’ 50	Amano Enzymes	Fat and Oil processing
Lipase AY ‘Amano’ 30/30G	Amano Enzymes	Cheese processing
Lipase R ‘Amano’, Lipase A “Amano” 12	Amano Enzymes	Dairy, cheese flavour
Lipase DF ‘Amano’ 15	Amano Enzymes	Fat and oil, Baking
Piccnate,	Gist-Brocades	Cheese processing
Lipolase 100L	Sigma-Aldrich/Novozymes	Removal of fat

(continued)

Table 10.2 (continued)

Product	Company	Application
Rohlase SEP	AB Enzyme	Grain processing
Rohlase PL-Extra	AB Enzyme	Vegetable oil processing
Maxpal A2	Sanovo	Egg processing
Lecitase™Ultra	Sigma-Aldrich/Novozymes	Oil processing
Lecitase Novo	Sigma-Aldrich/Novozymes	Oil degumming

10.5 Conclusion

Although lipases at present have a small share in global enzyme market, it has bright future prospects with innovative applications and products. However, application of lipases in the industry is still hampered by high cost of production, slow pace of research and reluctance of industry to adopt new processes. Industry must develop innovative cost-effective processes to enhance lipase yield and scale up the same. Development of new enzyme with better performance and benefits will increase the demand of lipases in the food industry. Development of new enzymes for oil degumming, customized vegetable oils with better nutritional values, human milk fat substitutes, diet control food and new areas will further drive lipase demand. Innovative immobilization techniques, protein engineering and genetic engineering should be used to improve the functional properties of lipases, which will expand their applications.

References

Abe K, Gomi K, Hasegawa F, Machida M (2006) Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. Mycopathologia 162:143–153

Ang YKWC, Liu KS, Huang YW (1999) Asian foods-science and technology. Technomic Publication Co., Lancaster, Pa, pp 181–183

Aravindan R, Anbumathi P, Viruthagiri T (2007) Lipase applications in food industry. Ind J Biotechnol 6:141–158

Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. Biochem J 343:177–183

Baldessari A, Iglesias LE (2012) Lipases in green chemistry: acylation and alcoholysis on steroids and nucleosides. In: Lipases Phospholipases, pp 457–469

Bloomer S, Adlercreutz P, Mattiasson B (1992) Facile synthesis of fatty acid esters in high yields. Enzyme Microb Technol 14:546–552

Bradoo S, Saxena RK, Gupta R (1999) Two acidothermotolerant lipases from new variants of *Bacillus* sp. World J Microbiol Biotechnol 15:97–102

Buisman GJH, Van-Heltersen CTW, Kramer GFH, Veldsnik JW, Derksen JTP (1998) Enzymatic esterification of functionalized phenols for the synthesis of lipophilic antioxidants. Biotechnol Lett 20:131–136

Casas-Godoy L, Duquesne S, Bordes F, Sandoval G, Marty A (2012) Lipases: an overview. Methods Mol Biol 861:3–30. https://doi.org/10.1007/978-1-61779-600-5_1

Cavalcanti-Oliveira E, da Silva PR, Ramos AP, Aranda DAG, Freire DMG (2011) Study of soybean oil hydrolysis catalyzed by *Thermomyces lanuginosus* lipase and its application to biodiesel production via hydroesterification. Enzyme Res 2011:1–8. https://doi.org/10.4061/2011/618692

Chang MK, Abraham G, John VT (1990) Production of cocoa butter-like fat from interesterification of vegetable oils. J Am Oil Chem Soc 67:832–834

Chang SW, Shaw JF, Shieh CJ (2003) Optimization of enzymatically prepared hexyl butyrate by lipozyme IM-77. Enzymatic synthesis of Hexyl Butyrate. Food Technol Biotech 41(3):237–243

Custry F, Fernander N, Shahani KM (2000) Effect of enzyme association on bread dough performance: a response surface study. Food Sci Technol lnt 6:117–266

David G (2017) Lipases industrial applications: focus on food and agroindustries. OCL 24(4): D403

David MHL, Lemmens HOJ, Gunther H, Roper HW (1989) Surface-active acylated alkylglycosides and their preparation by esterification in presence of an enzyme. European Patent 334:498

De Maria L, Vind J, Oxenbøll KM, Svendsen A, Patkar S (2007) Phospholipases and their industrial applications. Appl Microbiol Biotechnol 74:290–300

Dhake KP, Thakare DD, Bhanage BM (2013) Lipase: a potential biocatalyst for the synthesis of valuable flavour and fragrance ester compounds. Flavour Frag J 28:71–83

Farahat SM, Rabie AM, Faras AA (1990) Evaluation of the proteolytic and lipolytic activity of different *Penicillium roqueforti* strains. Food Chem 36:169–180

Fatima S, Ajmal R, Badr G, Khan RH (2014) Harmful effect of detergents on lipase. Cell Biochem Biophys 70(2):759–763. https://doi.org/10.1007/s12013-014-9978-4

Fekkes P, Driessen AJ (1999) Protein targeting to the bacterial cytoplasmic membrane. Microbiol Mol Biol Rev 63(1):161–173

Gandhi NN (1997) Applications of lipase. JAOCS 74(6)

Gerhartz W (1990) Industrial uses of enzymes. In: Enzymes in industry—production and application, VCH, Weinheim, pp 77–148

Ghanem EH, Al-Sayed HA, Saleh KM (2000) An alkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*. World J Microbiol Biotechnol 16:459–464

Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson S (1996) Microbial lipases: production and applications. Sci Prog 79(2):119–157

Gilbert EJ, Drozd JW, Jones CW (1991) Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginosa* EF2. J Gen Microbiol 137:2215–2221

Godfredson SE (1990) In: Fogarty WM, Kelly ET (eds) Microbial enzymes and biotechnology. Elsevier Applied Science, The Netherlands, pp 255–273

Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 64:763–781

Haelck L, Spener F (1989) Enzyme functionalization for alternate applications of natural fats. DECHEMA Biotech Conf 3:113–116

Huang WC, Chen CY, Wu SJ (2017) Almond skin polyphenol extract inhibits inflammation and promotes lipolysis in differentiated 3T3-L1 adipocytes. J Med Food 20:103–109

Jaeger KE, Rosenau F (2004) Overexpression and secretion of *Pseudomonas* lipases. Pseudomonas 3:491–508

Jo JC, Kim SJ, Kim HK (2014) Transesterification of plant oils using Staphylococcus haemolyticus L62 lipase displayed on Escherichia coli cell surface using the OmpA signal peptide and EstAb8 anchoring motif. Enzyme Microb Technol 67:32–39

Jooyandeh H, Amarjeet K, Minhas KS (2009) Lipases in dairy industry: a review. Food Sci. Technol 46(3):181–189

Kanwar L, Gogoi BK, Goswami PG (2002) Production of a Pseudomonas lipase in n-alkane substrate and its isolation using an improved ammonium sulfate precipitation technique. Bioresour Technol 84(3):207–211

Kapoor M, Gupta MN (2012) Lipase promiscuity and its biochemical applications. Process Biochem 47:555–569

Kuchner O, Arnold FH (1997) Directed evolution of enzyme catalysts. Trends Biotechnol 15 (12):523–530

Kumar S, Katiyar N, Ingle P, Negi S (2011) Optimization of lipase production using grease waste as substrate through EVOP-factorial design technique. Bioresour Technol 102:4909–4912

Kumar S, Mathur A, Singh V, Nandy S, Khare SK, Negi S (2012) Bioremediation of waste cooking oil using a novel lipase produced by *Penicillium chrysogenum* SNP5 grown in solid medium containing waste grease. Bioresour Technol 120:300–304

Kumar S, Yadav RK, Negi S (2014) A comparative study of immobilized lipase produced from Penicillium chrysogenum SNP5 on two different anionic carriers for its pH and thermostability. Ind J Biotechnol 13:301–305

Lampi AM, Damerau A, Li J, Moisio T, Partanen R, Forssell P, Piironen V (2015) Changes in lipids and volatile compounds of oat flours and extrudates during processing and storage. J Cereal Sci 62:102–109

Ling M, Huiping L (2005) The formation of flavouring and its influencing factors in cheese. Dairy Sci Technol 86(4):145–148

Liu IL, Tsai SW (2003) Improvements in lipase production and recovery form Acinetobacter radioresistens in presence of polypropylene powders filled with carbon sources. Appl Biochem Biotechnol 104:129–140

Lorenzo MD, Hidalgo A, Haas M, Bornscheuer UT (2005) Heterologous production of functional forms of *Rhizopus oryzae* lipase in *Escherichia Coli*. Appl Environ Microbiol 71(12):8974–8977

Macrae AR (1984) Microbial lipases as catalysts for interesterification of oils, biotechnol. Oils Fats Ind AOCS Monogr 11:189–198

Mahler GF, Kok RG, Cordenons A, Hellingwerf KJ, Nudel BC (2000) Effects of carbon sources on extracellular lipase production and lipA transcription in Acinetobacter calcoaceticus. J Ind Microbiol Biotechnol 24:25–30

Maugard T, Rejasse B, Legoy MD (2002) Synthesis of water-soluble retinol derivatives by enzymatic method. Biotechnol Prog 18:424–428

Moreau RA, Harron AF, Powell MJ, Hoyt JL (2016) A comparison of the levels of oil, carotenoids, and lipolytic enzyme activities in modern lines and hybrids of grain sorghum. J Am Oil Chem Soc 93:569–573

Nagao A, Kito M (1990) Manufacture of *O*-acylated amino acids as emulsifying agents for foods. Japanese Patent 2,17,156

Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F (1992) The alpha/beta hydrolase fold. Protein Eng 5:197–211

Ota Y, Machida H (1990) Manufacture of sucrose fatty acid esters with lipase, Japanese Patent 2,60,591

Patil KJ, Chopda MZ, Mahajan RT (2011) Lipase biodiversity. Indian J Sci Technol 4:971–982

Primozic M, Kavcic S, Knez Z, Leitgeb M (2016) Enzyme-catalyzed esterification of D, L-lactic acid in different SCF/IL media. J Supercrit Fluids 107:414–421

Quyen TD, Vu CH, Le GT (2012) Enhancing functional production of a chaperone-dependent lipase in Escherichia coli using the dual expression cassette plasmid. Microb Cell Fact 1 (11):29. https://doi.org/10.1186/1475-2859-11-29

Rashid N, Shimada Y, Ezaki S, Atomi H, Imanaka T (2001) Low temperature lipase from psychrotrophic *Pseudomonas* sp. Strain KB700A. Appl Environ Microbiol 67:4064–4069

Rashid FAA, Rahim RA, Ibrahim D, Balan A, Bakar NMA (2013) Purification and properties of thermostable lipase from a thermophilic bacterium, bacillus licheniformis IBRL-CHS2. J Pure Appl Microbiol 7:1635–1645

Rathi P, Saxena RK, Gupta R (2001) A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. Process Biochem 37:187–192

Rathi P, Goswami VK, Sahai V, Gupta R (2002) Statistical medium optimization and production of a hyperthermostable lipase from *Burkholderia cepacia* in a bioreactor. J Appl Microbiol 93:930–936

Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, Madhavan A, Rebello S, Pandey A (2018) Applications of microbial enzymes in food industry. Food Technol Biotechnol 56(1):16–30

Robert H (2015) Lipases in baking. Lipinov. Adebiotech, Romainville, FR

Romo-Sánchez S, Alves-Baffi M, Arévalo-Villena M, Ubeda-Iranzo J, Briones-Pérez A (2010) Yeast biodiversity from oleic ecosystems: study of their biotechnological properties. Food Microbiol 27(4):487–492. https://doi.org/10.1016/j.fm.2009.12.009

Rosenau F, Jaeger K (2000) Bacterial lipases from *Pseudomonas*: regulation of gene expression and mechanisms of secretion. Biochimie 82(11):1023–1032

Rosenau F, Tommassen J, Jaeger KE (2004) Lipase-specific foldases. ChemBioChem 5(2):152–161

Salaberria F, Palla C, Carrin ME (2017) Hydrolytic activity of castor bean powder: effect of gum arabic, lipase and oil concentrations. J Am Oil Chem Soc 94:741–745

Sanchez M, Prim N, Rendez-Gil F, Pastor FIJ, Diaz P (2002) Engineering of bakers yeasts, *Escherichia coli* and *Bacillus* hosts for the production of *Bacillus subtilis* lipase A. Biotechnol Bioeng 78:339–345

Seino H, Uchibori T, Nishitani T, Inamasu S (1984) Enzymatic synthesis of carbohydrate esters of fatty acids (I) esterification of sucrose, glucose, fructose and sorbitol. J Am Oil Chem Soc 61:1761–1765

Senanayake SN, Shahidi F (2002) Lipase-catalyzed incorporation of docosahexaenoic acid (DHA) into borage oil: optimization using response surface methodology. Food Chem 77:115–123

Sharma AK, Sharma V, Saxena J (2016) Isolation and screening of extracellular lipase producing fungi from soil. Am J Pharm Health Res 4(8):38–50

Shruthi H, Babu MM, Sankaran K (2010) TAT-pathway-dependent lipoproteins as a niche-based adaptation in prokaryotes. J Mol Evol 70(4):359–370. https://doi.org/10.1007/s00239-010-9334-2

Shukla AK (2014) Biodiversity in *Aspergillus nidulans* group on the basis of lipases profile. Int J Sci Res 3:1391–1394

Sun Q, Wang H, Zhang H, Luo H, Shi P, Bai Y, Lu F, Yao B, Huang H (2016) Heterologous production of an acidic thermostable lipase with broad-range pH activity from thermophilic fungus *Neosartorya fischeri* P1. J Biosci Bioeng 122(5):539–544. https://doi.org/10.1016/j.jbiosc.2016.05.003

Svendsen A (2000) Review: lipase protein engineering. Biochim Biophys Acta 1543(2):223–238

Thakur S (2012) Lipases, its sources, properties and applications: a review. Int J Sci Eng Res 3:1–29

Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, Van Dijl JM (2000) Signal peptide-dependent protein transport in *Bacillus* subtilis: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64(3):515–547

Valero F (2012) Heterologous expression systems for lipases: a review, vol 861, pp 161–178

Verma N, Thakur S, Bhatt AK (2012a) Microbial lipases: industrial applications and properties (a review). Int Res J Biol 1(8):88–92

Verma N, Thakur S, Bhatt AK (2012) Microbial lipases: industrial applications and properties (a review). Int Res J Biol Sci 1(8):88–92. ISSN 2278-3202

Villeneuve P, Muderhwa JM, Graille J, Haas MJ (2000) Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. J Mol Catal B Enzym 9:113–148

Wu X, You P, Su E, Xu J, Gao B, Wei D (2012) In vivo functional expression of a screened *P. aeruginosa* chaperone-dependent lipase in E. coli. BMC Biotechnol 12:58. https://doi.org/10.1186/1472-6750-12-58

Xiao Z, Hou X, Lyu X, Zhao JY, Xi L, Li J, Lu JR (2015) Enzymatic synthesis of aroma acetoin fatty acid esters by immobilized Candida antarctica lipase B. Biotechnol Lett 37:1671–1677

Xiao F, Li Z, Pan L (2017) Application of microbial lipase and its research progress. Progress Appl Microbiol 8–14

Xiaolong Xiong et al (2010) Lipase catalytic drug synthesis research progress. Chem Biol Eng 27 (8):1–8

Xie W, Khosasih V, Suwanto A, Kim HK (2012) Characterization of lipases from Staphylococcus aureus and Staphylococcus epidermidis isolated from human facial sebaceous skin. J Microbiol Biotechnol 22:84–91

Zaks A, Gross AT (1990) Production of monoglycerides by enzymatic transesterification. World Organization Patent 90, 04,033

Zin NBM, Yusof BM, Oslan SN, Wasoh H, Tan JS, Ariff AB, Halim M (2017) Utilization of acid pre-treated coconut dregs as a substrate for production of detergent compatible lipase by *Bacillus stratosphericus*. AMB Express 7:13. https://doi.org/10.1186/s13568-017-0433-y

Chapter 11
Amylases for Food Applications—Updated Information

Divya Balakrishnan, Swaroop S. Kumar and Shiburaj Sugathan

Abstract Discovering of new industrial applications from microorganisms is diverse as they came from variety of environmental niches. The majority of existing biotechnological applications are of microbial origin and enzymes are the most important among them. Microbial enzymes surpass those from animals and plant sources since their ease of production and genetic manipulation, diverse catalytic activities, etc. The role of enzymes in many processes has been known for a long time, in which the enzymes from microorganisms, used particularly for baking, brewing, alcohol production, cheese making etc. Starch represents one of the most pervasive and an important renewable biological resource that forms a major source of food to a large population. Starch hydrolysis forms the basis of many industrial processes and acid hydrolysis was significant during the earlier days. However, this was almost completely replaced by enzymatic hydrolysis, nowadays, since the availability and abundance of starch hydrolasing microorganisms, corrosion-free reaction, and specificity of the reaction. One of the major applications of these enzymes is in the food industry and starch hydrolysis yields a diverse range of products such as glucose, maltose and fructose syrups, cyclodextrins, fat mimetics substances, etc. They also find application as brewing and baking agents. Enzymatic liquefaction and saccharification of starch require higher temperatures; that demands novel thermostable amylases. In this chapter, we are discussing about various aspects of amylase enzymes, their sources, application in the food industry and future prospects of thermostable amylase from mesophilic organisms, etc.

Keywords Starch · Amylases · Glycoside hydrolase · Starch liquefaction Saccharification

D. Balakrishnan · S. Sugathan (✉)
Division of Microbiology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695562, Kerala, India
e-mail: drshiburaj@gmail.com

S. S. Kumar
Department of Biotechnology and Microbiology, Kannur University, Thalassery Campus, Kannur 670661, Kerala, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_11

11.1 Introduction

Amylolytic enzymes also known as amylases act on starch and related oligo/polysaccharides. The history of amylases was began with the discovery of the first starch degrading enzyme by Kirchhoff in 1811. Followed by this, many other digestive amylases and malt amylases were discovered in succession. Amylases are significant enzymes for their specific use not only in the industrial starch conversion but also in other commercial process. Last century witnessed the emergence of large-scale starch processing industries. For the last few decades, a change from starch acid hydrolysis toward its enzymatic hydrolysis for the production of modified starch and other products such as glucose and fructose syrups, dextrins, etc., has been noticed. Roughly about 30% of the global enzyme production is constituted by these starch-hydrolyzing enzymes. Apart from starch hydrolysis, these enzymes find their applications in industries such as food industry, detergent industry, paper and textile industry, etc. Amylases are one of the most widely distributed enzymes and it is also one of the most well-studied enzymes. Microbial amylases have potential benefit over chemical starch hydrolysis such as specificity and corrosion less mechanism. This is especially useful in the production of products with distinct qualities.

Prior to going in detail about amylases and their applications, let us go through the features of its natural substrate starch. As mentioned before, starch is one of the predominant naturally occurring carbohydrates on earth apart from cellulose. Potatoes, cassava, maize (corn), rice, and wheat are important starch-containing crops and are important constituents for the human diet and a large proportion of the world's population consume it as the major food (Van Der Maarel et al. 2002; Myat and Ryn 2013). Starch is frequently isolated and is used in food industries to impart the desirable functional properties, and to modify food texture and consistency (Kuttigounder et al. 2011). Apart from the usage of starchy plant material directly as food, starch is harvested and used as such or chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, and cyclodextrins (El-Fallal et al. 2012). Starch is widely used in the laundry sizing of fine fabrics in textile industry, skin cosmetics in cosmetic industry and for enhancing the paper strength and printing properties in paper industry. Amylolytic enzymes de-polymerize the starch, which forms the basis of these industries (Vaidya et al. 2015). Starch is also considered as a substrate in fuel production by enzymatic processes (Vengadaramana 2013), and the sugars produced can be fermented to produce ethanol.

Commercially, starch can be extracted in pure form from a variety of natural sources. Maize is the predominant source, but wheat, rice, potato, and sago also have significant contributions for the commercial production (Vengadaramana 2013). Native starch is a semicrystalline material synthesized as roughly spherical granules in plant tissues. It is a white, tasteless, and odorless powder that is insoluble in cold water or alcohol. Starch has been used as stiffening or thickening agent while dissolved in warm water. The origin of the word "starch" came from a

Middle English word "sterchen" which means to stiffen. The Greek word "amulon" means "not ground at a mill" from which the Latin word "Amylum" was derived meaning starch. Pure starch is a heterogeneous polysaccharide consisting predominantly of α-glucan in the form of amylose and amylopectin. These two polymers have different structure and physical properties (Mojsov 2012). Starch is a polymer formed of D-glucose units linked together by glycosidic bonds. At alkaline pH, the glycosidic linkage is stable, however, at acidic pH, the bond become instable resulting in hydrolysis (Aiyer 2005). Acid hydrolysis was routinely used for conversion of starch earlier.

The amylose and amylopectin molecules are polymers of α-D-glucose units but are fundamentally different. Amylose is usually a linear unbranched chain of glucose units ranging from 500 to 20,000 in number and it chiefly contains α-(1–4) linkage (approximately 99%). However, a small number of α-(1–6) branches (approximately 1%) and linked phosphate groups are also found. Due to the peculiar molecular shape and structure, amylose is not stable in aqueous solution. However, it has a considerable viscosity in alkaline solutions due to its molecular shape. The average amylose content in starch is 20–25% depending on the source (Amoozegar et al. 2003).

Amylopectin is a branched polymer consisting of linear chains of 10–60 glucose units linked by α(1–4) glycosidic bond and side chains of 15–45 glucose units linked by α(1–6) glycosidic bond. In this molecule, the branching points are roughly about 5%. However, it may vary depending on the source (Thompson 2000). Amylopectin is one among the largest molecule occurring in nature comprising of about 2,000,000 glucose units (Vengadaramana 2013). Side chains of amylopectin are ordered on the longer backbone to form a clustered appearance (Thompson 2000; Bertoft 2007). In aqueous solution, amylopectins are relatively stable due to branched molecules and are not able to form compact aggregates. Starch contains nearly 75–80% amylopectin depending on the source (Amoozegar et al. 2003).

11.2 Amylolytic Enzymes

As discussed above, starch is an important energy source for animals, plants, and microorganisms and its hydrolysis are achieved by α-glycoside hydrolases. Since these cleaves starch (amylose and amylopectin), they are called amylolytic enzymes often indicated as amylases. Amylolytic enzymes are the most widely distributed enzymes, making its presence in all three domains of life, i.e., Bacteria, Archaea, and Eucarya. Best known and characterized among the amylolytic enzymes are α-amylase, β-amylase and glucoamyase. Though they are similar in their functionalities, they show significant dissimilarity in structure and reaction mechanism owing to the larger evolutionary distance between them (Janecek 1994). Thus, they form their own independent glycoside hydrolase (GH) families such as α-amylases forming GH family 13, whereas β-amylases constituting GH family 14 and glucoamylases forming GH family 14 (Henrissat and Bairoch 1993).

11.2.1 α-Amylase Glycoside Hydrolase Family 13

α-amylases along with related enzymes constitute the GH family 13. Two related families GH family 70 and 77 are considered under α-amylases (Stam et al. 2006). These enzymes retain the α-configuration after hydrolysis. Apart from hydrolases belonging to class 3 (EC 3), the family also containing transferases and isomerases from classes 2 and 5, thus indicating that all the members of the family not necessarily cleave the glycoside bonds in starch. However, they exhibit certain common features thus clustering them in the same family (Horvathova et al. 2001). These features includes (i) they should contain same conserved regions (sequence similarity), (ii) catalytic centre should contain Asp, Glu and Asp at β strands (i) they have ability to act on α-glucosidic linkages; (iii) should hydrolyse or form by transglycosylation of α-glucosidic linkages; (v) should contain four conserved regions and (v) catalytic centre should contain Asp, Glu and Asp residues like Asp206, Glu230 and Asp297 of Taka-amylase A (Janecek 2002). Binding of substrate to the catalytic site makes the breakage of the glycoside links possible (Vaidya et al. 2015). Taka-amylase A, from *Aspergillus oryzae* was the first α-amylase enzyme to get structurally resolved (Matsuura et al. 1984). One of the most important characteristics of α-amylase family is its catalytic domain formed of (α/β) 8-barrel. C-terminal end of the β-barrel in (α/β) 8-structure constitute the active center. C-terminal ends of the three β-strands (β_4, β_5, and β_7) carries catalytic triad, i.e., Asp206, Glu230, and Asp297. The distance between carboxylate group and the catalytic triad ranges from 5 to 7 Å (Matsuura 2002).

Apart from α-amylase, few other enzymes such as isoamylase, pullanase, amylomaltase, and cyclodextrin glycosyltransferase also belong to this family (Horvathova et al. 2001). However, the glucosyltransferases and alternansucrase were placed under the GH family 70 as it is assumed that $(\beta/\alpha)_8$ barrel of the enzyme shows a slight variation from the α-amylases (MacGregor et al. 1996; Argüello-Morales et al. 2000). GH family 77 carries amylomaltases which exhibits low sequence similarities with α-amylases. However, they retained $(\alpha/\beta)_8$ barrel and catalytic triad (Asp-Glu-Asp) exclusive to α-amylases (Przylas et al. 2000). Three of the related α-amylases families together constitute clan GH-H (Stam et al. 2006). All these enzymes are α-retaining enzymes thus the resulting hydroxyl group retains the α-configuration. The reaction mechanism is double displacement two-step mechanism where covalent glycosil enzyme intermediate will be formed. One of the carboxylic acid residues acted as nucleophile and act upon the anomeric center of sugar to form glycosil-enzyme intermediate. Second carboxyl residue meanwhile acts as acid–base catalyst. In the first step, they protonate glycosidic oxygen and in the second step they protonate water. Figure 11.1 represents the double displacement retaining mechanism of α-amylases. During the reaction, an oxocarbenium ion-like transition state will also be achieved (square brackets) (Horvathova et al. 2001).

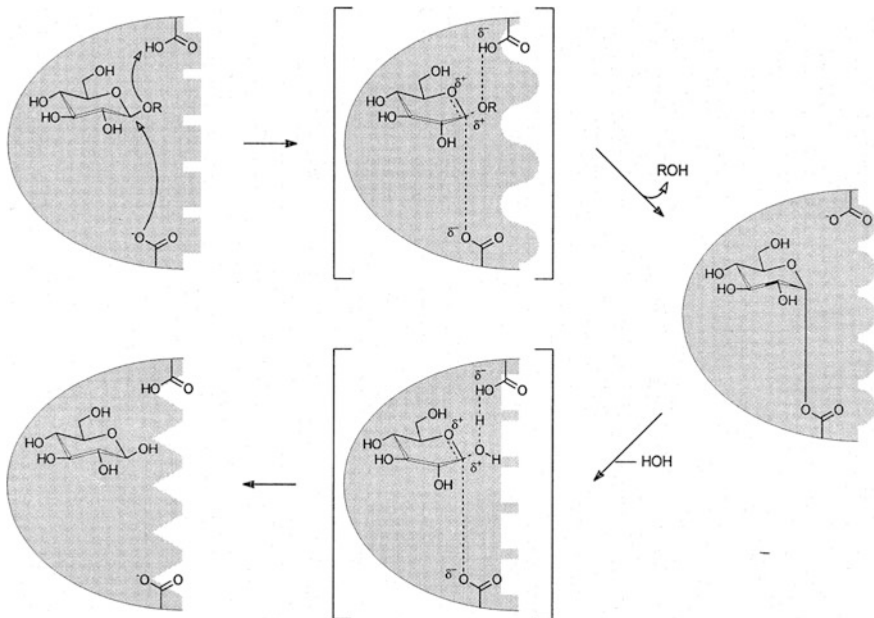


Fig. 11.1 Double displacement retaining mechanism of α-amylases (Horvathova et al. 2001)

11.2.1.1 α-Amylase

The α-amylases (1,4-α-D-glucan glucanohydrolases) acts upon 1,4-α-D-glucosidic bonds to cleave them, however, they cannot act upon 1,6-α-D-glucosidic branch points. These enzymes are endo-acting enzymes and are also called endo-amylases (Naidu and Saranraj 2013). Starch gets hydrolyzed to low molecular weight units such as maltotriose, maltose, and glucose by α-amylases (Mobini-Dehkordi and Javan 2012). Based on the degree of hydrolysis, the α-amylases can be categorized into two. (i) Saccharifying α-amylases that make 50–60% hydrolysis of starch. (ii) Liquefying α-amylases that could achieve about 30–40% starch hydrolysis. α-amylases are present in seeds with starch as reserve food and also microorganisms (Tiwari et al. 2015). Microbial amylases will be discussed below. Even though α-amylases are endo-acting, they are nonspecific and act randomly during hydrolysis. This yield many low molecular weight products such as maltose, maltotriose, and dextrins. Since these enzymes can act at random sites, α-amylases tends to be faster than β-amylase in enzymatic reaction (Liu et al. 2010) (Fig. 11.2).

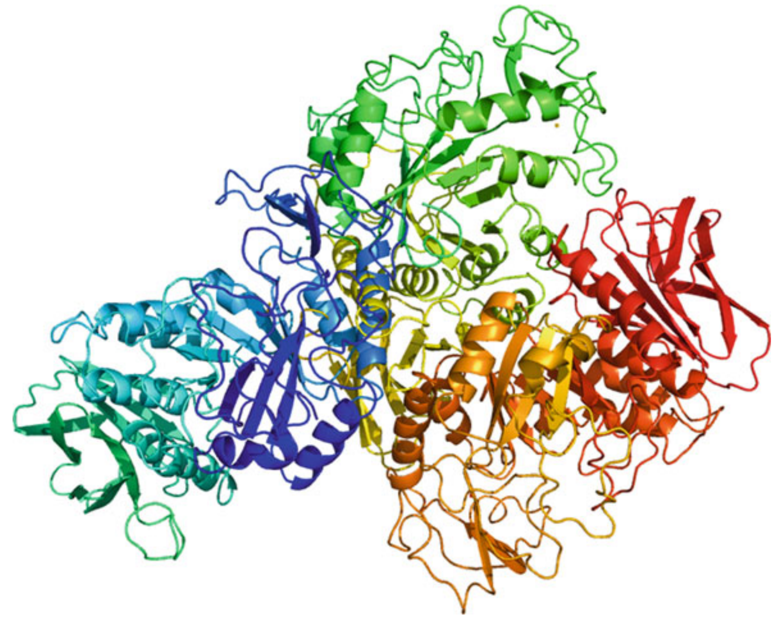


Fig. 11.2 Homology Model of soyabean α-amylase (pdb id: 2TAA) (Matsuura et al. 1984)

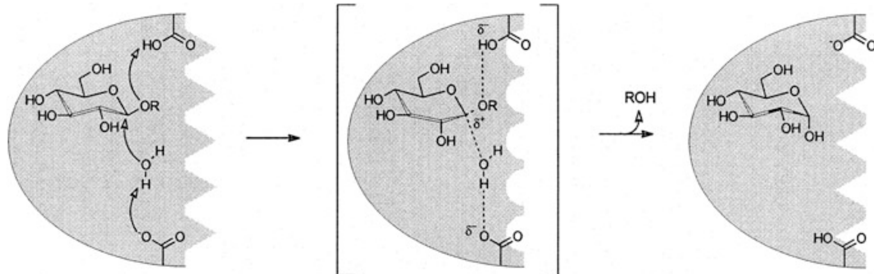


Fig. 11.3 Direct displacement inverting mechanism of β and γ-amylases (adopted from Horvathova et al. 2001)

11.2.2 GH Families 14 and 15: (β-Amylase and Glucoamylase)

Members of these two families show a similar reaction mechanism, although they are structurally distinct from each other. On the other hand, these enzymes have a different catalytic mechanism when compared to that of α-amylases. They exhibit an inverting reaction mechanism by which the configuration of the resulting hydroxyl group will be changed to β-configuration. The catalytic mechanism involves a simple direct displacement mechanism (Fig. 11.4). One of the carboxylic

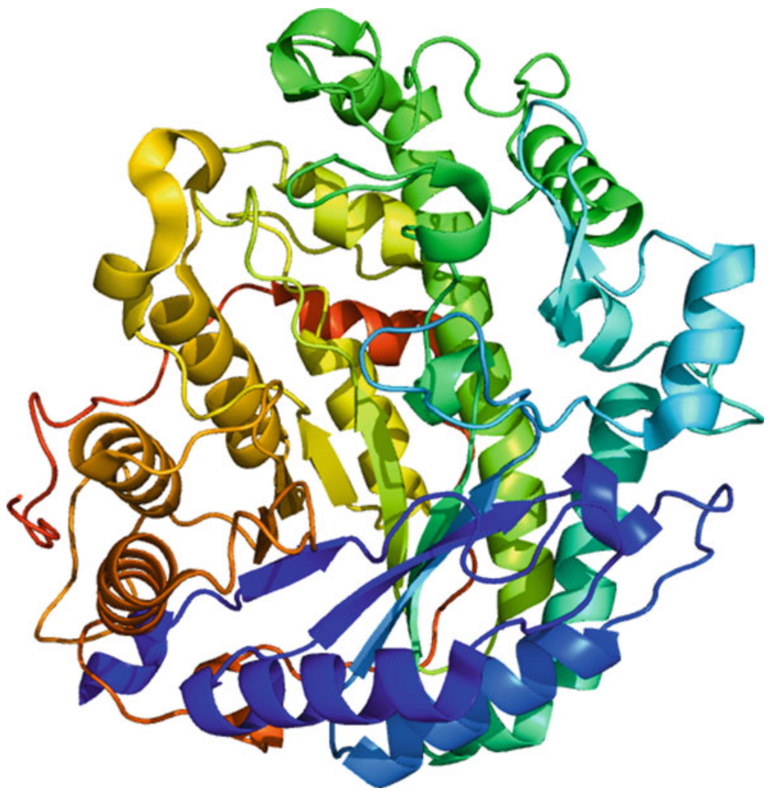


Fig. 11.4 Crystal structure of soyabean β-amylase mutant (pdb id: 1Q6G) (Hirata et al. 2004)

acid residues at the catalytic site acts as the base during the attack of water. Meanwhile, the second active site residue acts as the general acid during the cleavage of glycosidic bond. Here also, during the reaction an oxocarbenium ion-like transition state will be achieved (square brackets) (Horvathova et al. 2001). Two of the well-known members of these families are discussed below (Fig. 11.3).

11.2.2.1 GH Family 14/β-Amylase (EC 3.2.1.2)

β-amylase (1,4-D-glucan maltohydrolase) have wider distribution, produced by bacteria, fungi, and plants. However, animal tissue does not contain β-Amylase, but microflora of the gastrointestinal tract may possess these enzymes. They are also known as glycogenase or saccharogen amylase, and cleaves second α-(1,4) linkages at nonreducing end leaving maltose as the end product. Maltose formed has β-configuration in glucose units. Hence, they are β-amylase. They are also called exo-acting enzymes (Horvathova et al. 2001). Soybean β-amylase was first crystallized structure of β-amylase to be resolved (Mikami et al. 1994). They also carry a

TIM-barrel [$(\beta/\alpha)_8$-barrel] structure like α-amylase, however, they both are differing from each other (Horvathova et al. 2001). Catalytic domain of β-amylase carries two Glu residues (Mikami et al. 1994; Totsuka and Fukazawa 1996). The sweetness of ripened fruit is imparted by maltose formed by β-amylase. Seeds usually contain β-amylase apart from α-amylase. However, β-amylase occurs in inactive form before seed germination. Malt is produced by amylase in cereal grain (Kaplan and Guy 2004).

11.2.2.2 GH Family 15/Glucoamylase/γ-Amylase

γ-amylase is also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase, or 1,4-α-D-glucanglucohydrolase. They are generally multi-domain enzymes structurally very distinct from both α-amylases and β-amylases and consisting of a catalytic domain folded as a twisted $(\alpha/\alpha)_6$ barrel with a central funnel-shaped active site connected to a starch binding domain (Aleshin et al. 1992; Sevèík et al. 1998) Glucan 1,4-α-glucosidase acts on both α-(1–6) glycosidic linkages and terminal α(1–4) glycosidic linkages of starch molecules to yield glucose as the end product. Here also, catalysis is characterized by two Glu residues (Harris et al. 1993). Most of the γ-amylases are active at acidic pH, however, the search of newer and improved γ-amylases with ability to act on higher pH is in demand (Kumar and Satyanarayana 2009) (Fig. 11.5).

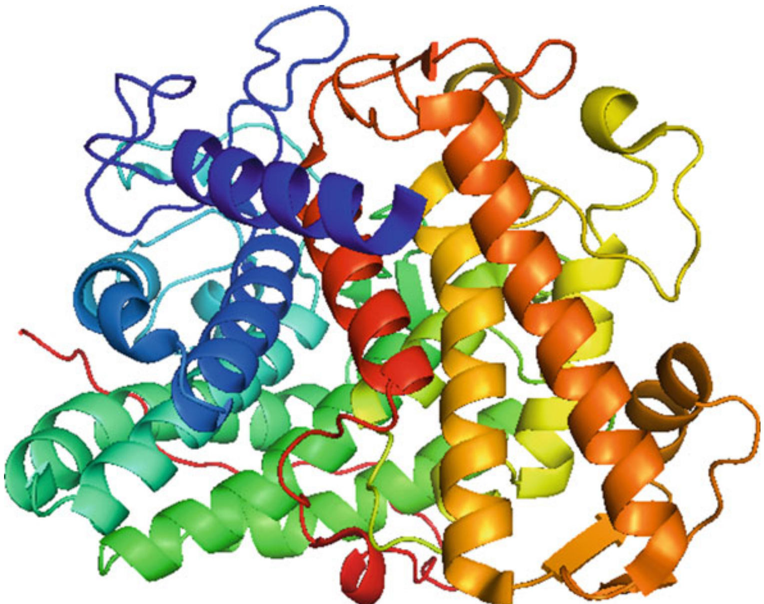


Fig. 11.5 Homology Model of glucoamylase from *aspergillus niger* (pdb id: 3EQA) (Hirata et al. 2004)

11.2.3 Types of Amylases

Another classification of amylases is based on the manner in which the glycosidic bond is attacked. Accordingly, starch-hydrolyzing enzymes are grouped into four such as (i) endo-amylases; (ii) exo-amylases; (iii) de-branching enzymes; and (iv) transferases which are used to hydrolyze starches particularly for the production of dextrins and glucose (Marc et al. 2002).

11.2.3.1 Endo-amylases

Endo-amylases are able to cleave α-(1–4) glycosidic bonds present in the inner part (endo) of the amylose or amylopectin chain. α-amylase is a well-known endo-amylase and characteristics were discussed above.

11.2.3.2 Exo-Amylases

The exo-amylases, either exclusively cleave α(1–4) glycosidic bonds such as β-amylase (EC 3.2.1.2) or cleave both α-(1–4) and α-(1–6) glycosidic bonds like amyloglucosidase or glucoamylase and α-glucosidase. These enzymes act upon the terminal glucose residues of starch molecules and thereby yielding maltose and limit dextrin (e.g., β-amylase) or glucose (glucoamylase and α-glucosidase) (Pandey et al. 2000). Two of the members of this group β-amylase and γ-amylase have already been discussed above.

11.2.3.3 The De-branching Enzymes

De-branching enzymes act on branching points and hydrolyze α-(1–6) glycosidic bonds. Few of the examples are pullulanase enzymes and isoamylase. Both act on amylopectin and hydrolyze to form linear polysachharides. Apart from that pullulanase could cleave α-(1–6) glycosidic bond in pullulan. These de-branching enzymes along with other amylolytic enzymes have been proved significant in the food industry, especially during saccharification process (Hii et al. 2012).

11.2.3.4 Transferases

Transferases not only hydrolyze the glycosidic linkage but they also transfer the glycoside bonds. They act by the cleavage of α-(1–4) glycosidic bond of donor molecule and transfer fragments of them to glycosidic acceptor molecule. Few of the members are amylomaltase and cyclodextrin glycosyltransferase that forms new

α-(1–4) glycosidic bond. However, new α-(1–6) glycosidic bond will be established by branching enzymes. They form cyclic molecule such as cyclodextrin (Horvathova et al. 2001).

11.3 Sources of Amylases

11.3.1 Bacterial Amylases

Bacteria are one of the major sources of amylase enzyme. Bacterial enzymes are more preferred since its ease of production, and genetic manipulation. Among bacterial amylolytic enzyme producers, *Bacillus* sp. is a predominant group and most of the commercially applied enzymes are obtained from the genus. Few of the industrially significant producers are *Bacillus licheniformis, Bacillus amyloliquefaciens,* and *Bacillus stearothermophilus* (Konsoula and Liakopoulou-Kyriakides 2007; Pandey et al. 2000). Among α-amylases from these strains, *B. licheniformis* was turned to be the most stable enzyme (Weemaes et al. 1996). *B. licheniformis* AI20 was reported with thermostable α-amylase of molecular weight 55 kDa (Abdel-Fattah et al. 2012). *Bacillus subtilis* 168 (1A1) is another bacterial amylase producer yielding a 55 kDa amylolytic enzyme with an optimum activity at 37 °C (Sumrin et al. 2011).

Amylases are preferably metal ion-dependent enzymes and few of them include divalent cations like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc. (Pandey et al. 2000). One or more Ca^{2+} has been reported near the active site of most of the α-amylases and is required for proper folding and stability of the enzyme making it vital for enzyme activity (Linden et al. 2002; Prakash and Jaiswal, 2010). However, the secondary calcium binding sites of amylases are assumed to be linked with thermostability. α-amylase of *B. amyloliquefaciens* with 17 Ca^{2+} showed stability toward thermal and surfactant denaturation (Saboury et al. 2005). An increase in enzyme activity was shown by α-amylase from *Bacillus* sp. ANT-6, whereas, the Zn^{2+} showed decremented activity (Burhan et al. 2003). However, few of the other enzymes showed calcium independency. *B. thermooleovorans* NP54 produced a thermostable α-amylase which showed calcium independency (Malhotra et al. 2000). Another calcium-deficient amylase was reported from *Bacillus* WN11 (Pandey et al. 2000).

Halophiles are also known to produces enzymes suiting the industrial needs where process occur at extreme conditions such as highly saline solutions (de Lourdes Moreno et al. 2013). Most of the halobacterial enzymes are assumed to be thermostable and can be stored at room temperature for longer periods. Halophilic amylases were reported from halophilic bacteria such as *Halo arculahispanica* and *Thalassobacillus sp. LY18* (Hutcheon et al. 2005; Li and Yu 2012). Alkaline and thermotolerant amylases were produced by *B. licheniformis* and *Bacillus halodurans* (Setyorini et al. 2006). Thermostable amylase of *Bacillus* sp. from salt farm showed maximum hydrolysis at a temperature of 110 °C and pH 8.0 (Pancha et al. 2010).

Archaea such as *Pyrococcus furiosus* and *Thermococcus kodakarensis* also produces amylase enzyme that is highly thermostable. *P. furiosus* yielded a 66 kDa amylase which showed significant thermal stability at 100 °C (Laderman et al. 1993). Amylase derived from *T. kodakarensis* was having a molecular weight of 80 kDa. The enzyme showed optimum activity at 95–100 °C and pH 3.5. Even though it was more active at acidic pH, the enzyme retained most of its activity in alkaline pH also (Ahmad et al. 2014). The bacterial amylase enzyme with its characteristics is shown in Table 11.1.

11.3.2 Actinobacterial Amylases

Streptomyces sp. is another important microbial producer of amylolytic enzymes. *Streptomyces erumpens* MTCC 7317 produced a thermostable α-amylase of molecular weight 54.5 kDa which is Ca^{2+} deficient (Kar and Ray 2008). Another amylolytic enzyme was reported from *Streptomyces* sp. MSC702 and enzyme derived was stable in presence of metal ions such as K^{+}, Co^{2+}, and Mo^{2+} and showed maximum stability at a temperature of 60 °C (Singh et al. 2014). *Streptomyces gulbargensis* DAS 131 produced amylase of molecular weight 55 kDa. The optimum pH of the enzyme was about 9.0 and optimum temperature was about 55 °C (Syed et al. 2009). *Streptomyces* strain A3, *Streptomyces avermitilis*, etc., were few among the other producers from the genus (Chakraborty et al. 2012; Hwang et al. 2013). *Streptomyces megasporus* produced thermostable amylase which showed optimum activity at about 85 °C (Dey and Agarwal 1999). *Streptomyces fragilis* DA7-7 a desert isolate showed thermostable amylase enzyme production that was stable up to 85 °C (Nithya et al. 2017). Amylases from actinomyces is shown in Table 11.2.

11.3.3 Fungal Amylases

Fungus represents another important microbial producer of amylase enzymes. The advantage of solid-state fermentation and economical production of enzyme makes fungus a better choice. Moreover the agro-industrial residues rich in starch undergo bioconversion upon solid-state fermentation using fungi. *Aspergillus* sp. is a known fungal producer of amylolytic enzymes. *Aspergillus niger* produced a 43 kDa amylase which showed enhanced the activity in presence of Ca^{2+} and Co (Varalakshmi et al. 2009), whereas *Aspergillus flavus* F2Mbb yielded amylase enzyme of molecular mass 56 kDa (Sidkey et al. 2011). A thermotolerant amylase was isolated from *A. penicillioides* which showed optimum activity at 80 °C. The enzyme was stabilized in presence of $CaCl_2$, while $ZnCl_2$, $FeCl_2$ and EDTA turned

Table 11.1 Bacterial amylolytic enzyme and properties

Organism	Mol wt (kDa)	pH	Temp/ optimal stability	Inhibitor	Stabilizer	Additional properties	References
Bacillus cereus GA6	55	9.0	22 ± 1 °C	Fe^{2+}, Zn^{2+}, H_2O_2 $CuSO_4$	Ca^{2+}	Km and *V*max value—0.27 mg/ml and 2600 U/ml	Roohi et al. (2013)
Bacillus sp. KR11	–	6.0	65	Hg^{2+}, Mg^{2+}, Ca^{2+}	–	–	Karmakar and Ray (2011)
Bacillus sp. MNJ23	70	9.0	50	Mg^{2+}, Na ions	–	–	Kumar et al. (2012)
B. subtilis (MTCC 121)	31	7.1	40	–	–	–	Raul et al. (2014).
B. subtilis KIBGE HAS	56	7.5	50	–	–	Km and *V*max value—2.68 mg/ml and 1773 U/ml	Bano et al. (2011)
B. subtilis	–	6.0	60	Co^{2+}, Hg^{2+}, and Cd^{2+}	Ca^{2+} and Mg^{2+}		Ozdemir et al. (2011)
Corallococcus sp. strain EGB	43	8.0	50	Ca^{2+}	–	Km and *V*max recombinant value—6.61 mg ml^{-1} and 44 301.5 μmol min^{-1} mg^{-1}	Li et al. (2015)
B. amyloliquefaciens	42	7.0	55	$FeCl_3$, SDS, EDTA	$CaCl_2$, $MnCl_2$	–	Rai and Solanki (2014)
B. subtilis (ATCC6633)	–	7.0	–	–	–	–	Maity et al. (2015)
B. thuringiensis subsp. *kurstaki*	51	6.0	60 °C	EDTA and SDS	Ag^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Mo^{2+}	Km and *V*max value—2.9 mg/ml and 0.053 μmol/min/ml	Smitha et al. (2015)
Bacillus sp. KR-8104	59	3.5	70 °C	No effect on Ca ions and EDTA	–	–	Sajedi et al. (2005)

(continued)

Table 11.1 (continued)

Organism	Mol wt (kDa)	pH	Temp/optimal stability	Inhibitor	Stabilizer	Additional properties	References
B. licheniformis Isolate AI20	55	7.0	70 °C	EDTA, EGTA and SDS	–	Km and *V*max value—454 U/mg and 0.709 mg/ml	Abdel-Fattah et al. (2012)
Thermoactinomyces vulgaris	135	6–7	50 °C	Ca ions	–	–	El-Sayed et al. (2013)
Bacillus subtilis	59	7.0	37 °C	–	–	Improved on treating N-methyl-N nitro-N-nitrosoguanidine	Bukhari and Rehman (2015)
B. subtilis	52	7.0	70 °C	EDTA	Cu^{2+}, Zn^{2+} and Ni^{3+}		Pavithra et al. (2014)
Bacillus sp. SI-136	26	10	80 °C	–	Mn^{2+}	–	Sarethy et al. (2012)
Anoxybacillus sp. AH1	85	7.0	60 °C	Zn^{2+} and Cu^{2+}	Mg^{2+} and Ca^{2+}	Km and *Vmax* values—0.102 µmol and 0.929 µmol/min	–
Lactobacillus plantarum MTCC 1407	75	–	–	–	–	–	Acer et al. (2016)
Anoxybacillus flavithermus	60	7.0	55 °C	–	–	Km: 0.005 mM and *V*max: 3.5 µmol min(−1)	Fincan et al. (2014)
Corynebacterium alkanolyticum ATH3	28	8.0	45 °C	–	–	–	Banerjee et al. (2016)
B. licheniformis B4-423	58	5.0	100 °C	–	–	–	Wu et al. (2018)
B. amyloliquefaciens BH072	68	6.0	60 °C	–	–	Km and *V*max 4.27 mg/ml and 987.34 U/mg	Du et al. (2018)

(continued)

Table 11.1 (continued)

Organism	Mol wt (kDa)	pH	Temp/ optimal stability	Inhibitor	Stabilizer	Additional properties	References
Anoxybacillus sp. YIM 342	68	9.0	80 °C	–	–	Km and *V*max 4.18 mg/ml and 7.48 μmol/min/mg	Zhang et al. (2016)
Exiguobacterium sp.	54	8.5	45 °C	–	–	–	Sen et al. (2016)
Geobacillus thermoleovorans	50	7.0	60 °C	–	–	–	Mehta and Satyanarayana (2014)
Halolactibacillus sp. SK71	78.5	8.0	70 °C	–	–	–	Yu and Li (2014)
Pyrococcus furiosus	66	–	Thermal stability at 100 °C	–	–	–	Laderman et al. (1993)
Thermococcus kodakarensis	80	3.5	95–100 °C	–	–	Broad pH range (3.0 to 8.5). Though maximum activity in acidic pH	Ahmad et al. (2014)

Table 11.2 Amylase enzymes from actinobacteria with its properties

Organism	Mol wt (kDa)	pH	Temp/ optimal stability (°C)	Inhibitor	Stabilizer	Additional properties	References
Streptomyces clavifer	55	6.0	60	–	–	–	Yassien and Asfour (2012)
Streptomyces sp. PDS1	44	7.0	40	–	–		Ragunathan and Padhmadas (2013)
S. erumpens MTCC 7317	54.5	6.0	50	Ca^{2+}	–	–	Kar et al. (2008)
S. gulbargensis DAS 131	55	9.0	45	–	–	–	Syed et al. (2009)
Streptomyces sp. MSC702	–	5.0	55	EDTA, SLS, Urea	–	Km and *V*max value—2.4 mg/ml, 21853.0 µmol/min/mg	Singh et al. (2014)
S. avermitilis	49	–	50	EDTA, Ca, Co, Cu, Al	–	–	Hwang et al. (2013)
Streptomyces strain A3	45	7.0	55	Ca ions	Mg,Ni, Fe, Mn, and Zn	SDS and Tween 80	Chakraborty et al. (2012)
S. fragilis DA7-7	51	–	–	–	–	Stable up to pH 9 and temperature 80 °C Km and *V*max value—0.624 mU/mg and 0.836 mg/ml	Nithya et al. (2017)
S. badius DB-1	57	6.0	50	–	–	–	Shivlata and Satyanarayana (2017)

to be its inhibitors (Ali et al. 2015). *Penicillium citrinum* HBF62 is another known fungal amylase enzyme producer which yielded 65 kDa amylolytic enzymes. The enzyme retained 50% of its activity up to 60 °C upon 34 h incubation (Metin et al. 2010). Details of fungal amylolytic enzymes are shown in Table 11.3:

Table 11.3 Fungal amylase enzymes with its properties

Organism	Mol wt (kDa)	pH	Temp/optimal stability (°C)	Inhibitor	Stabilizer	Additional properties	References
Aspergillus niger JGI24	43	9.5	30	–	–	Ca^{2+} and Co enhanced the activity	Varalakshmi et al. (2009)
Penicillium citrinum HBF62	65	5.5	55	PMSF, NBS, CMC	–	Km and *V*max value—0.2 mg/ml, 5000 U/mg	Metin et al. (2010).
A. flavus F2Mbb	56	6.4	30	–	–	Km and *V*max value—0.5 mg/ml and 17.7 mg/ml	Sidkey et al. (2011)
Talaromyces pinophilus	58	5.0	55	–	–	–	Xian et al. (2015)
A. penicillioides	42	9.0	80	$ZnCl_2$, $FeCl_2$, and EDTA	$CaCl_2$	–	Ali et al. (2015)
A. niger		6.0	30	–	–	–	Obafemi et al. (2018)
A. oryzae IFO-30103	51.3	5.5	50	–	Ca^{2+}	–	Dey and Banerjee (2015)
A. gracilis	35	5.0	60	–	–	Optimum activity at 30% NaCl Km and *V*max value—8.36 U/mg and 6.33 mg/ml	Ali et al. (2014)
Aspergillus japonicas	72	5.0	65	–	–	Mn^{2+}, Pb^{2+} enhanced activity Km, and *V*max value—059 mg/ml and 308.01 U/mg	Pasin et al. (2017)

11.4 Recombinant Amylase

Genetic manipulation of microorganisms brings out improved production of enzymes.α-amylase AmyM is a maltohexaose-forming exo-amylase produced by *Corallococcus* sp. strain EGB. The gene encoding α-amylase AmyM was identified and cloned in *E. coli*, overexpressed and purified using Ni-NTA affinity chromatography (Lia et al. 2015). Another recombinant protein α-amylase from *B. subtilis* was cloned and transformed into *Saccharomyces cerevisiae* (Afzal-Javan and Mobini-Dehkordi 2013). Recombinant production of α-amylase from *B. subtilis* PY22 was successfully cloned and overexpressed in *Pichia pastoris* (Karakaş

et al. 2010). Thermophilic alpha-amylase gene of *B. licheniformis* was transformed to *B. licheniformis* B0204 for hyperproduction (Niu et al. 2009). *amy* TO1 gene encoding amylase from *Streptomyces* strain sp. TO1 was successfully cloned into *S. lividans* for hyper production (Mellouli et al. 1999). amyR4 from *B. subtilis* KCC103 is a catabolite repression-resistant promoter of alpha-amylase and is useful in hyperproduction of recombinant enzymes in *B. subtilis* (Nagarajan and Krishnan 2010). Cloning and overexpression of thermo acidophilic, organic solvent-tolerant α-amylase from *Bacillus* sp. DR90 was successfully achieved in *E. coli* by Asoodeh et al. (2014). Double deletion mutants of *A. oryzae* which was lacking genes responsible for carbon catabolite repression showing enhance amylase enzyme production (Ichinose et al. 2014). Directed evolution has emerged as a pivotal tool gene manipulation experiments. Desired properties can be tailor-made by application of these techniques. pH stability and specific activity of α-amylase from *B. amyloliquefaciens* were enhanced by directed evolution. The mutant enzyme showed fivefold increased activity when compared with wild type (Bessler et al. 2003). Random mutagenesis was achieved by DNA shuffling to attain thermostability of a maltogenic amylase derived from *Thermus* sp. strain IM6501. The optimum temperature for amylolytic activity was incremented from 50 to 75 °C in the mutant strain (Kim et al. 2003). Acid stability of amylase from *B. licheniformis* was enhanced by site-directed mutagenesis (Liu et al. 2017). Recent advancements in improvising the characteristics of α-amylase such as pH stability, temperature stability and acid stability has helped in acquiring definite properties to the enzyme.

11.5 Applications of Microbial Amylase

Amylase is one of the most important hydrolytic enzymes used in all starch-based industries and has been in practice since 1984, as a pharmaceutical aid for the treatment of digestive disorders. Amylases are applied in all the industrial processes such as food, detergents, textiles and paper industries, for the hydrolysis of starch. Nowadays, chemical hydrolysis of starch is interchanged with enzymatic hydrolysis using microbial amylases in starch processing industries. One of the major applications of amylases is in food industry and starch processing industry.

11.5.1 Amylase in Food Industry

Starch is the major carbohydrate source and mainly of plant origin. Starch derivatives also have a momentous role in the food, beverage and feed industries, which include cyclodextrin, glucose syrup, hydrolysates, maltodextrin, and other modified starch. Production of starch derivatives is one of the growing industries, where starch modifying enzymes find substantial roles. Amylases find its applications in many

food processing industries, like brewing and baking sectors, preparation of fruit juices and starch syrups, etc. (Mobini-Dehkordi and Javan 2012).

Starch in dough can be broken down to α-limit dextrins, the intermediate product starch hydrolysis; along with fermentable sugars in bread baking process and further fermentation of these yield alcohol and CO_2 (Prakash and Jaiswal 2010). The presence of low molecular weight dextrins will reduce bread hardness. In wheat flour, the presence of β-amylases are abundant, have little activity on undamaged native starch granules, while α-amylases are absent. Starch hydrolysis in dough is by the combined action of heterogeneously supplied α-amylases and β-amylase. During milling process, the starch granules in flour are sufficiently damaged and make more susceptible to amylases. During baking process, gelatinization of the starch granules occurs, which together with the action of α-amylase cause liquefaction of the starch. Similarly, the β-amylase present in flour converts the dextrins to maltose, which is subsequently fermented by the baker's yeast. Only small amounts of fermentable sugars are available in the wheat flour. The enzymatic hydrolysis made available enough fermentable sugars in the dough to sustain vigorous yeast fermentation required to produce lively doughs and large loaf volumes. Fungal α-amylases, mainly from *A. oryzae* are usually used in bread baking to improve volume, color, and flavor, while bacterial α-amylases are used in the preparation of doughs for cakes, biscuits and crackers, where it adds more sweetness (Dekker 1994a).

There may be staling effects during storage of baked products, causing disagreeable changes affecting crumb firmness, crust crispness, moisture content of the crumb and loss of flavor, etc. Upon storage, the short amylopectin side chains present in soft, fresh bread, is gradually get crystallized to amylopectin network, which accounts for a major role in bread firming. Following starch crystallization, moisture migration within the crumb structure occurs, leads to increased crumb firmness and decreased crumb resilience. Bacterial α-amylases with intermediate thermostability are used in anti-staling agents. These limits recrystallisation of amylopectin, its network formation and consequent water immobilization and help to the retention of softness and improve the shelf life of baked food (Jana et al. 2013). The α-amylase from *B. stearothermophilus* has been employed in the baking industry as an anti-staling agent (Ogasahara et al. 1970; Mobini-Dehkordi and Javan 2012). However, overuse may result in gumminess of the bread, as it produces more branched dextrins. This can be reduced by the use of thermostable pullulanase along with amylase (Kulp et al. 1981). The pullulanase help to hydrolyze the branched dextrins produced by the α-amylase.

Sweetening agents are the major and expensive elements of large variety of confectionary products. Amylolytic enzymes enable starch from low-cost resources to be transformed into sugar syrup. Sucrose is used as a major sweetening agent and starch syrups (glucose) and dextrose occupy a second position. Glucose, fructose, maltose, and higher oligosaccharides, mainly derived from uncooked starch, mostly from cereal and tuber starches. Starch enzymatic hydrolysis using amylases in the starch liquefaction process converts starch into fructose and glucose syrups (Regulapati et al. 2007). The enzymatic conversion of starch is accomplished in a three-step procedure.

Step (i) Gelatinization: Production of viscous suspension by dissolving starch;
Step (ii) Liquefaction: Loss of viscosity by partial hydrolysis;
Step (iii) Saccharification: Further hydrolysis to form glucose and maltose.

The process liquefaction demands thermostable α-amylase that can act at high temperature ranging from 70 to 100 °C (Mobini-Dehkordi and Javan 2012). Due to thermostability, enzymes from *Bacillus* sp. are most preferred of the industrial applications. α-amylase from *B. amyloliquefaciens* was used previously, however, it has been substituted by α-amylase of *B. licheniformis* or *B. stearothermophilus* (Prakash and Jaiswal 2010).

High-maltose corn syrup (HMCS) is a food additive used as a sweetening agent as well as a preservative. HMCS contains little to no fructose and is less sweet than high-fructose corn syrup. The β-amylase or fungal α-amylase are used to produce glucose syrups containing over 50–70% maltose. HMCS is used in the production of hard candy. It is useful in frozen desserts, since maltose has a low freezing point. Another use of HMCSs is in food preservation, as it can inhibit bacterial growth and fermentation.

High-fructose corn syrup (HFCS) or high-fructose glucose syrup (HFGS) is a liquid alternative sweetener to sucrose (table), first introduced to the food and beverage industry in the 1970s. HFCS has become a major sweetener used extensively in different variety of processed foods and beverages like soft drinks, candies, jams and jellies, yogurts and breads. It has many advantages compared to sucrose, in its sweetness, solubility, acidity, relative cheapness, and that makes it more attractive to the food industry. It contains either 42% or 55% percent fructose depends upon applications and these are referred to in the industry as HFCS 42 and HFCS 55. The HFCS 42 is mainly used in processed foods, cereals, baked goods, and some beverages, while HFCS 55 is used primarily in soft drinks (https://www.fda.gov/Food/default.htm). Starch liquefaction and saccharification achieved using a combined α-amylase glucoamylase process to yield glucose syrup, containing mostly glucose and a third enzyme (glucose isomerase) is used to isomerize glucose in corn syrup to fructose to yield fructose syrup.

Chocolate syrup is prepared by treating cocoa slurries with amylases, in which chocolate starch is dextrinizing and thus syrup becoming thin. These syrups are having a high cocoa content and excellent stability and flow properties at room temperature. This is useful in the production of cocoa flavored frozen confections (Saini et al. 2017).

Maltooligosaccharides mixture, maltotetraose syrup, and anomalously linked oligosaccharide (Alo) mixture are used by food manufacturers as substitutes for sucrose and other saccharides. The replacement sucrose with these mixtures is useful in controlling microbial contamination as well as retrogradation of starchy foods because they have low water activity and high moisture-retaining capacity and also prevent crystallization of sucrose in foods. These products are attractive

due to lower viscosity, less sweet taste, and lower freezing point depression. Moreover, they have low-calorie content and have a great appeal to low-calorie dieters. Maltooligomer mixture is obtained by enzymatic hydrolysis of corn starch with α-amylase, β-amylase, and pullulanase. It usually contains 2.2% of glucose, 37.5% of maltose, 46.4% of maltotriose, and 14% of maltotetrose and larger malto oligosaccharides (Marc et al. 2002).

The "Alo mixture" (anomalously linked oligosaccharide) is a mixture of isomaltose, panose, isomaltotriose and branched oligosaccharide composed of four and five glucose residues. The Alo mixture has mildly sweet, low viscosity, high moisture-retaining capacity, and low water activity and makes it favorable for the food industry. It is manufactured by the dextrinization of starch is using thermostable bacterial α-amylase. In this process, the degree of hydrolysis (DE) of starch is kept between 6 and 10 and a reaction of saccharification and transglucosidation of dextrin is done by using soybean β-amylase and transglucosidase from *A. niger* (Prasanna 2005).

Maltotetraose, (G4) is an oligosaccharide of four units of α-D-glucopyranose linked by α-(1–4) bond. Maltotetraose-forming amylase is used to convert starch to maltotraose. This product finds potential applications in the food industries. It has high moisture retention power and can be used in baking to prevent retrogradation of starch ingredient. It is being used as a food additive to improve the texture, to reduce the sweetness of foods and in frozen food to depress the freezing point. Besides, the G4 syrup is a partially undigested and unabsorbed substrate in the small intestine, has shown a prebiotic effect by selectively promoting the growth and activity of beneficial bacteria, once it reaches the colon (Malabendu et al. 2013). The α-amylase used for G4 syrup production is mainly from *B. licheniformis* or *B. subtilis* (Vaidya et al. 2015).

Replacement of fat molecules in foods by lower caloric value fat mimic is a recent strategy in healthy dietary practices. Carbohydrate-based fat mimetics and starch hydrolysis products (SHP) are recommended as it is generating fatty mouthfeel as a phenomenon of rheology. Different starch hydrolytic enzymes like bacterial α-amylase, β-amylase, glucoamylase, and dextrozyme have been used to prepare fat mimics from corn starch (Ma et al. 2006). These fat mimetic substances find application in food industry. Low-fat mayonnaise (with 60% lower fat) produced by incorporating fat mimetics showed comparable sensory quality (Ma et al. 2006).

Food flavor stability is one of the highly desired factors in food and is highly relevant in with its quality and acceptability. Starch-based ingredients (modified starches, maltodextrins, *β*-cyclodextrins) are widely used in the food industry to retain its natural flavor, as carriers for aroma encapsulation or wall material for spray drying process. Amylase enzymes treated starch granules can create a more highly porous structures and such small starch granules have more ability to combine into potentially useful porous spheres when spray dried with small amounts of bonding agents (Madene et al. 2006). Maltodextrins are manufactured the enzymatic hydrolysis of cornflour and is a good candidate as wall material for encapsulation. Bangs and Reineccius (1981) demonstrated the retention of twelve

flavor compounds depends on the dextrose equivalents (DE) of the maltodextrins. Starches modified with octenyl succinic anhydride (OSA) have been used in the food industry and useful stabilizing and encapsulating agent. β-amylolysis is being used under appropriate conditions to modify the structure of gelatinized OSA-modified starches to increase its emulsification properties (Sweedman et al. 2013).

Another major application of amylase enzyme is in beer brewing. Beer is produced by yeast fermentation of sugars. Beer is traditionally based on barley, contain a large quantity of starch and before the yeast fermentation to produce alcohol, starch must be converted to fermentable sugars. Mashing (malting) is the process in which enzymatic degradation of starch into fermentable sugars (maltose) occurs and is a complex process that involves many enzymes like α-amylase, β-amylase, α-glucosidase, and limit dextrinase, etc. (Manners 1985). The α-amylase act on α-1,4 linkages at random, while β-amylases are exo-enzymes which attack the liquefied starch chains forming maltose units from the nonreducing end. Modern brewers usually supplement these enzymes and it is essential when grains other than barley are used. The thermostable α-amylases from *B. subtilis*, and those from *A. oryzae* (with glucoamylase activity), and glucoamylases from *A. niger*, are usually used in mashing for starch hydrolysis (Dekker 1994b).

Enzymes widely used in the maceration of fruit pulps and for clarification of fruit juices and has contributed to improving quality and yield of different types of juices. The application of enzymes fruit juice clarification will depend on the kind of polysaccharides present in different fruit juices. Pectinase plays a major role in clarification process (depectinization), whereas amylase is preferred where the presence of starch. These enzymes are used in combination with cellulose and hemicellulase to effectively reduce haziness in juices. Clarified apple juice is one of the most consumed fruit juices. Raw apple juice obtained as turbid, viscous and tends to settle during storage, due to the presence of polysaccharides (pectin and starch), tannins, proteins, etc. Amylases are used degrade starch present in the apple juice (Carrin et al. 2004). Amylase along with pectinases and other hydrolases are being used in the clarification of banana juice (0.02% amylase and 0.084% pectinase), Kiwifruit juice (0.025% amylases, 0.025% pectinases, 0.05% mash enzyme), and Passion Fruit Juice (0.001% amylase along with cellulase and pectinase) (Singh and Singh 2015).

11.5.2 Non Food Applications

Enzyme-based detergents are currently used in laundry, dishwashing, textile, and other such industries and they are also acknowledged as green chemicals. The amylase in the detergent mainly degrades the residues of starchy food like porridge, potatoes, gravies, custard, chocolate, etc., to dextrins (Kumar et al. 1998). The main advantage of the enzyme application in detergents is that they require only much

milder conditions than that with enzyme-free detergents (Kirk et al. 2002). The demand for α-amylase for automatic dishwashing detergents is now growing.

Modern production processes for textiles introduce a considerable strain on the warp during weaving. The yarn must, therefore, be prevented from breaking. Ease of availability, lower cost, and ease of removal makes starch, the most preferred sizing agent. Effective de-sizing without harming the fibers can be attained by α-amylases in starch-sized textiles, where it selectively removes the sizing agent (Feitkenhauer 2003).

The use of α-amylases in the pulp and paper industry is to provide modification on starch coated paper (Van Der Maarel et al. 2002). Another application of α-amylases in the textile industry is in making faded jeans. The process of enzyme fade is also called as bio washing or bio-bleaching retains the softness of the cloth while embossing shades in it (Kumar et al. 1998).

The α-amylases also find its usage in medical applications. Synthetic and natural biodegradable polymers have been a major focus of interest in pharmaceutical research. The biodegradable polymers are used to control the drug release rate from parenteral controlled delivery systems (Dumoulin et al. 1999). Starch-based biodegradable polysaccharide matrix is useful for drugs with lesser solubility. By incorporating α-amylase along with cross-linked amylose (CLA), the drug release can be controlled (Afzal-Javan and Mobini-Dehkordi 2013).

Starch and related polysaccharides in waste material produced by agro-industries causes environmental pollution. The starch pollution can be overcome either by application of purified microbial amylolytic enzymes or by using amylolytic microorganisms directly (Wu et al. 2008; Mobini-Dehkordi and Javan 2012).

Starch is the major substrate for bioethanol production since it is widely distributed and economical. Conventional bioethanol production converts starch to fermentable sugars called saccharification by amylolytic enzyme producers or enzymes, followed by fermentation of the sugar formed to ethanol by microorganisms such as *Saccharomyces cerevisiae, Saccharomycopsis fibuligera* etc. (Tesfaw and Assefa 2014; Chi et al. 2009).

11.6 Conclusion

Amylases share a major stake in the global enzyme market, and a wide range of applications demands newer enzymes with improved functionalities. Enzymes from *Bacillus* sp. have been industrially significant for quite a long time. In starch processing industries, thermally stable enzymes are in great demand since starch liquefaction occurs in higher temperatures. Apart from *Bacillus* sp. few members of Archaea has showed remarkable thermal stability. In fact, thermostable enzymes were obtained not only from extremophiles but mesophiles also. Enzymatic hydrolysis of starch yields high-quality products such as glucose and maltose syrups with definite characteristics. Fat mimetic substances produced by amylases could bring changes in the food industry for the production highly nutritious low-fat

diet. With large number of starchy agro-industrial waste, residues expelled to the environment, hyperproducers of amylolytic enzymes are needed. These microorganisms can bring rapid biomass conversion while yielding these enzymes. With advent of recombinant DNA technology, amylases with improved properties can be tailor-made to suit the needs of industry. Increasing demand of these enzymes makes the research on this topic fascinating.

References

Abdel-Fattah YR, Soliman NA, El-Toukhy NM, El-Gendi H, Ahmed RS (2012). Production, purification, and characterization of thermostable α-amylase produced by *Bacillus licheniformis* isolate AI20. J Chem

Acer Ö, Bekler FM, Pirinççioğlu H, Güven RG, Güven K (2016) Purification and characterization of thermostable and detergent-stable α-amylase from *Anoxybacillus* sp. AH1. Food Tech Biotechnol 54(1):70

Afzal-Javan F, Mobini-Dehkordi M, Saffar B (2013) Amplification, sequencing and cloning of iranian native *Bacillus subtilis* alpha-amylase gene in *Saccharomyces cerevisiae*. Jundishapur J Microbiol 6(8):1–7

Ahmad N, Rashid N, Haider MS, Akram M, Akhtar M (2014) Novel maltotriose-hydrolyzing thermoacidophilic type III pullulan hydrolase from *Thermococcus kodakarensis*. Appl Environ Microbiol 80(3):1108–1115

Aiyer PV (2005) Amylases and their applications. Afr J Biotechnol 4(13)

Aleshin A, Golubev A, Firsov LM, Honzatko RB (1992) Crystal structure of glucoamylase from *Aspergillus awamori* var. X100 to 2.2-A resolution. J Biol Chem 267(27):19291–19298

Ali I, Akbar A, Anwar M, Prasongsuk S, Lotrakul P, Punnapayak H (2015) Purification and characterization of a polyextremophilic α-Amylase from an obligate halophilic *Aspergillus penicillioides* isolate and its potential for souse with detergents. In: BioMed research international

Ali I, Akbar A, Yanwisetpakdee B, Prasongsuk S, Lotrakul P, Punnapayak H (2014) Purification, characterization, and potential of saline waste water remediation of a polyextremophilic α-amylase from an obligate halophilic *Aspergillus gracilis*. In: BioMed research international

Amoozegar MA, Malekzadeh F, Malik KA (2003) Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. J Microbiol Method 52(3):353–359

Argüello-Morales MA, Remaud-Simeon M, Pizzut S, Sarçabal P, Willemot R, Monsan P (2000) Sequence analysis of the gene encoding alternansucrase, a sucrase glucosyltransferase from Leuconostoc mesenteroides NRRL B-1355. FEMS Microbiol Lett 182(1):81-85

Asoodeh A, Emtenani S, Emtenani S, Jalal R, Housaindokht MR (2014) Molecular cloning and biochemical characterization of a thermoacidophilic, organic-solvent tolerant α-amylase from a *Bacillus strain* in *Escherichia coli*. J Mol Catal B Enzym 99:114–120

Banerjee G, Mukherjee S, Bhattacharya S, Ray AK (2016) Purification and characterization of extracellular protease and amylase produced by the bacterial strain, *Corynebacterium alkanolyticum* ATH3 isolated from fish gut. Arab J Sci Eng 41(1):9–16

Bangs WE, Reineccius GA (1981) Influence of dryer infeed matrices on retention of volatile flavor compounds during spray-drying. J Food Sci 47:254–259

Bano S, Qader SAU, Aman A, Syed MN, Azhar A (2011) Purification and characterization of novel α-amylase from *Bacillus subtilis* KIBGE HAS. AAPS PharmSciTech 12(1):255–261

Bertoft E (2007) Composition of clusters and their arrangement in potato amylopectin. Carbohyd Polym 68(3):433–446

Bessler C, Schmitt J, Maurer KH, Schmid RD (2003) Directed evolution of a bacterial α-amylase: toward enhanced pH-performance and higher specific activity. Protein Sci 12(10):2141–2149

Bukhari DA, Rehman A (2015) Purification and characterization of α-amylase from *Bacillus subtilis* isolated from local environment. Pak J Zool 47(4)

Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochem 38(10):1397–1403
Carrin ME, Ceci LN, Lozano JE (2004) Characterization of starch in apple juice and its degradation by amylases. Food Chem 87:173–178
Chakraborty S, Khopade RGA, Mahadik K, Kokare C (2012) Study on calcium ion independent α-amylase from haloalkaliphilic marine *Streptomyces* strain A3. Indian J Biotechnol 11(4): 427–437
Chi Z, Chi Z, Liu G, Wang F, Ju L, Zhang T (2009) *Saccharomycopsis fibuligera* and its applications in biotechnology. Biotechnol Adv 27(4):423–431
de Lourdes Moreno M, Pérez D, García MT, Mellado E (2013) Halophilic bacteria as a source of novel hydrolytic enzymes. Life 3(1):38–51
Dekker RFH (1994a) Enzymes in food and beverage processing-1. Food Aust 46(3):136–139
Dekker RFH (1994b) Enzymes in food and beverage processing-2 Food Aust 46(4):179–181
Dey S, Agarwal SO (1999) Characterization of a thermostable alpha-amylase from a thermophilic *Streptomyces megasporus* strain SD12. Indian J Biochem Biophys 36:150–157
Dey TB, Banerjee R (2015) Purification, biochemical characterization and application of α-amylase produced by *Aspergillus oryzae* IFO-30103. Biocatal Agric Biotechnol 4(1):83–90
Du R, Song Q, Zhang Q, Zhao F, Kim RC, Zhou Z, Han Y (2018) Purification and characterization of novel thermostable and Ca-independent α-amylase produced by *Bacillus amyloliquefaciens* BH072. Int J Biol Macromol 115:1151–1156
Dumoulin Y, Cartilier LH, Mateescu MA (1999) Cross-linked amylose tablets containing α-amylase: an enzymatically-controlled drug release system. J Controll Release 60(2–3):161–167
El-Fallal A, Dobara MA, El-Sayed A, Omar N (2012) Starch and microbial α-amylases: from concepts to biotechnological applications. In: Carbohydrates-comprehensive studies on glycobiology and glycotechnology. InTech
El-Sayed AK, Dobara MIA, El-Fallal AA, Omar NF (2013) Purification, sequencing, and biochemical characterization of a novel calcium-independent α-amylase AmyTVE from *Thermoactinomyces vulgaris*. Appl Biochem Biotechnol 170(3):483–497
Feitkenhauer H (2003) Anaerobic digestion of desizing wastewater: influence of pretreatment and anionic surfactant on degradation and intermediate accumulation. Enzyme Microb Technol 33 (2–3):250–258
Fincan SA, Enez B, Özdemir S, Bekler FM (2014) Purification and characterization of thermostable α-amylase from thermophilic *Anoxybacillus flavithermus*. Carbohyd Polym 102:144–150
Harris EMS, Aleshin AE, Firsov LM, Honzatko RB (1993) Refined structure for the complex of 1-deoxynojirimycin with glucoamylase from *Aspergillus awamori* var. X100 to 2.4 Å. resolution. Biochemistry 32(6):1618–1626
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
Hii SL, Tan JS, Ling TC, Ariff AB (2012) Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res
Hirata A, Adachi M, Sekine A, Kang YN, Utsumi S, Mikami B (2004) Structural and enzymatic analysis of soybean β-amylase mutants with increased pH optimum. J Biol Chem 279(8):7287–7295
Horvathova V, Janecek S, Sturdik E (2001) Amylolytic enzymes: molecular aspects of their properties. Gen Physiol Biophys 20(1):7–32
Hutcheon GW, Vasisht N, Bolhuis A (2005) Characterisation of a highly stable α-amylase from the halophilic archaeon *Haloarcula hispanica*. Extremophiles 9(6):487–495
Hwang SY, Nakashima K, Okai N, Okazaki F, Miyake M, Harazono K, Ogino C, Kondo A (2013) Thermal stability and starch degradation profile of α-amylase from *Streptomyces avermitilis*. Biosci Biotechnol Biochem 77(12):2449–2453

Ichinose S, Tanaka M, Shintani T, Gomi K (2014) Improved α-amylase production by *Aspergillus oryzae* after a double deletion of genes involved in carbon catabolite repression. Appl Microbiol Biotechnol 98(1):335–343

Jana M, Maity C, Samanta S, Pati BR, Islam SS, Mohapatra PKD, Mondal KC (2013) Salt-independent thermophilic α-amylase from *Bacillus megaterium* VUMB109: an efficacy testing for preparation of maltooligosaccharides. Ind Crops Prod 41:386–391

Janeček Š (1994) Parallel β/α-barrels of α-amylase, cyclodextrin glycosyltransferase and oligo-1, 6-glucosidase versus the barrel of β-amylase: evolutionary distance is a reflection of unrelated sequences. FEBS Lett 353(2):119–123

Janeček Š (2002) How many conserved sequence regions are there in the α-amylase family. Biologia 57(Suppl 11):29–41

Kaplan F, Guy CL (2004) β-Amylase induction and the protective role of maltose during temperature shock. Plant Physiol 135(3):1674–1684

Kar S, Ray RC (2008) Statistical optimization alpha-amylase production by *Streptomyces erumpens* MTCC 7317 cells in calcium alginate beads using response surface methodology. Polish J Microbiol 57(1):49–57

Kar S, Ray RC, Mohapatra UB (2008) Alpha-amylase production by *Streptomyces erumpens* MTCC 7317 in solid state fermentation using response surface methodology (RSM). Polish J Microbiol 57(4):289-96

Karakaş B, İnan M, Certel M (2010) Expression and characterization of *Bacillus subtilis* PY22 α-amylase in *Pichia pastoris*. J Mol Catal B Enzym 64(3–4):129–134

Karmakar M, Ray RR (2011) A maltotriose producing thermostable amylase from *Bacillu*s sp KR11. J Microbiol Biotechnol Res 1(3):91–99

Kim YW, Choi JH, Kim JW, Park C, Kim JW, Cha H, Lee SB, Oh BH, Moon TW, Park KH (2003) Directed evolution of *Thermus* maltogenic amylase toward enhanced thermal resistance. Appl Environ Microbiol 69(8):4866–4874

Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13(4):345–351

Konsoula Z, Liakopoulou-Kyriakides M (2007) Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. Biores Technol 98(1):150–157

Kulp K, Ponte JG Jr, D’Appolonia BL (1981) Staling of white pan bread: fundamental causes. Crit Rev Food Sci Nutr 15(1):1–48

Kumar CG, Malik RK, Tiwari MP (1998) Novel enzyme-based detergents: an Indian perspective. Curr Sci 75(12):1312–1318

Kumar MDJ, Jayanthisiddhuraj AB, Monica DD, Balakumaran MD, Kalaichelvan PT (2012) Purification and characterization of α-amylase and α–galactosidase from *Bacillus* Sp. MNJ23 produced in a concomitant medium. Am Eurasian J Agric Environ Sci 12(5):566–573

Kumar P, Satyanarayana T (2009) Microbial glucoamylases: characteristics and applications. Crit Rev Biotechnol 29(3):225–255

Kuttigounder D, Lingamallu JR, Bhattacharya S (2011) Turmeric powder and starch: selected physical, physicochemical, and microstructural properties. J Food Sci 76(9):C1284–C1291

Laderman KA, Davis BR, Krutzsch HC, Lewis MS, Griko YV, Privalov PL, Anfinsen CB (1993) The purification and characterization of an extremely thermostable alpha-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 268(32):24394–24401

Li X, Yu HY (2012) Characterization of an organic solvent-tolerant α-amylase from a halophilic isolate, *Thalassobacillus* sp. LY18. Folia Microbiol 57(5):447–453

Li Z, Wu J, Zhang B, Wang F, Ye X, Huang Y, Huang Q, Cui Z (2015) Characterization of a novel maltohexaose-forming α-amylase AmyM from *Corallococcus* sp. strain EGB. Appl Env Microbiol, pp. AEM-03934

Linden A, Mayans O, Meyer-Klaucke W, Antranikian G, Wilmanns M (2002) Differential regulation of a hyperthermophilic α-amylase with a novel (Ca, Zn)-dimetal centre by zinc. J Biol Chem

Liu Y, Huang L, Jia L, Gui S, Fu Y, Zheng D, Guo W, Lu F (2017) Improvement of the acid stability of *Bacillus licheniformis* alpha amylase by site-directed mutagenesis. Process Biochem 58:174–180

Liu Y, Lu F, Chen G, Snyder CL, Sun J, Li Y, Wang J, Xiao J (2010) High-level expression, purification and characterization of a recombinant medium-temperature α-amylase from *Bacillus subtilis*. Biotech Lett 32(1):119

Ma Y, Cai C, Wang J, Sun DW (2006) Enzymatic hydrolysis of corn starch for producing fat mimetics. J Food Eng 73(3):297–303

MacGregor EA, Jespersen HM, Svensson B (1996) A circularly permuted alpha-amylase-type alpha/beta-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett 378 (3):263-266

Madene A, Jacquot M, Scher J, Desobry S (2006) Flavour encapsulation and controlled release—a review. Int J Food Sci Technol 41:1–21

Maity S, Mallik S, Basuthakur R, Gupta S (2015) Optimization of solid state fermentation conditions and characterization of thermostable alpha amylase from *Bacillus subtilis* (ATCC 6633). J Bioprocess Biotech 5:218

Malabendu J, Chiranjit M, Saptadip S, Bikas RP (2013) Salt-independent thermophilic α-amylase from *Bacillus megaterium* VUMB109: an efficacy testing for preparation of maltooligosaccharides. Ind Crops Prod 41:386–390

Malhotra R, Noorwez SM, Satyanarayana T (2000) Production and partial characterization of thermostable and calcium-independent α-amylase of an extreme thermophile Bacillus thermooleovorans NP54. Lett Appl Microbiol 31(5):378–384

Manners DJ (1985) Some aspects of the metabolism of starch. Cereal Foods World 30:722–727

Marc JEC, Maarel V, Veen BV, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol 94:137–155

Matsuura Y (2002) A possible mechanism of catalysis involving three essential residues in the enzymes of alpha-amylase family. Biologia 57(Supp 2):21–28

Matsuura Y, Kusunoki M, Harada W, Kakudo M (1984) Structure and possible catalytic residues of Taka-amylase A. J Biochem 95(3):697–702

Mehta D, Satyanarayana T (2014) Domain C of thermostable α-amylase of *Geobacillus thermoleovorans* mediates raw starch adsorption. Appl Microbiol Biotechnol 98(10):4503–4519

Mellouli L, Guerineau M, Bejar S, Virolle MJ (1999). Regulation of the expression of amy TO1 encoding a thermostable α-amylase from Streptomyces sp. TO1, in its original host and in *Streptomyces lividans* TK24. FEMS Microbiol Lett 181(1):31–39

Metin K, Ateşlier ZBB, Bıyık HH (2010) Purification and characterization of-amylase produced by *Penicillium citrinum* HBF62. Afr J Biotech 9(45):7692–7701

Mikami B, Degano M, Hehre EJ, Sacchettini JC (1994) Crystal structures of soybean beta-amylase reacted with beta-maltose and maltal: active site components and their apparent roles in catalysis. Biochemistry 33(25):7779–7787

Mobini-Dehkordi M, Javan FA (2012) Application of alpha-amylase in biotechnology. J Biol Today's World 1(1):15–20

Mojsov K (2012) Microbial alpha-amylases and their industrial applications: a review. Int J f Manag IT Eng (IJMIE) 2(10):583–609

Myat L, Ryu G (2013) Extrusion with thermostable α-amylase injection as pretreatment method for ethanol production from corn starch. J Microb Biochem Technol 5(2):47–53

Nagarajan DR, Krishnan C (2010) Use of a new catabolite repression resistant promoter isolated from *Bacillus subtilis* KCC103 for hyper-production of recombinant enzymes. Protein Expr Purif 70(1):122–128

Naidu MA, Saranraj P (2013) Bacterial amylase a review. Int J Pharm Biol Arch 4(2):274–287

Nithya K, Muthukumar C, Kadaikunnan S, Alharbi NS, Khaled JM, Dhanasekaran D (2017) Purification, characterization, and statistical optimization of a thermostable α-amylase from desert actinobacterium *Streptomyces fragilis* DA7–7. *3* Biotech 7(5):350

Niu DD, Shi GY, Wang ZX (2009) Genetic improvement of alpha-amylase producing Bacillus licheniformis by homolog-mediated alpha-amylase gene amplification. Chin J Biotechnol 25 (3):375–380

Obafemi YD, Ajayi AA, Olasehinde GI, Atolagbe OM, Onibokun EA (2018) Screening and partial purification of amylase from *Aspergillus Niger* isolated from deteriorated tomato (*Lycopersicon Esculentum* mill.) fruits. Afr J Clin Exp Microbiol 19(1):47–57

Ogasahara K, Imanishi A, Isemura T (1970) Studies on thermophilic α-amylase from *Bacillus stearothermophilus*. J Biochem 67(1):65–75

Özdemir S, Matpan F, Güven K, Baysal Z (2011) Production and characterization of partially purified extracellular thermostable α-amylase by Bacillus subtilis in submerged fermentation (SmF). Prep Biochem Biotechnol 41(4):365–381

Pancha I, Jain D, Shrivastav A, Mishra SK, Shethia B, Mishra S, Mohandas VP, Jha B (2010) A thermoactive α-amylase from a *Bacillus* sp. isolated from CSMCRI salt farm. Int J Biol Macromol 47(2):288–291

Pandey A, Nigam PR, Sccol C, Soccol T, Singh V, Mohan DR (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31:135–152

Pasin TM, Benassi VM, Heinen PR, de Lima Damasio AR, Cereia M, Jorge JA, de Moraes MDLT (2017) Purification and functional properties of a novel glucoamylase activated by manganese and lead produced by *Aspergillus japonicus*. Int J Biol Macromol 102:779–788

Pavithra S, Ramesh R, Aarthy M, Ayyadurai N, Gowthaman MK, Kamini NR (2014) Starchy substrates for production and characterization of *Bacillus subtilis* amylase and its efficacy in detergent and bread making formulations. Starch 66(11–12):976–984

Prakash O, Jaiswal N (2010) α-Amylase: an ideal representative of thermostable enzymes. Appl Biochem Biotechnol 160(8):2401–2414

Prasanna VA (2005) Amylases and their applications. Afr J Biotech 4(13):1525–1529

Przylas I, Tomoo K, Terada Y, Takaha T, Fujii K, Saenger W, Sträter N (2000) Crystal structure of amylomaltase from *Thermus aquaticus*, a glycosyltransferase catalysing the production of large cyclic glucans1. J Mol Biol 296(3):873–886

Ragunathan R, Padhmadas R (2013) Production, purification and characterization of-amylase using *Streptomyces* spp. PDS1 and *Rhodococcus* spp. Isolated from Western Ghats. Int J Curr Microbiol Appl Sci 2(8):206–214

Rai S, Solanki MK (2014) Optimization of thermostable alpha-amylase production via mix agricultural-residues and *Bacillus amyloliquefacien*s. Not Sci Biol 6(1):105–111

Singh R, Singh RK (2015) Role of enzymes in fruit juices clarification during processing: a review. Int J Biol Technol 6(1):1–12

Raul D, Biswas T, Mukhopadhyay S, Kumar Das S, Gupta S (2014) Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. Biochem Res Int

Regulapati R, Malav NP, Gummadi NS (2007) Production of thermostable c-amylases by solid state fermentation: a review. Am J Food Technol 2:1–11

Roohi R, Kuddus M, Saima S (2013) Cold-active detergent-stable extracellular α-amylase from *Bacillus cereus* GA6&58; biochemical characteristics and its perspectives in laundry detergent formulation. J Biochem Technol 4(4):636–644

Saboury AA, Ghasemi S, Dahot MU (2005) Thermodynamic study of magnesium ion binding to alpha-amylase. Indian J Biochem Biophys 42:326–329

Saini R, Saini HS, Dahiya A (2017) Amylases: characteristics and industrial applications. J Pharmacogn Phytochem 6(4):1865–1871

Sajedi RH, Naderi-Manesh H, Khajeh K, Ahmadvand R, Ranjbar B, Asoodeh A, Moradian F (2005) A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. Enzyme Microb Technol 36(5–6):666–671

Sarethy IP, Saxena Y, Kapoor A, Sharma M, Seth R, Sharma H, Sharma SK, Gupta S (2012). Amylase produced by Bacillus sp. SI-136 isolated from sodic-alkaline soil for efficient starch desizing. J Biochem Technol 4(2):604–609

Sen SK, Jana A, Bandyopadhyay P, Mohapatra PKD, Raut S (2016) Thermostable amylase production from hot spring isolate *Exiguobacterium* sp: a promising agent for natural detergents. Sustain Chem Pharm 3:59–68
Setyorini E, Takenaka S, Murakami S, Aoki K (2006) Purification and characterization of two novel halotolerant extracellular proteases from Bacillus subtilis strain FP-133. Biosci Biotechnol Biochem 70(2):433–440
Ševčík J, Solovicová A, Hostinová E, Gašperík J, Wilson KS, Dauter Z (1998) Structure of glucoamylase from *Saccharomycopsis fibuligera* at 1.7 Å resolution. Acta Crystallogr Sect D 54(5):854–866
Shivlata L, Satyanarayana T (2017) Characteristics of raw starch-digesting α-amylase of *Streptomyces badius* DB-1 with transglycosylation activity and its applications. Appl Biochem Biotechnol 181(4):1283–1303
Sidkey NM, Abo-Shadi MA, Balahmar R, Sabry R, Badrany G (2011) Purification and characterization of α-amylase from a newly isolated *Aspergillus flavus* F2Mbb. Int Res J Microbiol 2(3):096–103
Singh R, Kumar V, Kapoor V (2014) Partial purification and characterization of a heat stable α-amylase from a thermophilic actinobacteria, *Streptomyces* sp. MSC702. Enzyme Res
Smitha RB, Sajith S, Priji P, Unni KNN, Roy TAN, Benjamin S (2015) Purification and characterization of amylase from *Bacillus thuringiensis* subsp. *kurstaki*. Bt Res 6
Stam MR, Danchin EG, Rancurel C, Coutinho PM, Henrissat B (2006) Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of α-amylase-related proteins. Protein Eng Des Sel 19(12):555–562
Sumrin A, Ahmad W, Ijaz B, Sarwar MT, Gull S, Kausar H, Shahid I, Jahan S, Asad S, Hussain M, Riazuddin S (2011) Purification and medium optimization of α-amylase from *Bacillus subtilis* 168. Afr J Biotech 10(11):2119–2129
Sweedman MC, Hasjim J, Tizzotti MJ, Schäfer C, Gilbert RG (2013) Effect of octenylsuccinic anhydride modification on β-amylolysis of starch. Carbohydr Polym 97(1):9–17
Syed DG, Agasar D, Pandey A (2009) Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*. J Ind Microbiol Biotechnol 36(2):189–194
Tesfaw A, Assefa F (2014) Current trends in bioethanol production by *Saccharomyces cerevisiae*: substrate, inhibitor reduction, growth variables, coculture, and immobilization. Int Sch Res Not
Thompson DB (2000) On the non-random nature of amylopectin branching. Carbohyd Polym 43 (3):223–239
Tiwari SP, Srivastava R, Singh CS, Shukla K, Singh RK, Singh P, Singh R, Singh NL, Sharma R (2015) Amylases: an overview with special reference to alpha amylase. J Global Biosci 4:1886–1901
Totsuka A, Fukazawa C (1996) Functional analysis of Glu380 and Leu383 of soybean β-amylase: a proposed action mechanism. Eur J Biochem 240(3):655–659
Vaidya S, Srivastava PK, Rathore P, Pandey AK (2015) Amylases: a prospective enzyme in the field of biotechnology. J Appl Biosci 41(1):1–18
Van Der Maarel MJ, Van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol 94 (2):137–155
Varalakshmi KN, Kumudini BS, Nandini BN, Solomon J, Suhas R, Mahesh B, Pavitha AP (2009) Production and characterization of α-amylase from *Aspergillus niger* JGI24 isolated in Banglore. Polish J Microbiol 58(1):29–36
Vengadaramana A (2013) Industrial important microbial alpha-amylase on starch-converting process. Scholars Acad J Pharm 2(3):209–221
Weemaes C, De Cordt S, Goossens K, Ludikhuyze L, Hendrickx M, Heremans K, Tobback P (1996) High pressure, thermal and combined pressure–temperature stabilities of α-amylases from Bacillus species. Biotechnol Bioeng 50(1):49–56
Wu CH, Mulchandani A, Chen W (2008) Versatile microbial surface-display for environmental remediation and biofuels production. Trends Microbiol 16(4):181–188

Wu X, Wang Y, Tong B, Chen X, Chen J (2018) Purification and biochemical characterization of a thermostable and acid-stable alpha-amylase from *Bacillus licheniformis* B4-423. Int J Biol Macromol 109:329–337

Xian L, Wang F, Luo X, Feng YL, Feng JX (2015) Purification and characterization of a highly efficient calcium-independent α-amylase from *Talaromyces pinophilus* 1-95. PLoS ONE 10(3): e0121531

Yassien MAM, Asfour HZ (2012) Improved production, purification and some properties of α-amylase from *Streptomyces clavifer*. Afr J Biotech 11(80):14603–14611

Yu HY, Li X (2014) Characterization of an organic solvent-tolerant thermostable glucoamylase from a halophilic isolate, *Halolactibacillus* sp. SK 71 and its application in raw starch hydrolysis for bioethanol production. Biotechnol progress 30(6):1262–1268

Zhang F, Yang X, Geng L, Zhang Z, Yin Y, Li W (2016) Purification and characterization of a novel and versatile α-amylase from thermophilic *Anoxybacillus* sp. YIM 342. Starch 68(5–6): 446–453

Chapter 12
α-L-Arabinofuranosidase: A Potential Enzyme for the Food Industry

Abhijeet Thakur, Kedar Sharma and Arun Goyal

Abstract Cellulose, hemicellulose, pectin, and lignin are the major components of plant cell wall. Hemicellulose is the second most abundant carbohydrate polymer on the earth. Hemicelluloses are branched, hetero-polysaccharides formed by β-(1 → 4)-linked backbones of hexoses like glucose (xyloglucan), galactose (galactan), mannose (mannan) or pentoses like xylose (xylan), and arabinose (arabinan). Xylan contains the backbone of 1,4-linked-β-D-xylopyranose with various side-chain substitutions such as arabinose, acetic acid, glucuronic acid, ferulic, acid, and *p*-coumaric acid. L-arabinose side chain is found in hemicelluloses like arabinan, arabinoxylan, oat spelt xylan, and arabinogalactan. The extent of side-chain substitution depends on the source of the xylan, which makes its structure complex and hinders its enzymatic hydrolysis. α-L-arabinofuranosidase hydrolyzes arabinose side chain present at α-1,2-, α-1,3-, and α-1,5-positions in arabinoxylan, thus potentiating other xylanolytic enzymes to act efficiently on the backbone. Therefore, α-L-arabinofuranosidase has potential application in agro-industrial processes because of its functioning synergistically with other hemicellulases. α-L-arabinofuranosidases are used for improving bread quality, for wine flavor, for clarification of fruit juices, as supplement for feedstock for enhancing digestion, in the production of medicinal compounds, and in the production of oligosaccharide and modification of their side chains. This chapter presents a comprehensive overview of α-L-arabinofuranosidase, sources, production, and its applications in food processing.

A. Thakur · K. Sharma · A. Goyal (✉)
Carbohydrate Enzyme Biotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
e-mail: arungoyl@iitg.ac.in

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_12

12.1 Introduction

The cell wall of the plant is a semirigid and complex structure. It is the outer most layer of the cell that plays a key role in exchange of the substances necessary for cell metabolism, and also in excretion of other waste substances from the cell. The cell wall is crucial for maintaining the cell structure and cell protection (Carpita and Gibeaut 1993). Cell wall acts as a buffer and between protoplasm and environment, and is essential for cell signaling and cell–cell adhesion. It is a source of nutrition, fiber, and energy. Plant cell wall is mainly composed of carbohydrates and also contains protein, lignin, and water. Carbohydrate component of the cell wall is cellulose, hemicellulose, and pectin. Cellulose is a linear homopolymer of glucose, whereas, the hemicelluloses are an important group of polysaccharides, which are interconnected and also connected to cellulose and pectins. The most significant hemicelluloses are xylans, arabinoxylans, mannans, galactomannans, galactomannans, arabinogalactan II, and xylogucans. The major constituent of the primary walls of nonwoody plant cells is pectin and is rich in most vegetables and fruits, providing the strength and flexibleness to the cell wall. The principal components of pectin are homogalacturonan and the substituted rhamnogalacturonan I and rhamnogalacturonan II (Dhillon et al. 2016).

Hemicellulose resides in an assembly of diverse polysaccharides, which are assembled through a biosynthetic pathway distinct from that of cellulose. The vital biological role of hemicelluloses is to give strength to the cell wall by interaction with cellulose and lignin (Scheller and Ulvskov 2010). The amount of hemicellulose of the dry weight of wood is between 20 and 40%. Significant differences in the composition and content between the stem, branches, roots, and bark of hemicelluloses have been reported. Xylan belongs to the second most abundant structural component of the secondary walls of dicot plants. Xylan has a backbone of β-(1 → 4)-linked xylose residues and are found with variable degree of substitution based on the plant species and tissue types (Fig. 12.1). The common side-chain substitutions on the xylan backbone are arabinose, acetic acid, glucuronic acid, ferulic acid, *p*-coumaric acid, or *4-O*-methyl glucuronic acid (Ordaz-Ortiz and Saulnier 2005). Depending on the relative richness of their substitutions within the xylan, it is further categorized into glucuronoxylan, arabinoxylans and xyloglucans, etc. Arabinose and uronic acid chains stabilize the xylan structure against alkali-catalyzed deterioration. Hemicellulose contains various more prominent substitutions in the side chains as an alternative for hydroxyl groups present at positions C2, C3, and C6 (Sjostrom 2013).

Glucuronoxylan is a polymer of linear D-xylopyranosyl residues connected by β-(1 → 4) glycosidic bonds. The glucuronate residues (generally methylated at C4 position) are attached by α(2 → 3) linkage to many of the xylose residues of the backbone. At position C2, most of the xylans have a substitution of single 4-O-methyl-α-D-glucuronic acid residues. This structure is usually referred to as 4-O-methyl-D-glucurono-D-xylan (Sjostrom 2013). Xyloglucan has a backbone of β-(1 → 4)-glucose residues, linked with β-(1 → 6)-D-xylose side chains. The

Fig. 12.1 Structure of heteroxylan showing different substitutions

primary cell wall of dicotyledonous plants consists of xyloglucan. Xyloglucan has a property to bind with cellulose microfibrils and therefore, xyloglucan–cellulose interactions are important determinants of the mechanical strength as well as the growth of cell wall (Cosgrove 2005). Arabinoxylan (AX) is a non-starch polysaccharide, mainly found in cereal plants, considered as dietary fiber. Arabinoxylans have the β-(1 → 4)-linked xylose backbone with the substitution of one or more L-arabinofuranosyl units, at position C2 or C3 (Fig. 12.2). AX is located in primary cell walls of grasses and monocot plants cereals like wheat, rye, barley, oat, rice, corn, and sorghum (Brett and Waldron 1990). L-arabinosyl residues are extensively found in these polymers as side chains. The presence of L-arabinosyl side chain hinders the enzymes, in the hydrolysis of hemicelluloses and pectins causing a technological bottleneck for various industrial processes (Saha 2000).

Degradation of plant cell wall makes a nutrient pool available for recycling. Nature has gifted a diverse group of microorganisms with enzymes to disrupt the plant cell wall polysaccharides (Ochiai et al. 2007). Principally fungi and bacteria are responsible for the cell wall polysaccharides deterioration. Few insects and molluscs also release enzymes that act on the plant cell wall (Lynd et al. 2002). Due to the heterogeneous nature of hemicellulose, a single enzyme is not sufficient for the complete hydrolysis. Therefore, several enzymes are required for complete degradation of plant biomass. Some enzymes act on hemicellulose main chain and some act on the side chain. Side-chain-removing enzymes are essential as they act synergistically and enhance the rate of hydrolysis by other glycoside hydrolases.

Hemicellulases are a divergent group of enzymes which hydrolyze hemicelluloses, Hemicellulases include endo-β-1,4-xylanase, β-1,4-xylosidase, α-L-arabinofuranosidase, α-1,5-arabinanase, α-glucuronidase, β-1,4-mannanase, β-1,4-mannosidase, α-galactosidase, β-glucosidase, endo-galactanase, acetyl mannan esterase, acetyl xylan

Fig. 12.2 Arabinoxylans and mode of action of α-L-arabinofuranosidase

esterase, ferulic acid esterase, and *p*-coumaric acid esterase (Beg et al. 2001). Arabinose side chain is widely distributed in hemicellulose and pectins linked with cellulose and, thus to break down the integrity of plant cell wall it is necessary to remove this side chain. α-L-arabinofuranosidase is one of the important hemicellulases, which cleaves α-L-arabinofuranosidic bonds (1 → 2, 1 → 3 and 1 → 5) and plays a vital role toward complete degradation of hemicellulose and pectins by acting synergistically with other hemicellulases and pectinolytic enzymes (Margolles-Clark et al. 1996). α-L-arabinofuranosidase has several food applications such as in bread making, clarification of fruit juices, in brewing industry for enriching flavor of wine, for extraction of coffee, starch, and plant oils. It is also used in making healthy agro-based fodder and grain feed, paper and pulp industry, bioethanol production, and production of therapeutic compounds (Fig. 12.3). Arabinofuranosidase releases arabinose that inhibits intestinal sucrase and acts as antiglycemic agent (Seri et al. 1996). It produces arabinoxylan oligosaccharide that acts as a potential prebiotic (Numan and Bhosle 2006).

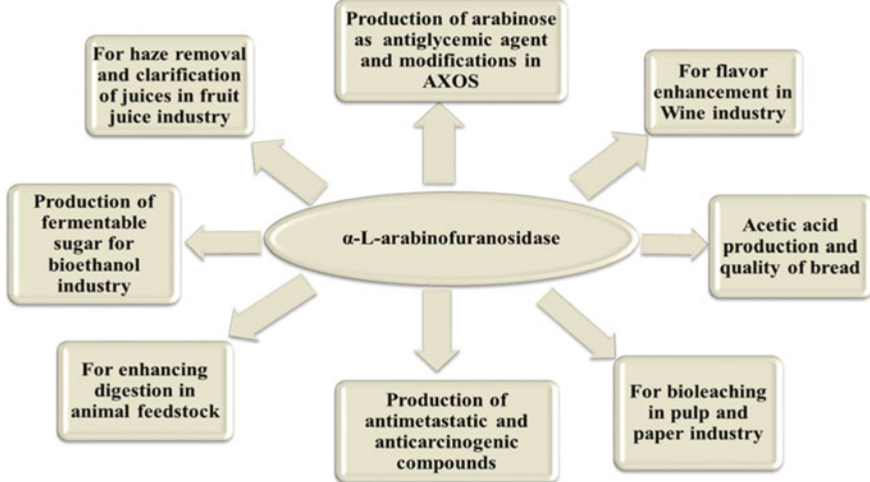


Fig. 12.3 Applications of α-L-arabinofuranosidase

12.2 Arabinoxylan

Arabinoxylan (AX) is an elemental part of plant cell wall, which is a non-starch polysaccharide and is nondigestible. All main cereal grains, including rye, wheat, rice, barley, oats, sorghum, and maize are rich in AX (Izydorczyk and Biliaderis 1995). Moreover, it is also present in other plants such as psyllium (Fischer et al. 2004), rye grass, and bamboo shoots (Dotsenko et al. 2017; Zelaya et al. 2017). In the common cereal grains, the maximum amount of AX is present in rye followed by wheat, barley, oats, rice, and sorghum (Table 12.1) (Dervilly-Pinel et al. 2001; Stone et al. 2009; Izydorczyk and Dexter 2008; Rao and Muralikrishna 2007; Freitas et al. 2003; Hashimoto et al. 1987; Holtekj⊘len et al. 2008). In cereals, starchy endosperm, aleurone, bran tissues, and some cereal's husk contain arabinoxylans in bulk. The content of arabinoxylan also depends on genetic and environmental factors (Holtekj⊘len et al. 2008). It has been reported that arabinoxylan content in wheat, rye, and barley contains genotypic and environmental variations (Lempereur et al. 1997). However, the deciding factor for variation in arabinoxylan content and its molecular structure is genus and species they belongs.

α-L-arabinofuranosyl residues are linked to a few of the xylopyranosyl unit of backbone at C2, C3 or at both C2, C3 positions (Fig. 12.2). Due to these conformations of arabinofuranosyl residues, four different molecular structures of AXexist: mono substituted xylopyranosyl at C2 or C3, disubstituted xylopyranosyl at C2, C3 and without any substitution the xylopyranosyl unit. The most of AX contains higher number of arabinofuranosyl substitution as mono substitution, while, a few AX contains two or more arabinofuranosyl residues attached by $\alpha(1 \rightarrow 2)$, $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 5)$linkages. The secondary walls in the pericarp

Table 12.1 Arabinose and xylose composition in different cereals

Cereals	Arabinose quantity (%)	Arabinose/Xylose ratio	References
Rye	6.9–7.6	0.7–1.1	(Zelaya et al. 2017)
Wheat	5.5–7.1	0.6	(Dervilly-Pinel et al. 2001)
Barley	4–5.4	0.7	(Stone et al. 2009)
Rice	2–3	0.8–0.9	(Izydorczyk and Dexter 2008)
Maize	1–2	0.8	(Rao and Muralikrishna 2007)
Oats	2–2.7	–	(Freitas et al. 2003)
Sorghum	1.8	0.9	(Hashimoto et al. 1987)

and testa tissues of bran contain other substitution of 4-O-methyl glucuronic acid at C2 position along with arabinose substitution at C3 position and make a structure called (arabino)glucuronoxylans (AGX) (27). AX from rice, sorghum, finger millets, and maize bran are more complicated in comparison to that from the common cereals (Rao and Muralikrishna 2004; Ebringerova and Heinze 2000). They may contain ample amounts of glucuronic acid residues linked at O-2 and are specified as (glucurono)arabinoxylans(GAX) (Ebringerova and Heinze 2000). Sorghum GAX contains one additional substitution of arabinofuranosyl residue at position C3 of the xylan backbone along with the arabinose substitution at position C2. The side-chain substitution in corncob AGX contains traces of xylopyranose, galactopyranose and α-D-glucuronic acid or 4-O-methyl-α-D-glucuronic sugar units in addition to arabinose units. The terminal xylose residue of corncob AGX is substituted with one arabinose residue at C2 position (Faulds et al. 1995).

AX exhibits various important characteristics like viscosity improvement, formation of a gel, stabilization of foam, absorption of water, restoration of fat, and prebiotic properties (Izydorczyk and Biliaderis 1995). As an ingredient of dietary fiber in cereals, AX enhances the nutritional value of foods by offering both soluble and insoluble fiber. Moreover, presence of the phenolic content in AX molecular structures, imparts antioxidant properties (Katapodis et al. 2003). The dietary fibers are essential for human health as they can lower down the blood cholesterol, regulate the level of blood glucose, show anticancer activity (Kendall et al. 2010), and are also effective against the proliferation of colorectal cancer (Samuelsen et al. 2011). On enzymatic treatment of AX arabino-xylooligosaccharides (AXOS) are produced which are described as potential prebiotics (Grootaert et al. 2007; Hughes et al. 2007). Due to the presence of AX in the majority of cereals, it constitutes a compelling part of ingested human dietary fiber. Agricultural by-products with less economic value, such as stalks, distiller's grain, seed cake, straw, hulls and husk of various grains, or banana peels, are potential sources of AX and other xylans.

12.3 α-L-arabinofuranosidase

α-L-arabinofuranosidase is of two types endo α-L-arabinofuranosidase (EC 3.2.1.99) and exo α-L-arabinofuranosidase (EC 3.2.1.55). Endo α-L-arabinofuranosidase (EC 3.2.1.99) cleaves α(1 → 5) arabinofuranosidic linkages in arabinans, whereas, exo α-L-arabinofuranosidase (EC 3.2.1.55) cleaves α(1 → 2), (1 → 3), and (1 → 5)-linked arabinose side chain from arabinose-substituted polysaccharides (Wilkens et al. 2017). Exo α-L-arabinofuranosidase (EC 3.2.1.55) are found in glycoside hydrolase (GH) families 2, 3, 43, 51, 54, and 62 (http://www.cazy.org/). The biochemical properties of recombinant α-L-arabinofuranosidase from various fungi and bacteria are listed in (Table 12.2) (Shinozaki et al. 2014; Couturier et al. 2011; Pérez and Eyzaguirre 2016; Sakamoto et al. 2011; Yang et al. 2015; Ravanal and Eyzaguirre 2015; Culleton et al. 2014; Cartmell et al. 2011; Shin et al. 2003; Margolles and Clara 2003; de Camargo et al. 2018; Ahmed et al. 2013; Birgisson et al. 2004; Debeche et al. 2000). α-L-arabinofuranosidase hydrolyzes arabinofuranosyl residue present at the nonreducing end of AX (Fig. 12.2). As a result, arabinose is released in an exolytic mode from the substrates containing (1 → 2) and (1 → 3) α-L-arabinofuranosidic linkages (Saha 2000). The arabinose substitution in hemicellulose interact with cellulose, pectin, and lignin make the structure complicated and provide strength to the plant cell wall. The substitution of arabinose residue in hemicellulose and pectin hinders the activity of hemicellulolytic and pectinolytic enzymes. Therefore, α-L-arabinofuranosidase can be used in synergistic manner for the enhancement of plant biomass hydrolysis (Rye and Withers 2000; Shallom et al. 2002). Enzymatic hydrolysis releases soluble sugars, which is utilized by prokaryotic and eukaryotic microorganisms for the production of several value-added products like xylitol, bioethanol, etc. (Margolles-Clark et al. 1996). α-L-arabinofuranosidase does not differentiate on the basis of backbone to which arabinofuranosyl residues is attached and thus shows a wide range of substrate specificity (Rahman et al. 2003).

α-L-arabinofuranosidases from different families show variation in substrate specificity in terms of the linkage(s) they hydrolyze. The family GH1 and GH30 arabinofuranosidases show exolytic mode of action acting against pNP-arabinofuranose and α-(1 → 5) arabino-oligosaccharides (Suzuki et al. 2013; Zhou et al. 2012). GH93 family enzymes release arabinobiose from the nonreducing end of α-(1 → 5)-L-arabinan (Carapito et al. 2009). α-L-arabinofuranosidases from family GH51 and GH54 remove branching arabinose residues from both arabinan and arabinoxylans (Taylor et al. 2006). GH3 arabinofuranosidases display both α-L-arabinofuranosidase and β-D-xylopyranosidase activities with pNP substrates (Lee et al. 2003). The family GH43 arabinofuranosidases display different types of activities such as endo α-L-arabinanase (Mckie et al. 1997), exo α-(1 → 5)-L-arabinanase (Bourgois et al. 2007) and arabinofuranosidase (Ahmed et al. 2013). Endo α-L-arabinanase (Mckie et al. 1997) and exo α-(1 → 5)-L-arabinanase (Bourgois et al. 2007) cleave (1 → 5)-linked arabinose from

Table 12.2 Biochemical properties of α-L-arabinofuranosidase from different microorganisms

Organism	Mol mass (kDa)	Optimum temp (°C)	Optimum pH	GH family	References
Fungus					
Penicillium chrysogenum 31B	31.0	35	5.0	43	(Wilkens et al. 2017)
Podospora anserine	37	37	5.0	62	(Shinozaki et al. 2014)
Aspergillus fumigatus	43.5	42	4.5–5.0	62	(Couturier et al. 2011)
Penicillium chrysogenum	35.0	40–50	5.0	62	(Pérez and Eyzaguirre 2016)
Humicola insolens	50.6	50	5.0	43	(Sakamoto et al. 2011)
Penicillium purpurogenum ABF 4	68.0	50	4.6	54	(Yang et al. 2015)
Aspergillus vadensis	53.0	60	3.5	54	(Ravanal and Eyzaguirre 2015)
Bacterium					
Cellvibrio japonicas	–	25	7.0	43	(Culleton et al. 2014)
Bifidobacterium breve K-110	60	45	4.5	–	(Cartmell et al. 2011)
Bifidobacterium longum	260	45	6	51	(Shin et al. 2003)
Clostridium thermocellum B8	50	50	5–6	43	(Margolles and Clara 2003)
Clostridium thermocellum	34	50	5.4	43	(de Camargo et al. 2018)
Thermomicrobia bacterium	350	70	6.0	51	(Ahmed et al. 2013)
Thermobacillus xylanilyticus	56	75	5.6–6.2	51	(Birgisson et al. 2004)

arabinan. α-L-arabinofuranosidase removes L-arabinose residues that are (1 → 2) and (1 → 3) linked to monosubstituted β-D-xylose backbone (Ahmed et al. 2013).

α-L-arabinofuranosidase hydrolyzes glycosidic bond by acid–base catalysis by employing two modes of action, first by either overall retention or by an inversion of the anomeric configuration (de Groot et al. 2003). It hydrolyzes the glycosidic linkage by a double displacement mechanism in two steps. Glycosylation is the first step in which, glycosidic oxygen is protonated and leaving group is stabilized by acid–base residue, which works as a general acid. Nucleophilic residue (glutamic acid) attacks the anomeric carbon of the scissile bond, and a covalent substrate enzyme intermediate is formed with the opposite anomeric configuration of the

glycosyl residue of the substrate. In the subsequent step of deglycosylation, a water molecule activated by acid–base residue behaves like a general base. The anomeric center of the substrate enzyme intermediate is attacked by activated water molecule along the same direction of the original bond, releasing the L-arabinose, and maintain anomeric configuration with overall retention (Ferchichi et al. 2003; Hövel et al. 2003). The arabinose residue in the side chain of hemicelluloses and pectins form cross-linking within the plant cell wall structure. The occurrence of arabinose side chains also hinders the structure and function of hemicelluloses and pectins (DeVries et al. 2000). Due to their intrinsically more flexible water-hungry furanose conformations, they reduce the interaction between polymer chains.

12.4 α-L-Arabinofuranosidase in the Food Industry

12.4.1 Quality Enhancement in Bread Making

Wheat and rye flour are majorly used for bread making. AX content in wheat is 1.5–3% and 7–8% in rye, out of which water-soluble AX is present in very little quantity (Shewry et al. 2010; Gebruers et al. 2010). The arabinose residue present in arabinoxylan increases the water uptake and absorbs around 30% of water in dough, which reduces the volume of dough and changes its texture. In sourdough fermentation, the solubilization of AX during fermentation contributes the constructive effects and enhances the bread quality (Yang et al. 2017). Pentosans are one of the necessary additives for bread which improve its texture (Yegin et al. 2018). Wheat flour enzymes slightly hydrolyze the pentosans, added to the dough. The hydrolysis of pentosans is further carried out by exogenic xylanolytic enzymes including α-L-arabinofuranosidase added to the dough (Fessas and Schiraldi 1998), resulting in the release of free pentosans, which are used by a sourdough lactic acid bacterium. During the sourdough fermentation, the arabinofuranosidases release arabinose and also synergistically enhance the activity of other enzymes. The soluble carbohydrates availability increases, thus acidification and acetic acid production also increases. The quality of bread is improved by α-L-arabinofuranosidase, pentosanase, and other enzymes, which have been investigated as natural supplements (Gobbetti et al. 2000). The treatment with arabinofuranosidase releases the trapped water in AX, which improves dough handling, crumb structure and increases the dough volume and thus delays the staling of bread and enhances the bread shelf life. The improved bread quality and shelf life gives economic benefits to the bread industries (Bosetto et al. 2016; Jiménez and Martinez-Anaya 1999). *Thermoascus aurantiacus* produces enzyme cocktail comprising xylanase, xylosidase, and arabinofuranosidase which release xylose and arabinose during solid-state fermentation after prolonged incubation. The enzyme mixture causes significant increase in volume of the wheat dough. The bread volume increased by 22%, whereas, crumb firmness and amylopectin retrogradation was reduced by 25 and 17%, respectively (Oliveira et al. 2014).

12.4.2 Flavor Enhancement in Juices and Wine

Aromatic compounds are one of the important constituents of wine. Fermenting microbes like yeast and bacteria produce aromatic compounds during fruits fermentation. The fruits used in the wine industry including grapes produce volatile compounds along with some nonvolatile compounds. These volatile compounds are responsible for aroma in wine. The nonvolatile compounds lose their aromatic properties because they are attached to carbohydrates as glycoconjugates and are thus flavorless (Zhu et al. 2017). The glycoconjugates are precursor of glycosidic aroma in wine. Glycoconjugates exist in the form of di-glycosides that are attached to monosaccharide moieties such as α-L-arabinofuranose, α-L-arabinopyranose, α-L-rhamnopyranose, β-D-apiofuranose, β-D-glucopyranose, or β-D-xylopyranose (Guo et al. 1993; Williams et al. 1982). Terpene is one of the important aromatic molecules, which is generally attached to α-L-arabinofuranose. α-L-arabinofuranosidase hydrolyze monoterpene and give rise to volatile terpenes and L-arabinose (Mateo and Di Stefano 1997; Belda et al. 2017).

Fruits are rich in carbohydrate polysaccharides such as pectin and arabinan. The polysaccharides, proteins, and polyphenols form precipitates during storage. α-L-arabinofuranosidase is used for clarification of juices in the fruit juice industry. For clarification of fruit juices, the pectinolytic enzymes are used in combination with α-L-arabinofuranosidase. The addition of α-L-arabinofuranosidase removes arabinose side chain from hemicelluloses that help in the degradation of pectin and arabinan by pectinolytic enzymes and α-1,5 arabinanase, respectively (Churms et al. 1983; De Vries et al. 1982). The enzymatic treatment enhances the solubility of juices by reducing the haze formation. The treatment of apple, grape, orange and peach juices with α-L-arabinofuranosidase and xylanase together cause improved clarity, reducing sugar content, and juice yield (İlgü et al. 2018).

12.4.3 Arabinoxylan and Arabinoxylan Oligosaccharide (AXOS) as Prebiotic

The arabinofuranosidase cleaves the arabinosyl residue present in the side chains of arabinoxylan backbone. Arabinoxylan oligosaccharide (AXOS) of varying backbone lengths and substitution of arabinose side chains can be produced by the action of xylanolytic enzymes. The break down of xylan produces short-chain AXOS which are not completely hydrolyzed, and these oligosaccharides are beneficial for the growth of probiotic bacteria. The degree of substitution and polymerization varies in AXOS depending on the source of arabinoxylan, which may be further modified by arabinofuranosidase and xylosidase treatment (Falck et al. 2018). The elucidation of the relationship between structure and functions of AXOS with changing polymerization and substitution is an interesting area for investigation. Several species of bacteria found in human alimentary canal have been

reported for producing the efficient enzymes to hydrolyze AX (Van Laere et al. 2000). Species, like lactobacilli, bacteroides, and nonpathogenic clostridia, found in the intestine are efficient in hydrolyzing complex carbohydrates. It was reported that species *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* belonging to the *Bacteroides fragilis* group play a crucial role in the metabolism of carbohydrates, as they secrete several carbohydrate-active enzymes (Berg et al. 1978; Cummings and Macfarlane 1991). AXOS produced from rye and rye bran is reported to affect positively on the growth of various *Lactobacillus* strains (Koistinen et al. 2018). Besides prebiotic effect, AX and AXOS play a vital role in human health by lowering cholesterol level and the risk of type II diabetes and obesity by reducing postprandial glucose level (Amrein et al. 2003; Lu et al. 2000; Möhlig et al. 2005).

12.4.4 Production of Sugar Alcohols from Agricultural Wastes

Agricultural wastes majorly contain cellulose, hemicellulose, and lignin. The enzymatic production of sugar alcohols from lignocellulosic biomass is a promising alternative to the chemical process. The saccharification of plant polysaccharides to monomeric sugars by enzymatic hydrolysis is reported (Sartori et al. 2015). Cocktail of enzymes including cellulases, hemicellulases are required for complete hydrolysis of plant polysaccharides. Cellulases (endoglucanase, cellobiohydrolase, and glucosidase) convert cellulose to glucose whereas, hemicellulases like xylanase, xylosidase, and arabinofuranosidase release xylose and arabinose from hemicellulose present in agricultural waste. Released sugars undergo hydrogenation reaction in the presence of metal catalyst for production of sugar alcohols, like xylitol, arabitol, galactitol, sorbitol, mannitol, etc. (Tathod and Dhepe 2015). These sugar alcohols are used as low-calorie sweeteners as they have very low glycemic index (Grembecka 2015). They also impart health benefits as they are non-cariogenic, improve dental health, promote the absorption of calcium and B vitamins, etc. (Grembecka 2015). L-arabinose released by arabinofuranosidase is converted to xylitol (Sakakibara et al. 2009).

12.5 Conclusion and Future Prospective

α-L-arabinofuranosidase acts synergistically with other carbohydrate-active enzymes for complete degradation of lignocellulosic biomass into its basic reducing or monomeric sugar units. α-L-arabinofuranosidase has great potential for application in fuel, animal feed, therapeutic and pharmaceutical, and food industries. Treatment of dough with α-L-arabinofuranosidase delays the staling of bread, thereby adding the quality factor to the bread reducing the enormous losses to the industries. Several aromatic compounds present in the form of glycoconjugates

need the treatment of these enzymes to add flavor in alcoholic beverages. In fruit juice processing, this enzyme in combination with others hydrolyzes the precipitate formed thereby, increasing the solubility and clarity of juices. The action of xylanolytic enzyme on AX produces AXOS with varying degree of polymerization and substitutions. AXOS displays potential as prebiotic conferring several health benefits to the host. Arabinose released by the action of α-L-arabinofuranosidase is used as artificial sweetener and can be used as an antiglycemic agent. The demand for healthy food is on the rise. AXOS are more diverse in comparison with the other commercially available oligosaccharides. Therefore, further research is required to investigate the health-promoting effects of AXOS with varying structures. There is also a need to find more efficient α-L-arabinofuranosidases for industrial perspective by genome mining from new novel organisms.

References

Ahmed S, Luis AS, Bras JL, Ghosh A, Gautam S, Gupta MN, Fontes CM, Goyal A (2013) A novel α-L-arabinofuranosidase of family 43 glycoside hydrolase (*Ct*43Ara*f*) from *Clostridium thermocellum*. PLoS ONE 8(9):e73575

Amrein TM, Gränicher P, Arrigoni E, Amadò R (2003) *In vitro* digestibility and colonic fermentability of aleurone isolated from wheat bran. LWT-Food Sci Technol 36(4):451–460

Beg Q, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56(3–4):326–338

Belda I, Ruiz J, Esteban-Fernández A, Navascués E, Marquina D, Santos A, Moreno-Arribas M (2017) Microbial contribution to wine aroma and its intended use for wine quality improvement. Molecules 22(2):189

Berg JO, Nord CE, Wadström T (1978) Formation of glycosidases in batch and continuous culture of *Bacteroides fragilis*. Appl Environ Microbiol 35(2):269–273

Birgisson H, Fridjonsson O, Bahrani-Mougeot FK, Hreggvidsson GO, Kristjansson JK, Mattiasson B (2004) A new thermostable α-L-arabinofuranosidase from a novel thermophilic bacterium. Biotech Lett 26(17):1347–1351

Bosetto A, Justo PI, Zanardi B, Venzon SS, Graciano L, dos Santos EL, Simão RDCG (2016) Research progress concerning fungal and bacterial β-xylosidases. Appl Biochem Biotechnol 178(4):766–795

Bourgois TM, Van Craeyveld V, Van Campenhout S, Courtin CM, Delcour JA, Robben J, Volckaert G (2007) Recombinant expression and characterization of XynD from Bacillus subtilis subsp. subtilis ATCC 6051: a GH 43 arabinoxylan arabinofuranohydrolase. Appl Microbiol Biotechnol 75(6):1309–1317

Brett C, Waldron K (1990) Cell wall structure and the skeletal functions of the wall. Physiology and. Springer, Dordrecht, pp 4–57

Carapito R, Imberty A, Jeltsch JM, Byrns SC, Tam PH, Lowary TL, Varrot A, Phalip V (2009) Molecular basis of arabinobio-hydrolase activity in phytopathogenic fungi. Crystal structure and catalytic mechanism of *fusarium graminearum* GH93 exo-alpha-L-arabinanase. J Biol Chem

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3(1):1–30

Cartmell A, McKee LS, Peña MJ, Larsbrink J, Brumer H, Kaneko S, Ichinose H, Lewis RJ, Viksø-Nielsen A, Gilbert HJ, Marles-Wright J (2011) The structure and function of an

arabinan-specific α-1,2-arabinofuranosidase identified from screening the activities of bacterial GH43 glycoside hydrolases. J Biol Chem 286(17):15483–15495

Churms SC, Merrifield EH, Stephen AM, Walwyn DR, Polson A, van der Merwe KJ, Spies HS, Costa N (1983) An L-arabinan from apple-juice concentrates. Carbohyd. Res. 113(2):339–344

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6(11):850

Couturier M, Haon M, Coutinho PM, Henrissat B, Lesage-Meessen L, Berrin JG (2011) *Podospora anserina* hemicellulases potentiate the *Trichoderma reesei* secretome for saccharification of lignocellulosic biomass. Appl Environ Microbiol 77(1):237–246

Culleton H, McKie VA, de Vries RP (2014) Overexpression, purification and characterisation of homologous α-L-arabinofuranosidase and endo-1,4-β-D-glucanase in *Aspergillus vadensis*. J Ind Microbiol Biotechnol 41(11):1697–1708

Cummings JH, Macfarlane GT (1991) The control and consequences of bacterial fermentation in the human colon. J Appl Microbiol 70(6):443–459

de Camargo BR, Claassens NJ, Quirino BF, Noronha EF, Kengen SW (2018) Heterologous expression and characterization of a putative glycoside hydrolase family 43 arabinofuranosidase from *Clostridium thermocellum* B8. Enzyme Microb Technol 109:74–83

de Groot MJ, van de Vondervoort PJ, de Vries RP, Ruijter GJ, Visser J (2003) Isolation and characterization of two specific regulatory Aspergillus niger mutants shows antagonistic regulation of arabinan and xylan metabolism. Microbiology 149(5):1183–1191

De Vries JA, Rombouts FM, Voragen AGJ, Pilnik W (1982) Enzymic degradation of apple pectins. Carbohyd Polym 2(1):25–33

Debeche T, Cummings N, Connerton I, Debeire P, O'Donohue MJ (2000) Genetic and biochemical characterization of a highly thermostable α-L-arabinofuranosidase from *Thermobacillus xylanilyticus*. Appl Environ Microbiol 66(4):1734–1736

Dervilly-Pinel G, Rimsten L, Saulnier L, Andersson R, Åman P (2001) Water-extractable arabinoxylan from pearled flours of wheat, barley, rye and triticale. Evidence for the presence of ferulic acid dimers and their involvement in gel formation. J Cereal Sci 34(2):207–214

DeVries RP, Kester HC, Poulsen CH, Benen JA, Visser J (2000) Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides. Carbohyd Res 327 (4):401–410

Dhillon A, Fernandes VO, Dias FM, Prates JA, Ferreira LM, Fontes CM, Centeno MSJ, Goyal A (2016) A new member of family 11 polysaccharide lyase, rhamnogalacturonan lyase (*Ct*RGLf) from *Clostridium thermocellum*. Mol. Biotechnol. 58(4):232–240

Dotsenko G, Meyer AS, Canibe N, Thygesen A, Nielsen MK, Lange L (2017) Enzymatic production of wheat and ryegrass derived xylooligosaccharides and evaluation of their in vitro effect on pig gut microbiota. Biomass Convers Biorefinery 8:1–11

Ebringerova A, Heinze T (2000) Xylan and xylan derivatives-biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. Macromol Rapid Commun 21(9):542–556

Falck P, Linares-Pastén JA, Karlsson EN, Adlercreutz P (2018) Arabinoxylanase from glycoside hydrolase family 5 is a selective enzyme for production of specific arabinoxylooligosaccharides. Food Chem 242:579–584

Faulds CB, Kroon PA, Saulnier L, Thibault JF, Williamson G (1995) Release of ferulic acid from maize bran and derived oligosaccharides by *Aspergillus niger* esterases. Carbohyd Polym 27 (3):187–190

Ferchichi M, Rémond C, Simo R, O'Donohue MJ (2003) Investigation of the functional relevance of the catalytically important Glu28 in family 51 arabinosidases. FEBS Lett 553(3):381–386

Fessas D, Schiraldi A (1998) Texture and staling of wheat bread crumb: effects of water extractable proteins and 'pentosans'. Thermochim Acta 323(1–2):17–26

Fischer MH, Yu N, Gray GR, Ralph J, Anderson L, Marlett JA (2004) The gel-forming polysaccharide of psyllium husk (Plantago ovata Forsk). Carbohyd Res 339(11):2009–2017

Freitas RA, Gorin PAJ, Neves J, Sierakowski MR (2003) A rheological description of mixtures of a galactoxyloglucan with high amylose and waxy corn starches. Carbohyd Polym 51(1):25–32

Gebruers K, Dornez E, Bedo Z, Rakszegi M, Courtin CM, Delcour JA (2010) Variability in xylanase and xylanase inhibition activities in different cereals in the healthgrain diversity screen and contribution of environment and genotype to this variability in common wheat. J Agric Food Chem 58(17):9362–9371

Gobbetti M, Lavermicocca P, Minervini F, De Angelis M, Corsetti A (2000) Arabinose fermentation by *Lactobacillus plantarum* in sourdough with added pentosans and α-L-arabinofuranosidase: a tool to increase the production of acetic acid. J Appl Microbiol 88 (2):317–324

Grembecka M (2015) Sugar alcohols-their role in the modern world of sweeteners: a review. Eur Food Res Technol 241(1):1–14

Grootaert C, Delcour JA, Courtin CM, Broekaert WF, Verstraete W, Van de Wiele T (2007) Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. Trends Food Sci Technol 18(2):64–71

Guo W, Sakata K, Watanabe N, Nakajima R, Yagi A, Ina K, Luo S (1993) Geranyl 6-O-β-d-xylopyranosyl-β-d-glucopyranoside isolated as an aroma precursor from tea leaves for oolong tea. Phytochemistry 33(6):1373–1375

Hashimoto S, Shogren MD, Pomeranz Y (1987) Cereal pentosans: their estimation and significance. I. Pentosans in wheat and milled wheat products. Cereal Chem 64(1):30–34

Holtekj⊘len AK, Uhlen AK, Knutsen SH (2008) Barley carbohydrate composition varies with genetic and abiotic factors. Acta Anaesthesiol Scand Section B-Soil Plant Sci 58(1):27–34

Hövel K, Shallom D, Niefind K, Belakhov V, Shoham G, Baasov T, Shoham Y, Schomburg D (2003) Crystal structure and snapshots along the reaction pathway of a family 51 α-l-arabinofuranosidase. EMBO J 22(19):4922–4932

Hughes SA, Shewry PR, Li L, Gibson GR, Sanz ML, Rastall RA (2007) In vitro fermentation by human fecal microflora of wheat arabinoxylans. J Agric Food Chem 55(11):4589–4595

İlgü H, Sürmeli Y, Şanlı-Mohamed G (2018) A thermophilic α-l-Arabinofuranosidase from *Geobacillus vulcani* GS90: heterologous expression, biochemical characterization, and its synergistic action in fruit juice enrichment. Eur Food Res Technol 244:1–10

Izydorczyk MS, Biliaderis CG (1995) Cereal arabinoxylans: advances in structure and physicochemical properties. Carbohyd Polym 28(1):33–48

Izydorczyk MS, Dexter JE (2008) Barley β-glucans and arabinoxylans: molecular structure, physicochemical properties, and uses in food products—a review. Food Res Int 41(9):850–868

Jiménez T, Martinez-Anaya MA (1999). Enzymes, a key to improve bread and dough quality: degradation by products and relationship with quality. In: 17th ICC conference of the cereal across the continents, vol 6, No. 9, p. 168

Katapodis P, Vardakou M, Kalogeris E, Kekos D, Macris BJ, Christakopoulos P (2003) Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. Eur J Nutr 42(1):55–60

Kendall CW, Esfahani A, Jenkins DJ (2010) The link between dietary fibre and human health. Food Hydrocolloids 24(1):42–48

Koistinen VM, Mattila O, Katina K, Poutanen K, Aura AM, Hanhineva K (2018) Metabolic profiling of sourdough fermented wheat and rye bread. Sci Rep 8(1):5684

Lee RC, Hrmova M, Burton RA, Lahnstein J, Fincher GB (2003) Bifunctional family 3 glycoside hydrolases from barley with α-L-arabinofuranosidase and β-d-xylosidase activity characterization, primary structures, and COOH-terminal processing. J Biol Chem 278(7):5377–5387

Lempereur I, Rouau X, Abecassis J (1997) Genetic and agronomic variation in arabinoxylan and ferulic acid contents of durum wheat (*Triticum durumL.*) grain and its milling fractions. J Cereal Sci 25(2):103–110

Lu ZX, Walker KZ, Muir JG, Mascara T, O'Dea K (2000) Arabinoxylan fiber, a byproduct of wheat flour processing, reduces the postprandial glucose response in normoglycemic subjects. Am J Clin Nutr 71(5):1123–1128

Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microb Mol Biol Rev 66(3):506–577

Margolles A, Clara G (2003) Purification and functional characterization of a novel α-L-arabinofuranosidase from *Bifidobacterium longum* B667. Appl Environ Microbiol 69(9):5096–5103

Margolles-Clark E, Tenkanen M, Nakari-Setälä TIINA, Penttilä MERJA (1996) Cloning of genes encoding alpha-L-arabinofuranosidase and beta-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62(10):3840–3846

Mateo JJ, Di Stefano R (1997) Description of the β-glucosidase activity of wine yeasts. Food Microbiol 14(6):583–591

Mckie VA, Gary W, Millward-Sadler SJ, Hazlewood GP, Laurie JI, Gilbert HJ (1997) Arabinanase A from *Pseudomonas fluorescens subsp.* cellulosa exhibits both an endo-and an exo-mode of action. Biochem J 323(2):547–555

Möhlig M, Koebnick C, Weickert MO, Lueder W, Otto B, Steiniger J, Twilfert M, Meuser F, Pfeiffer AFH, Zunft HJ (2005) Arabinoxylan-enriched meal increases serum ghrelin levels in healthy humans. Horm Metab Res 37(05):303–308

Numan MT, Bhosle NB (2006) α-L-arabinofuranosidases: the potential applications in biotechnology. J Ind Microbiol Biotechnol 33(4):247–260

Ochiai A, Itoh T, Kawamata A, Hashimoto W, Murata K (2007) Plant cell wall degradation by saprophytic Bacillus subtilis strains: gene clusters responsible for rhamnogalacturonan depolymerization. Appl Environ Microbiol 73(12):3803–3813

Oliveira DS, Telis-Romero J, Da-Silva R, Franco CML (2014) Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread qualitiy. Food Chem 143:139–146

Ordaz-Ortiz JJ, Saulnier L (2005) Structural variability of arabinoxylans from wheat flour. Comparison of water-extractable and xylanase-extractable arabinoxylans. J Cereal Sci 42 (1):119–125

Pérez R, Eyzaguirre J (2016) *Aspergillus fumigatus* produces two arabinofuranosidases from glycosyl hydrolase family 62: comparative properties of the recombinant enzymes. Appl. Biochem. Biotechnol. 179(1):143–154

Rahman AS, Kato K, Kawai S, Takamizawa K (2003) Substrate specificity of the α-L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1. Carbohyd Res 338(14):1469–1476

Rao MS, Muralikrishna G (2004) Structural analysis of arabinoxylans isolated from native and malted finger millet (*Eleusine coracana*, ragi). Carbohyd Res 339(14):2457–2463

Rao RSP, Muralikrishna G (2007) Structural characteristics of water-soluble feruloyl arabinoxylans from rice (*Oryza sativa*) and ragi (finger millet, *Eleusine coracana*): Variations upon malting. Food Chem 104(3):1160–1170

Ravanal MC, Eyzaguirre J (2015) Heterologous expression and characterization of α-L-arabinofuranosidase 4 from *Penicillium purpurogenum* and comparison with the other isoenzymes produced by the fungus. Fungal Biol 119(7):641–647

Rye CS, Withers SG (2000) Glycosidase mechanisms. Curr Opin Chem Biol 4(5):573–580

Saha BC (2000) α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 18(5):403–423

Sakakibara Y, Saha BC, Taylor P (2009) Microbial production of xylitol from L-arabinose by metabolically engineered Escherichia coli. J Biosci Bioeng 107(5):506–511

Sakamoto T, Ogura A, Inui M, Tokuda S, Hosokawa S, Ihara H, Kasai N (2011) Identification of a GH62 α-L-arabinofuranosidase specific for arabinoxylan produced by *Penicillium chrysogenum*. Appl Microbiol Biotechnol 90(1):137–146

Samuelsen AB, Rieder A, Grimmer S, Michaelsen TE, Knutsen SH (2011) Immunomodulatory activity of dietary fiber: arabinoxylan and mixed-linked beta-glucan isolated from barley show modest activities in vitro. Int J Mol Sci 12(1):570–587

Sartori T, Tibolla H, Prigol E, Colla LM, Costa JAV, Bertolin TE (2015). Enzymatic saccharification of lignocellulosic residues by cellulases obtained from solid state fermentation using *Trichoderma viride*. BioMed Res Int

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

Seri K, Sanai K, Matsuo N, Kawakubo K, Xue C, Inoue S (1996) L-arabinose selectively inhibits intestinal sucrase in an uncompetitive manner and suppresses glycemic response after sucrose ingestion in animals. Metabolism 45(11):1368–1374

Shallom D, Belakhov V, Solomon D, Gilead-Gropper S, Baasov T, Shoham G, Shoham Y (2002) The identification of the acid-base catalyst of α-arabinofuranosidase from *Geobacillus stearothermophilus* T6, a family 51 glycoside hydrolase. FEBS Lett 514(2–3):163–167

Shewry PR, Piironen V, Lampi AM, Edelmann M, Kariluoto S, Nurmi T, Fernandez-Orozco R, Andersson AA, Åman P, Fras A, Boros D (2010) Effects of genotype and environment on the content and composition of phytochemicals and dietary fiber components in rye in the HEALTHGRAIN diversity screen. J Agric Food Chem 58(17):9372–9383

Shin HY, Park SY, Sung JH, Kim DH (2003) Purification and Characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. Appl Environ Microbiol 69(12):7116–7123

Shinozaki A, Kawakami T, Hosokawa S, Sakamoto T (2014) A novel GH43 α-L-arabinofuranosidase of *Penicillium chrysogenum* that preferentially degrades single-substituted arabinosyl side chains in arabinan. Enzyme Microb Technol 58:80–86

Sjostrom E (2013) Wood chemistry: fundamentals and applications. Elsevier, Amsterdam

Stone B, Morell MK, Khan K, Shewry PR (2009). Wheat: chemistry and technology, 4th edn

Suzuki H, Murakami A, Yoshida KI (2013) Motif-guided identification of a glycoside hydrolase family 1 α-L-arabinofuranosidase in *Bifidobacterium adolescentis*. Biosci Biotechnol Biochem 77(8):1709–1714

Tathod AP, Dhepe PL (2015) Efficient method for the conversion of agricultural waste into sugar alcohols over supported bimetallic catalysts. Biores Technol 178:36–44

Taylor EJ, Smith NL, Turkenburg JP, D'souza S, Gilbert HJ, Davies GJ (2006) Structural insight into the ligand specificity of a thermostable family 51 arabinofuranosidase, Araf51, from *Clostridium thermocellum*. Biochem J 395(1):31–37

Van Laere KM, Hartemink R, Bosveld M, Schols HA, Voragen AG (2000) Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. J Agric Food Chem 48(5):1644–1652

Wilkens C, Andersen S, Dumon C, Berrin JG, Svensson B (2017) GH62 arabinofuranosidases: structure, function and applications. Biotechnol Adv 35(6):792–804

Williams PJ, Strauss CR, Wilson B, Massy-Westropp RA (1982) Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. Phytochemistry 21(8):2013–2020

Yang X, Shi P, Ma R, Luo H, Huang H, Yang P, Yao B (2015) A new GH43 α-arabinofuranosidase from *Humicola insolens* Y1: biochemical characterization and synergistic action with a xylanase on xylan degradation. Appl Biochem Biotechnol 175(4):1960–1970

Yang W, Jiang Z, Liu L, Lin Y, Wang L, Zhou S (2017) The effect of pentosanase on the solubilisation and degradation of arabinoxylan extracted from whole and refined wheat flour. J Sci Food Agric 97(3):1034–1041

Yegin S, Altinel B, Tuluk K (2018) A novel extremophilic xylanase produced on wheat bran from *Aureobasidium pullulans* NRRL Y-2311-1: effects on dough rheology and bread quality. Food Hydrocolloids 81:389–397

Zelaya VM, Fernández PV, Vega AS, Mantese AI, Federico AA, Ciancia M (2017) Glucuronoarabinoxylans as major cell walls polymers from young shoots of the woody bamboo *Phyllostachys aurea*. Carbohyd Polym 167:240–249

Zhou J, Bao L, Chang L, Zhou Y, Lu H (2012) Biochemical and kinetic characterization of GH43 β-D-xylosidase/α-L-arabinofuranosidase and GH30 α-L-arabinofuranosidase/β-D-xylosidase from rumen metagenome. J Ind Microbiol Biotechnol 39(1):143–152

Zhu F, Du B, Ma Y, Li J (2017) The glycosidic aroma precursors in wine: occurrence, characterization and potential biological applications. Phytochem Rev 16(3):565–571

Chapter 13
Agro-Industrial By-Products in the Synthesis of Food Grade Microbial Pigments: An Eco-Friendly Alternative

Bishwambhar Mishra, Sunita Varjani and G. Karthikeya Srinivasa Varma

Abstract Food industry is mainly dependent upon various colors to prepare the food items attractive and appealing to the consumers. Chemically synthesized food colorant used as the additives in foods causes the risk of toxicity and hazardous effects to the consumers. The application of biosynthesized natural colorant as food additives is quite safer, nontoxic, and nonhazardous in nature. Currently, the researchers have developed the value-added products like microbial pigments by utilizing various agro-industrial waste products through the fermentation processes. This can make the whole process cost-effective and environmental friendly. The current chapter describes the utilization of cheaply available agro-industrial residues for the production of microbial pigment which can be explored further for its application in the food Industries. In this chapter, the attention of researchers, academicians, and the food industry professionals have drawn to the stimulating findings in the research field of microbial pigments considering various basic approaches in the related topic like microbial source of pigments, strain improvements, fermentative production, metabolic engineering, and future aspects.

Keywords Food colorant · Agro-industrial waste · Fermentation
Microbial pigments · Eco-friendly

B. Mishra (✉)
Department of Biotechnology, Centurion University of Technology and Management,
Bhubaneswar 752050, Odisha, India
e-mail: mishra.bishwambhar@gmail.com

S. Varjani
Gujarat Pollution Control Board, Sector-10A, Gandhinagar 382010, Gujarat, India

G. Karthikeya Srinivasa Varma
Department of Biotechnology, Sreenidhi Institute of Science and Technology, Hyderabad
501301, Telangana, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment,
and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_13

13.1 Introduction

Agro-industrial wastes are rich in natural carbon and oxygen sources and bioactive compounds. The waste generated during the production has disposable problems as well as loss of raw materials which are not used to the limit. The perception toward the waste has been changed over the years and is considered as a valuable resource now. The waste products can be used as a feedstock for fermentation to develop products which are used instead of its conventional applications, i.e., feed for cattle. Various processes have been introduced and developed in order to utilize the agro-industrial residues for the production of bulk chemicals and value-added products (Araujo et al. 2010).

Chemically synthesized pigments are the potential source of cancer as they are carcinogenic in nature. Therefore, people are shifting toward the use of edible coloring agents which are naturally occurring and safe (Chadni et al. 2017). The potential source of biopigment production is microbial sources like bacteria, yeast, and mold because of their advantages over similar pigments produced from plants. Various biopigments such as quinines, violancein, monascins, flavins, and carotenoids were produced from microorganisms (Duran et al. 2007). The microbial pigments which are produced by microorganisms are independent of weather conditions, grow fast, and are also cost-effective when compared to the production of pigments from plants (Eisenman and Casadevall 2012). A broad range of waste from agro-industries such as corn steep liquor (Hamano and Kilikian 2006), jackfruit seed (Sumathy et al. 2007), grape waste (Silvana et al. 2008), etc., are a potential source of carbon, nitrogen, and mineral sources for the production of pigments from microorganisms.

13.1.1 History of Microbial Pigment

Addition of the color to foods is an old practice but use of microbial pigments is a recent phenomenon. Dyeing dates back to the Indus valley period (2600-1900BC) as there were findings of red colored clothes and traces of madder dye in the ruined site of Indus valley civilization at Mohenjo-Daro and Harappa. The record found in China dated 2600 BC was found to be the oldest one which was written with natural dye (Heer and Sharma 2017). The techniques for the production of natural dyes became more sophisticated over time. The dyes were prepared from crushed fruits and vegetables, berries, and other plants through boiling.

Natural and biological pigments were the sole source of colors available before the onset of synthetic pigments. In the year 1856, Perkin had introduced many synthetic pigments which were very cheap and convenient to be used, due to which the use of natural dye was decreased (Joshi et al. 2003).

Synthetic molecules such as pthalocyanine that range from blue-green, arylides that are reddish yellow and quinacridones ranging from orange to violet, have stopped the usage of naturally occurring pigments (Kamla et al. 2012). In addition

Table 13.1 Chronological development of microbial pigments (Joshi et al. 2003)

S. No.	Year	Development
1	1856	Coaltar dyes were synthesized; Perkin's mauve pigment was discovered
2	1884	Production of red rice wine and red Chinese rice by utilizing *Monascus* sp.
3	1954	Production of Carotenoid pigment by *Cryptococcus* which was marketed for the first time
4	1963	Production of Carotenoid pigments commercially from *Rhodotorula* sp. for the first time
5	1970	Astaxanthin was isolated from *Phaffia rhodozyma* for the first time
6	Early 1980s	Beta-carotene from *Dunaliella salina* was started
7	1985	Beta-carotene products were commercially produced by Betatene Limited Corporation and cultivation of *Dunaliella salina* on large scale for its production

to that, with the advancement in organic chemistry, the production of these chemically synthesized pigments has become relatively cheaper. Therefore, the application of natural dye was decreased drastically. The historical development of various activities related to microbial pigments has been summarized in Table 13.1.

13.1.2 Synthetic Pigments Versus Microbial Pigments

Natural and synthetic dyes are used extensively in various fields such as textile and paper industry, food additives, and agricultural purposes (Kamla et al. 2012). As synthetic dyes are carcinogenic in nature, therefore the use of natural materials as starting products is encouraged for production. Natural pigments tend to increase the marketability of products and are advantageous compared to synthetic pigments, i.e., they act as antioxidants and anticancer agents. Bacterial pigments are used in the production of cosmetics, food, textiles, etc., and are of a good market value if the product is prepared using natural pigments (Li et al. 2017).

Natural products which impart color to the substances such as alkaloids, flavonoids, etc., were used as colors, flavors by humans. As there was limited availability of natural products, people have shifted toward the use of synthetic compounds and it has gained more importance. As the research in the field on pigments has increased, they have found out that synthetic pigments are toxic in nature and now people are demanding the use of natural pigments (Konstantina and Triantafyllos 2016). The new techniques of production of natural pigments have been discovered using microorganisms and are expected to continue as a source of natural pigments in future. The major sources of the biopigments have been illustrated in Fig. 13.1.

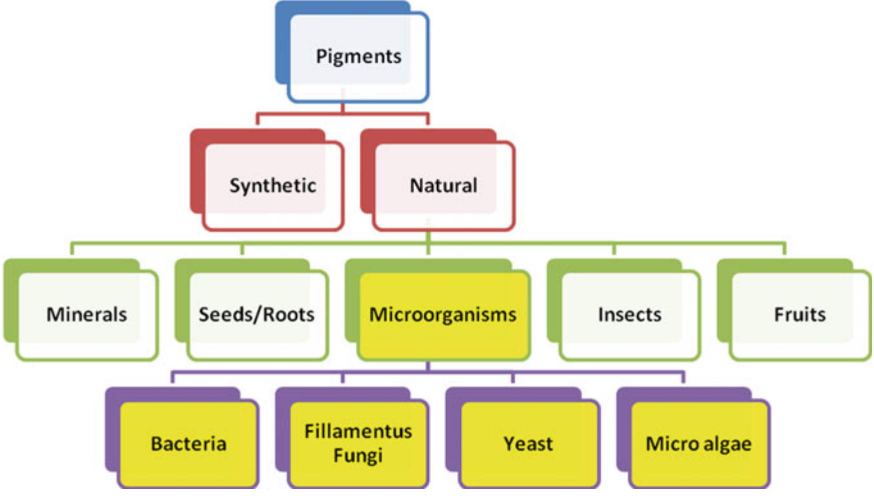


Fig. 13.1 Various source of the pigments

13.1.3 Microbial Pigments as Natural Colorants and Its Significance

Microorganisms are the most prevailing creatures exist on this planet. They are directly or indirectly linked with whatever food we take and are responsible for the fermentative production of specific food products. They can be used as a source of food in the form of amino acids, organic acids, single-cell proteins, pigments, vitamins, and enzymes. Therefore, the pigments obtained from microbes act as a good alternative. The microbial cells synthesize a wide range of pigments, hence, they act as a favorable source of food additives used as colorants (Mata-Gómez et al. 2014). The most natural significant pigments are carotenoids, tetrapyrroles, flavanoids, and astaxanthin. Some microalgae and cyanobacteria synthesize beta-carotene which is frequently used in the industries. Astaxanthin has a great commercial value which is used in pharmaceutical, aqua culture, and feed industries. The strain of *Phaffia rohodozoa* and *Haematococcus pluviais* are used as the key producer of this astaxanthin.

Microorganisms such as *Streptomyces, Serratia, Cordyceps*, *Monascus*, and *Paecilomyces* produce high yields of pigments. Some of the microbial strains like *Penicillium atrovenetum Penicillium herquei*, *Rhodotorula*, *Sarcina*, *Cryptococcus*, *Phaffia rhodozyma*, *Bacillus* sp., *Monascus purpureus*, *Achromobacter, Yarrowia*, and *Phaffia* produces a major source of blue and yellow-red pigments (Raina et al. 2011). Most of the fungi and bacteria were extensively studied for their potential to synthesize the food colorants. Natural pigments are found to be showing various types of anticancer activity. In addition to this, it contains pro-vitamin A which is an essential nutrient. These pigments also possess some important properties such as

stability to heat, light, and pH (Sumathy et al. 2007). Hence, the food industry has explored the usage of microbial and fermentation technology to produce colors applied in various kinds of food products. It also helps in overcoming the public apprehension over the effects of synthetic colors in food products. The natural colorants will not only be valuable to the human beings but also to the environment as they preserve the biodiversity and the harmful chemicals released by the synthetic colorants could be stopped. The natural colorants are used in breakfast cereals, sauces, baby foods, pastas, fruit drinks, processed cheese, and some energy drinks. Hence, the natural colors not only add visual appealing colors to foods but also enhance the probiotic benefits in food products. They are also eco-friendly in nature.

13.2 Pigment-Producing Microorganisms Based on Color and Appearance

The pigments produced by the organisms as reminiscent of its secondary metabolism are known as biopigments. These biopigments have many commercial and synthetic applications. Biological pigments can be categorized into various categories based on natural occurrences and structural affinities. The summary of different types of microbial pigments with respect to the source organisms has been summarized in Table 13.2.

Table 13.2 Summary of different types of microbial pigments with respect to the source organisms

Group	Source organism	Name of the pigment	Color/ Appearance	References
Bacteria	*Paracoccus carotinifaciens*	Astaxanthin	Pink-red	Venil et al. (2013)
	Corynebacterium insidiosum	Indigoidine	Blue	Kamla et al. (2012)
	Streptoverticillium rubrireticuli	Prodigiosin	Red	Darshan et al. (2015)
	Staphylococcus aureus	Zeaxanthin	Golden yellow	Liu et al. (2005)
	Serratia marcescens	Prodigiosin	Red	Deorukhkar et al. (2007)
	Pseudomonas aeruginosa	Pyocyanin	Blue-green	Fouly et al. (2015)
	Janthinobacterium lividum	Violacein	Purple	Pantanella et al. (2007)

(continued)

Table 13.2 (continued)

Group	Source organism	Name of the pigment	Color/ Appearance	References
Fungi	*Aspergillus* sp.	β- carotene	Orange-red	Álvarez et al. (2006)
	Fusarium sporotrichioides	Lycopene	Red	Dufossé and de Echanove (2005)
	Haematococcus pluvialis	Astaxanthin	Red	Dawidziuk et al. (2017)
	Monascus sp.	Monascorubramin	Red-orange	Wild et al. (2002)
	Monascus roseus	Canthaxanthin	Orange-pink	Venil et al. (2009)
	Blakeslea trispora	Lycopene	Red	Jin-Feng et al. (2012)
	Neurospora crassa	β-Carotene	Yellow-orange	Socaciu et al. (2007)
	Pacilomyces farinosus	Anthraquinone	Red	Velmurugan et al. (2010)
Yeast	*Saccharomyces neoformans*	Melanin	Black	Eisenman et al. (2012)
	Phaffia rhodozyma	Astaxanthin	Pink-red	Bjerkeng et al. (2007)
	Rhodotorula glutinis	Torularhodin	Orange-red	Buzzini et al. (200)
Microalgae	*Hematococcus* sp.	Canthaxanthin	Yellow-orange	Christaki et al. (2013)
	Dunaliella salina	β-Carotene	Red	Hejazi et al. (2004)
	Chlorococcum sp.	Leutein	Orange	Del Campo et al. (2000)

13.3 Microbial Pigment Production Technology

There are two fundamental approaches for the biotechnological production of natural colors; to find out the sources of the colors and enhance or increase their capacity of color production. In order to maximize the yield and to get good sources of the colorant, it is the primary step to optimize the various process parameters or to implement the strain improvement strategy. The mass production of colorants from the microorganisms can be done with well-optimized fermentation technology along with metabolic engineering (Nielsen and Olsson 2002). With the advancement in the gene technology, a heterologous expression system can be constructed, which are already known or novel pigment producers (Sumathy 2009).

13.3.1 Fermentation

The pigmentation and growth of microorganism are significantly affected by two types of fermentation, i.e., submerged fermentation and solid-state fermentation. The easy isolation and production of color pigment lead to the advancements in fermentation techniques. Generally, for large-scale fermentation, submerged fermentation is used.

However, due to its natural potential and advantages, solid-state fermentation systems appear to be more promising (Arunachala and Narmadhapriya 2011). The microbial pigments can be produced either by submerged fermentation or solid-state fermentation. In solid substrate fermentation (SSF) process, the biosynthesis of the microbial pigments occurs on the surface of the solid substrate (Grossart et al. 2009; Araujo et al. 2010). This solid-state fermentation technique saves wastewater and yields higher amount of metabolites. In the submerged fermentation, microorganisms are cultivated and grown in liquid medium aerobically with proper agitation to attain a homogenous growth of cells and media components (Cho et al. 2002; Heer and Sharma 2017).

The various parameters such as nitrogen source, carbon source, pH, aeration rate, and temperature are required for the pigment production. The high cost of synthetic media has led the researchers to develop a low-cost technique or process and extraction procedure for the production of microbial pigments. Various efforts have been made to utilize the waste from the agricultural and industrial by-products for the large-scale production of the microbial pigments.

13.3.2 Recovery and Separation

As new research areas were developed, i.e., biological and pharmacological properties, the necessities for both quality and quantity for the microbial pigments have increased. The techniques involved in the purification and separation process of prodigiosin (red pigments from *Vibrio psychroerythrus*), still many things to be considered that constrain the large-scale production. The downstream processing for prodigiosin was based upon the extraction using organic solvents which is a convention method (Wang et al. 2004). As the prodigiosin produced by *Serratia marcescens* was bound to the bacterial envelopes, the process involved a complicated and time-consuming one. Only a small part of prodigiosin was released into the broth. As organic solvents were used, a large amount of the solvent was exhausted which resulted in a very low yield to get the product with high purity.

Various isolation and purification techniques were used such as nonionic adsorption resins which separate the peptides, acids, proteins, nucleic acids and other compounds (Raina et al. 2011).

The compound which is to be separated could be directly adsorbed on the selected resin obtained from the culture broth. This resulted in the elimination of

cell separation and pigment extraction steps. This technology lowered the cost of operation as the consumption of reusable adsorbents extraction solvents has decreased. Wang et al. (2004) reported an effective adsorption method for the separation and purification of prodigiosin directly from the broth culture with increased recovery. The recovery of this process was higher (83%) as compared to the silica gel chromatography (50%) and conventional extraction.

To meet the demand for violacein (indole produced from bacteria), it is extracted using simple technical processes from different groups of bacteria economically. Bhaskaran (2011) had reported that *Pseudoalteromonas* sp. (DSM 13623) has given 13 folds more yield and the crude dye was extracted from the cell mass by slurrying with hot methanol. The same finding was disclosed in Patent Nos. DE000010063712, AT000000369438, EP000001341925, WO002002050299, US020040053375. In a research, it was found that carotenoids can be extracted directly from the sunflower oil rather than conventional organic solvents which could solve the possible toxic reactions occurred due to trace concentration of solvents such as acetone or hexane (Dufosse 2006). Various technological advancements are still required to improve the recovery and separation of bacterial pigments from the culture broth which may reduce the energy and the cost involved in the process. Schematic representation of production, characterization, and applications of various pigments has been illustrated in Fig. 13.2.

13.3.3 Strain Improvement

Multiple rounds of random mutagenesis and selection were done to achieve strain improvement (Portnoy et al. 2012). In the latest decade, the development of gene deletion approaches (Bloor and Cranenburgh 2006) enabled the efficient genome DNA inactivation and greatly improved the metabolic engineering of bacteria. A more integrated and systematic approach for the strain development became prevalent using biotechnological process. The motivation to develop strains has been developed as the wild strains produced less concentration of pigments and this was too low for economic process. The hallmark of all the commercial fermentation process was to develop and improve the microbial strains for the production of industrial products. The common mutagens involved in the strain improvement are ultraviolet (UV), 1-methyl-3-nitro-1-nitrosoguanidine (NTG), and ethyl methane-sulfonate (EMS) which has shown a several-fold enhancement of pigments (Chen et al. 2003).

13.3.4 Enzymes Responsible for the Synthesis of Pigments

There are so many reasons for the microorganisms to produce pigments. These may be photosynthesis, UV light protection, defense mechanisms, and stress, etc. All these

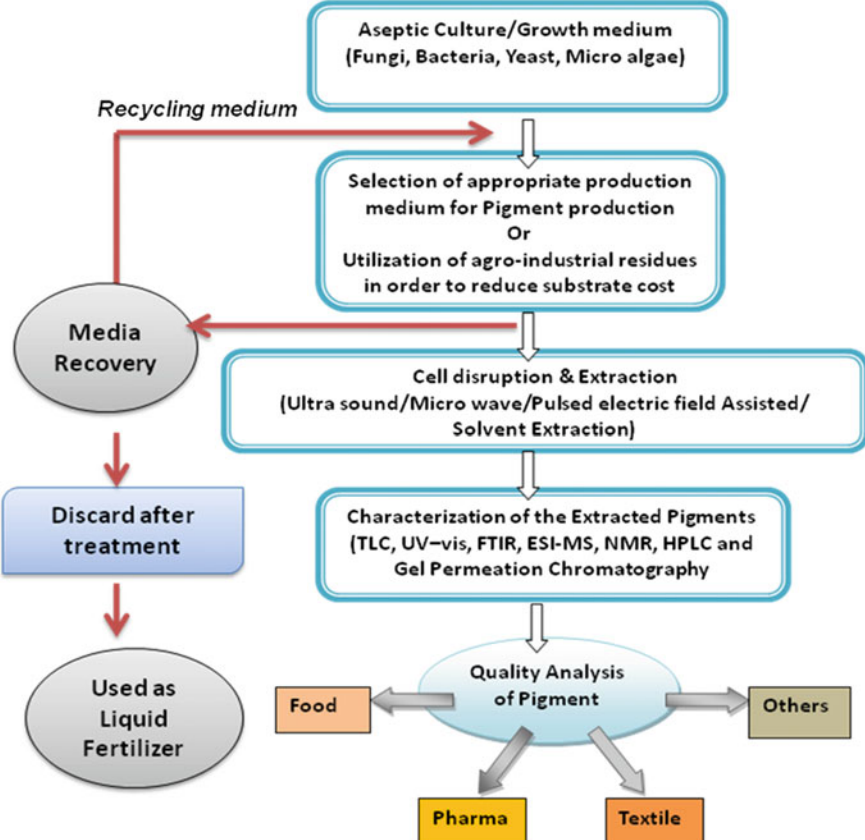


Fig. 13.2 Schematic representation of production, characterization, and applications

causes are mediated through the enzymatic pathways. It was found that the enzyme β-carotene hydroxylase can synthesize zeaxanthin synthesis (Delphine and Wim 1999). Wang et al. (2017) had reported that β-carotene ketolase is responsible for the synthesis of β-carotene in the *Yarrowia lipolytica*. Various classes of the genes such as (CHYb) gene associated with the enzyme hydroxylase are responsible for the synthesis of Astaxanthin in *C. zafingiensis*. Nitrogen sources are a critical factor for protein synthesis. It was reported that in nitrogen deficit condition, the synthesis of LCYb enzyme has increased which enhances the pigment expression rate in the cyanobacterial species. Few species of Monascus showed low glucoamylase and alpha-amylase activity (Sumathy et al. 2007). The activity of these hydrolyzing enzymes resulted in the high pigment yield. Different categories of downstream enzymes, i.e., desaturases and cyclases were functional on this nonnatural substrate, which led to the production of a series of novel carotenoids in the C30 and C40 carotenoid pathways. Recently, different enzymes have been identified, but PSY,

BKT, and PDS are found to be mostly targeted for the overexpression of different carotenoids (Saini et al. 2018).

13.3.5 Metabolic Engineering Approach

To reduce the costs of production, a strategy was used by producing specific hyper-produced strains. Techniques which were efficient are used to obtain the mutant strains (Malisorn and Suntornsuk 2009). An easy technique is used, i.e., random selection mutagenesis which uses color to distinguish. Many chemicals such as 1-methyl-3-nitroguanidine or antymicin A, and ethyl methanesulfonate and physical methods such as gamma radiations, UV light were used. Metabolic engineering is the process in which the improvement of the cellular properties are done through the modification of particular biochemical reactions or the initiation of new reactions, with the help of recombinant DNA technology (Park et al. 2007). Currently, the modifications in non-corotenoid microorganisms are conducted in order to produce carotenoids and are considered a very useful tool. Specific microorganisms are selected in order to produce excessive levels of important molecules which are directly linked in improving the public health using biotechnology (Ye and Bhatia 2012). The selection of the appropriate and specific microorganism is the first step involved in biotechnological process. This can be done using the process mutagenesis which improves the strains and simultaneously increases the production of the metabolites (Palágyi et al. 2001).

Several authors have reported the use and application of metabolic engineering in yeasts, i.e., *Saccharomyces cerevisiae* (Ungureanu et al. 2012) and *Candida utilis* (Mata-Gómez et al. 2014). The yeasts aforementioned have been modified successfully for the catenoid production: B-Carotene, Astaxanthin, or lycopene through the insertion of carotenogenic genes from microorganisms such as *Agrobacterium aurantiacum, Erwinia uredovora*, or *Xanthophyllomyces dendrorhus.* The mentioned yeasts are useful in food industries and are generally considered as safe organisms by the Food and Drug Administration (US) (Araya-Garay et al. 2012).

Bacteria such as *Escherichia coli* were engineered for the production of carotenoids. Nonetheless, *S. cerevisiae* is considered as safe yeast and has advantages such as easy manipulation of genes using accepted host-vector systems. However, naturally, the yeast *S. cerevisiae* does not produce carotenoids as it produces geranyl diphosphate. The yeast is to be integrated with two important carotenoid genes, i.e., phytoene synthase (crtYB) and phyotene desaturase (ctrl), from the microorganism, *Xanthophyllomyces dendrorhous.* These microorganisms produce carotenoids (Veerwaal et al. 2007).

Being characterized in its physiology, genetic system, and regulatory networks (Ye and Bhatia 2012), the yeast is an appropriate organism to be engineered for the production of carotenoids. An attempt was done to produce B-carotene in *Sacharomyces cerevisiae* using bacterial genes. It was successful in the production of B-carotene, however, the production level was low (103 g/g dry wt.). Veerwaal

et al. (2007) have studied the expression of the carotenogenic genes which encode a bifunctional lycopene synthase and phytoene synthase from *X. dendrorhus*. The production of B-carotene concentration produced was 5.9 mg/g with the improved strain. *S. cerevisiae* was also engineered for the production of other carotenoids. Bhataya et al. (2009) used various genes to encode (CrtI) phytoene synthase and bifunctional phytoene synthase/Lycopene class (CrtYB) from *X. dendrorhus. Pichia pastoris* is another noncarotenogenic yeast that was also studied for carotenoid production. It was also able to grow organic materials. Araya-Garay et al. (2012) designed and constructed two plasmids which contain two genes encoding B-carotene and lycopene.

13.4 Utilization of Agro-Industrial Waste for Production of Microbial Pigments

The cost of bioprocess highly influenced the substrates which are used for the bioproduct production. This resulted in a need of cheap and efficient substrates for production of bioproducts economically. Detailed information regarding the utilization of agro-industrial wastes for the biosynthesis of specific pigments has been summarized in Table 13.3.

Table 13.3 Summary of agro-industrial wastes in the production of microbial pigments

S. No.	Name of the pigment	Name of the microorganism	Name of the agro-industrial waste	References
1	Beta-carotene	*Rhodotorula rubra*	Soap stock	Sajad et al. (2017)
2	Bostrycin	*Nigrospora* sp. *no. 407*	Cane molasses	Yi-Hsuan et al. (2017)
3	Carotenoid	*Dietzia maris NIT-D*	Sugarcane bagasse hydrolysate	Gargi et al. (2015)
4	Carotene	*Blakeslea trispora*	Waste cooking oil	Konstantina et al. (2016)
5	Beta-carotene, Torulene and Torularhodin	*Rhodotorula mucilaginosa CRUB 0195*	Cane molasses, corn syrup, raw malt extract	Diego and van María (2006)
6	Carotenoid	*Microbacterium* sp.	Olive mill waste	Borroni et al. (2016)
7	Carotenoid	*Rhodotorula mucilaginosa*	Cassava bagasse	Manimala and Murugesan (2017)
8	Carotenoid	*Rhodotorula glutinis*	Hydrolyzed mung bean waste flour	Tinoi et al. (2006)

(continued)

Table 13.3 (continued)

S. No.	Name of the pigment	Name of the microorganism	Name of the agro-industrial waste	References
9	Melanin pigment	*Streptomyces griseorubens DKR4*	Banana stalks; coconut husk, rice husk, and rice flour	Santhanalakshmi et al. (2017)
10	Monascus pigment	*Monascus purpureus KACC 42430*	Corn cob powder	Palanivel et al. (2011)
11	Red Pigment	*Monascus Purpureus*	Bug damaged wheat	Serap et al. (2013)
12	Monascus pigment	*Monascus purpureus*	Grape waste	Silvana et al. (2008)
13	Monascus pigment	*Monascus purpureus*	Jackfruit seed	c
14	Red and Yellow Pigment	*Monascus purpureus*	Chicken feathers waste	Orak et al. (2018)
15	Yellow, orange and red pigments	*Monascus purpureus* ATCC 16365; *Penicillium purpurogenum* CBS 113139	Orange processing wastes	Kantifedaki et al. (2018)
16	Red pigment	*Monascus ruber*	Corn steep liquor	Hamano et al. (2006)
17	Yellowish-orange pigment	*Chryseobacterium artocarpi* CECT 8497	Pineapple waste	Claira et al. (2016)

13.4.1 Fruit and Vegetable Industry

During processing of vegetables and fruits, huge amounts of waste have been generated in form of pulp, peel, waste water, seeds, etc. The waste products contain several important nutrients which can be utilized by the microorganisms for their growth as well as fermentation. To study this, an investigation was carried out on jackfruit seed to determine its feasibility as substrate supplemented with various sources of carbon for production of the red pigment by *Monascus purpureus* in solid-state fermentation (Ungureanu et al. 2012). Various key sources of agro-industrial waste were utilized for the production of microbial pigments. This is illustrated in Fig. 13.3.

Durian seed (*Durio zibethinus*) which is obtained from the Durian King fruit, in considered as an agro-industrial residue and is usually discarded. The Durian seeds were found to be substrate for the production of angkak, i.e., fermented steam rice

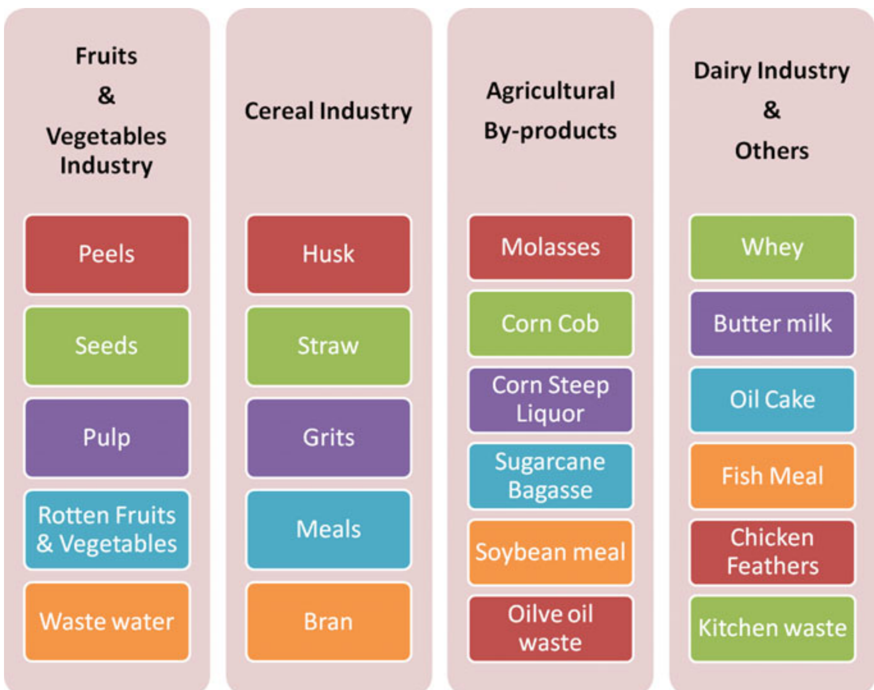


Fig. 13.3 Types of agro-industrial waste generated from different industries

using *Monascus sp.* which yields 50 mg/kg of Monacolin K. The Minnow peel powder was utilized and was found to be a good substrate for growth of MTCC 369 *Monascus purpureus,* which resulted in an adequate amount of pigment production. The waste extracted from apple juice pigment production, Apple pomace, is rich in minerals, sugar, and acids and was utilized for the microbial pigment production (Claira et al. 2016). The pigment produced by *Monascus pupureusin* in submerged fermentation process using waste from grape fruit as a growth substrate is optimized by using response surface techniques and factorial design (Silvana et al. 20018). The tomato waste is considered valuable because of its richness in biological active compounds such as crude protein and fat, and carbohydrates which provide a medium for growth of yeast. To maximize the productivity and yield of carotenoids by *Monascus purpureus* MTCC 369, the optimization of tomato waste was adjunct with other nutrients using response surface modeling (Rashmi and Padmavathi 2014).

13.4.2 Cereal Industry

Corn steep liquor (CSL) is a by-product which is obtained by corn wet-milling industry. This industry uses traditional methods for the production of microbial products such as ethanol, penicillin and lactic acid. The by-product is used as the nitrogen source but also contains adequate sugars, vitamins, amino acids, and ash. As nutrition content of corn steep liquor (CSL) is high, it has stimulated interests of many researchers in the utilization and development of various microbial pigments. CSL has been perceived as a good source of salts and nitrogen for the production of red pigment by *Monascus ruber*, hence substituting salts and yeast extracts (Subhasree et al. 2011). Waste stream dissolved and derived from corn is being used as culture medium for the production of pigments from fungal. Similarly, the carotenoid production was optimized by *Rhodotorula glutinis* using hydrolyzed waste flour from mung bean as a substrate and was reported (Srianta et al. 2012).

13.4.3 Agro-Industrial Residues

The processing of crops and the post-harvesting operations resulted in production of various by-products such as molasses, bran, cobs, husk, bagasse, etc., along with the major project. The corn steep liquor (CSL) and cassava liquid waste from agro-industrial without or with mannitol supplementation act as a low-cost growth medium for *S. marcescens* and the prodigiosin production was reported. Results from the production of prodigiosin using effluent from agro-industrial resulted in an increase of production thus reducing the cost associated with the pollutant which also contributed to the improvement of environment (Malisorn and Suntornsuk 2008). The production of pigments by KACC 42430 *Monascus purpureus* in SSF using corn cob powder as substrate was investigated and was reported that a yield of 25.42 OD Units/g dry fermented substrate was obtained by optimizing the process parameters (Mendez et al. 2011). The effect of several nitrogen sources, i.e., part powder, taro leaves, and green gram waste okra were observed individually for the pigment production. Among all, pea pod powder resulted in higher pigment contents with a yield 6.93 AU/g (Reeba 2014). The pigments from Monascus were produced using cheap agro-industrial waste which includes a carbon source which can be utilized as a courant in foodstuffs and foods. The production of red pigment using *Monascus purpureus* was conducted through various experiments by combining the resources from carbon sources (Sugarcane bagasse) and nitrogen sources. Similarly, sugarcane bagasse and corn were used as starch source for the microbial pigment production using Monascus (Mendez et al. 2011).

13.4.4 Dairy Industry

In the dairy industry, Whey is one of the major waste products. It is a by-product during the manufacturing of cheese and is obtained after the precipitation and removal of milk. Whey is lactose and protein-rich by-product, and is used as a growth substrate for different lactose-utilizing microbes. Various filamentous fungi strains were being used for the production of yellow pigments for their growth in soya protein and cheese. Submerged fermentation using coconut water and whey which are rich in amino acids, minerals, and fatty acids were being carried out for yellowish-pink pigment production using MTCC 1446 *Rhodotorula rubra* (Lopes et al. 2013).

13.4.5 Miscellaneous

The nitrogen source, i.e., peptone, is one of the most expensive medium constituents. In this view, various experiments were conducted to prepare peptone from waste items such as chicken feathers. The feathers undergo acid hydrolysis and were investigated for the usability of peptone by using it as a substrate for the carotenoid and biomass production by *Rhodotorula glutinous* (Taskin et al. 2011). Various synthetic and natural media like powdered sesame seed broth, peanut seed broth, maltose, and glucose sugar substrates and various oils like sesame, coconut, peanut, etc., were compared to the study of prodigiosin production by *S. marcescens*. Among all the substrates for the prodigiosin production, peanut seed broth was found to be cheapest (Shahitha and Poornima 2012). The efficiency of various natural substrates was studied for the production of progigiosin from *S. marcescens* MBB05. The natural substrates, namely coconut powder and oil, fenugreek powder, mustard powder and oil, peanut powder, peanut oil, sesame oil, white sesame powder, and black sesame powder. Compared to the optimized basal medium, the production was 4.5 times higher with natural substrates (Pradeep et al. 2013).

13.5 Applications of Microbial Pigments in Food Industries

The major applications of microbial pigments have been summarized in Table 13.4.

Table 13.4 Applications of various microbial pigments in the food industries

S. No.	Name of the pigment	Applications	Reference
1	Melanin	Anticancer agent and antioxidant	El-Naggar and El-Ewasy (2017)
2	Carotenoid	Photo-sensitizers in dye-sensitized solar cells	Ordenes Aenishanslins et al. (2016)
3	Violacein	Antifungal agent	Sasidharan et al. (2015)
4	Pyocyanin	Antimicrobial agent	El-Fouly et al. (2015)
5	Carotenoid	Antioxidant	Correa Llanten et al. (2012)
6	Asperversin	Antifungal agent	Miao et al. (2012)
7	Anthraquinone	Antioxidant	Li et al. (2017)
8	Red pigment	Dye textile having antimicrobial activity	Chadni et al. (2017)
9	Benzoquinon	Anticancer agent	Zheng et al. (2017)
10	Anthraquinone	Dyeing of wool fabrics	Nagia and El-Mohamedy (2007)
11	Violacein	Antitumor, antimicrobial, and antiparasitic agent	Duran et al. (2007)
12	Riboflavin	Baby foods, breakfast cereals, sauces, pastas, processed cheese, fruit drinks, vitamin-enriched milk products, and some energy drinks	Jelena et al. (2014)
13	Beta-carotene	coloring agent in foodstuffs	Francielo et al. (2013), Chidambaram et al. (2013)
14	Canthaxanthin	Antioxidants and inhibit the oxidation of lipids	Paola et al. (2000)
15	Carotenoids	Potent antioxidants and are widely used as food colorants	Joanna and Kvetoslava (2014)
16	Prodigiosin	Antimalarial, antibacterial, antineoplastic, antibiotic activity	Lapenda et al. (2015)
17	Phycocyanin	Dietary supplement which is rich in proteins	Stahmann et al. (2000), Kamala et al. (2012), Raisainen et al. (2002)
18	Arprink red	Meat and meat product analogues, milk products, ice cream, and confectionary	Abhishek et al. (2015)
19	Monascus	Coloring and flavoring agent used as food additives in sausages, meats, and red wines	Abhishek et al. (2015), Chung et al. (2008)
20	Lycopene	Used to manufacture the colorant from cheap corn fiber material as substrate	Venil et al. (2014)

13.6 Major Challenges

The extraction process of pigments still remains a challenge to manufacture as the concentrated and purified form of the pigments is difficult to extract. The pigments produced by the microorganisms are usually intracellular or extracellular. If the pigment produced is intracellular, various methods are required to disrupt the cell, hence increasing the downstream processing steps. The microbial pigments stability depends on the stress by the environment or the exposure of the pigments to UV light (Mohammed et al. 2018). Several drawbacks were reported during the microbial pigment production, extraction, and purification. The use of organic solvents and high temperatures leads to unwanted isomer formation and degradation. A number of microbial pigments have not been authorized for the use of food colorant by regulatory authorities, for example, Monascus pigments are a mixture of azaphilone pigments and its constituents are toxic in mature and are not used as a colorant in the food industry (Daehwan and Seockmo 2018).

13.7 Future Orientation

The use of natural colorants instead of synthetic started with increase in knowing the side effects of the use of the synthetic colorants. Another important factor is the leniency and flexibility of the governments in the use of natural colorants. There are many advantages when the colorants are produced using fermentation. They include cheaper production, higher yields, easier extraction, no seasonal variations, and no lack of raw materials. The interest in use of colorants produced by bacteria is increasing rapidly, and is a possible alternate source of colorants used in textile, foods, pharma, etc. Hence, biotechnology plays an important role for large-scale production and fermentation of colorants.

There is an urgent need for the development of novel strains which can produce higher yields using less and cheap substrate with fewer down streaming steps. Certain pigments produced from microorganisms are extremely sensitive to light, pH, and variations in temperature, hence exhibiting poor stability and degradation. This facet needs the focus of the investigation. The commercialization of microbial pigments as food colorants requires investment from key private and public stakeholders. This can overcome the challenges faced in production of the microbial pigments and develop technologies for the production, extraction, and purification of novel pigments from various microbial sources.

13.8 Conclusion

There are many environmental hazards and health issues caused by synthetic dyes. In contrast, the pigments produced from microbial agents are eco-friendly and can be used as antioxidants, food colorants, and bioindicators, in textile industry, anticancer, and antimicrobial agents. Although so much extensive research has been conducted in the last decade to bring microbial pigments to the market, the output is still not enough to meet the market demand. Continuous efforts in discovering the novel microbial strains for pigment production, strain improvement, optimizing to decrease the production cost, metabolic engineering, and recombinant DNA technology were carried out to eradicate the toxic synthetic dyes in the coming future.

Acknowledgements Author, Bishwambhar Mishra acknowledges the Department of Biotechnology, Centurion University of Technology and Management, Bhubaneswar, Odisha for providing the good infrastructure and smart digital library facilities.

References

Araujo HWCD, Fukushima K, Takaki GMC (2010) Prodigiosin production by *Serratia marcescens* UCP 1549 using renewable-resources as a low cost substrate. Molecules 15:6931–6940

Araya-Garay JM, Ageitos JM, Vallejo JA, Veiga-Crespo P, Sánchez Perez A, Villa TG (2012) Construction of a novel *Pichia pastoris* strain for production of xanthophylls. AMB Exp 2:24–32

Arunachala C, Narmadhapriya D (2011) Monascus fermented rice and its beneficial aspects: a new review. Asian J Pharm Clin Res 4:29–31

Bhaskaran K (2011) Process for the production of violacein and its derivative deoxy violacein containing bioactive pigment from chromobacterium sp. (MTCC5522). WO2011110932 A1

Bhataya A, Schmidt-Dannert C, Lee PC (2009) Metabolic engineering of *Pichia pastoris* X-33 for lycopene production. Process Biochem 44:1095–1102

Bloor AE, Cranenburgh RM (2006) An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. Appl Environ Microbiol 72:2520–2525

Borroni V, Gonzalez MT, Carelli AA (2016) Bioproduction of carotenoid compounds using two-phase olive mill waste as the substrate. Process Biochem 54:128–134

Chadni Z, Rahaman MH, Jerin I, Hoque KMF, Reza MA (2017) Extraction and optimisation of red pigment production as secondary metabolites from *Talaromyces verruculosus* and its potential use in textile industries. Mycology 8:48–57

Chen Y, Li D, Lu W, Xing J, Hui B, Han Y (2003) Screening and characterization of astaxanthin-hyper-producing mutants of *Haematococcus pluvialis*. Biotechnol Lett 25:527–529

Cho YJ, Hwang HJ, Kim SW, Song CH, Yun JW (2002) Effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by *Paecilomyces sinclairii* in a batch bioreactor. J Biotechnol 95:13–23

Claira AA, Amira A, Chidambaram KV, Ali Reza K, Wan AA (2016) Utilization of agro-industrial waste for the production of yellowish orange pigment from *Chryseobacterium artocarpi* CECT 8497. Int Biodeterior Biodegradation 113:342–349

Correa Llanten DN, Amenabar MJ, Blamey JM (2012) Antioxidant capacity of novel pigments from an Antarctic bacterium. J Microbiol 50:374–379

Daehwan K, Seockmo K (2018) Beneficial effects of Monascus sp. KCCM 10093 pigments and derivatives: a mini review. Molecules 23. https://doi.org/10.3390/molecules23010098

Darshan N, Manonmani HK (2015) Prodigiosin and its potential applications. J Food Sci Technol 52:5393–5407

Dawidziuk A, Popiel D, Luboinska M, Grzebyk M, Wisniewski M, Koczyk G (2017) Assessing contamination of microalgal astaxanthin producer Haematococcus cultures with high-resolution melting curve analysis. J Appl Genet 58:277–285

Del Campo JA, Moreno J, Rodríguez H, Vargas MA, Rivas J, Guerrero MG (2000) Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis sp.* (Chlorophyta). J Biotechnol 76:51–59

Delphine L, Wim V (1999) The zeaxanthin biosynthesis enzyme β-carotene hydroxylase is involved in myxoxanthophyll synthesis in *Synechocystis sp*. PCC 6803 FEBS Lett 454:247–251

Diego L, van María B (2006) Biomass and carotenoid pigment production by patagonian native yeasts. World J Microbiol Biotechnol 22:687–692

Dufossé L, de Echanove MC (2005) The last step in the biosynthesis of aryl carotenoids in the cheese ripening bacteria Brevibacterium linens ATCC 9175 (*Brevibacterium aurantiacum sp. nov*.) involves a cytochrome P450-dependent monooxygenase. Food Res Int 38:967–973

Duran N, Justo GZ, Ferreira CV, Melo PS, Cordi L, Martins D (2007) Violacein: properties and biological activities. Biotechnol Appl Biochem 48:127–133

Eisenman HC, Casadevall A (2012) Synthesis and assembly of fungal melanin. Appl Microbiol Biotechnol 93:931–940

El-Fouly MZ, Sharaf AM, Shahin AAM, El-Bialy HA, Omara AMA (2015) Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. J Radiat Res Appl Sci 8:36–48

El-Naggar NE, El-Ewasy SM (2017) Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. Sci Rep 14:421–429

Frases S, Salazar A, Dadachova E, Casadevall A (2007) *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. Appl Environ Microbiol 73:615–621

Gargi G, Surabhi C, Debjani D (2015) Studies on the stability of a carotenoid produced by a novel isolate using low cost agro-industrial residue and its application in different model systems. LWT—Food Sci Technol 63:780–790

Grossart HP, Thorwest M, Plitzko I, Brinkhoff T, Simon M, Zeeck A (2009) Production of a blue pigment (glaukothalin) by marine *rheinheimera spp*. Int J Microbiol 2009:1–7

Hamano PS, Kilikian BV (2006) Production of red pigments by *Monascus Ruber* in culture media containing corn steep liquor. Braz J Chem Eng 23:443–449

Heer K, Sharma S (2017) Microbial pigments as a natural color: a review. Int J Pharm Sci Res 8:1913–1922

Hejazi MA, Holwerda E, Wijffels RH (2004) Milking microalga *Dunaliella salina* for beta-carotene production in two-phase bioreactors. Biotechnol Bioeng 85:475–481

Jelena L, Marko D, Slavica S, Gordana K, Nataša T, Snežana M, Tatjana J (2014) Assessment of thiamine content in some dairy products and rice milk. CyTA-J Food 12:203–209

Jin-Feng W, Xiu-Ji L, Rui-Sang L, Hong-Mei L, Ya-Jie T (2012) Optimization of the mated fermentation process for the production of lycopene by *Blakeslea trispora* NRRL 2895 (+) and NRRL 2896 (−). Bioprocess Biosyst Eng 35:553–564

Joanna F, Květoslava B (2014) Role of carotenoids as antioxidants in human health and disease. Nutrients 6:466–488

Joshi VK, Attri D, Bala A, Bhushan S (2003) Microbial pigments. Ind J Biotechnol 2:362–369

Kamla M, Jayanti T, Sneh G (2012) Microbial pigments: a review. Int J Microb Resour Technol 2012:361–365

Kantifedaki A, Kachrimanidou V, Mallouchos A, Papanikolaou S, Koutinas AA (2018) Orange processing waste valorisation for the production of bio-based pigments using the fungal strains *Monascus purpureus* and *Penicillium purpurogenum*. J Clean Prod 185:882–890

Konstantina N, Triantafyllos R (2016) Waste cooking oil: a new substrate for carotene production by *Blakeslea trispora* in submerged fermentation. Biores Technol 203:198–203

Lapenda JC, Silva PA, Vicalvi MC, Sena KX, Nascimento SC (2015) Antimicrobial activity of prodigiosin isolated from Serratia marcescens UFPEDA 398. World J Microbiol Biotechnol 31:399–406

Li F, Xue F, Yu X (2017) GC-MS, FTIR and Raman analysis of antioxidant components of red pigments from *Stemphylium lycopersici*. Curr Microbiol 74:532–539

Lopes FC, Tichota DM, Pereira JQ, Segalin J, Rios AO, Brandelli A (2013) Pigment production by filamentous fungi on agro-industrial byproducts: an eco-friendly alternative. Appl Biochem Biotechnol 171:616–625

Malisorn C, Suntornsuk W (2008) Optimization of b-carotene production by *Rhodotorula glutinis* DM28 in fermented radish brine. Bioresour Technol 99:2281–2287

Malisorn C, Suntornsuk W (2009) Improved β-carotene production of Rhodotorula glutinis in fermented radish brine by continuous cultivation. Biochem Eng J 43:27–32

Manimala MRA, Murugesan R (2017) Studies on carotenoid pigment production by yeast *Rhodotorula mucilaginosa* using cheap materials of agro-industrial origin. Pharma Innov J 6:80–82

Mata-Gómez LC, Julio CM, Alejandro MZ, Cristóbal NA (2014) Biotechnological production of carotenoids by yeasts: an overview. Microbial Cell Fact 13:12

Mendez A, Perez C, Montanez CJ, Mrtinez G, Aguilar NC (2011) Red pigment production by *Pencillium purpurogenum* GH2 is influenced pH and temperature. Biomed Biotechnol 12:961–968

Miao FP, Li XD, Liu XH, Cichewicz RH, Ji NY (2012) Secondary metabolites from an algicolous *Aspergillus versicolor* strain. Mar Drugs 10:131–139

Mohammed AAQ, Saravanan S, Sowrirajan S (2018) Effect of certain compounds on the pigmentation characteristis of some bacterial isolates. Int J Curr Microbiol App Sci 7:2464–2483

Nagia FA, El-Mohamedy RSR (2007) Dyeing of wool with natural anthraquinone dyes from *Fusarium oxysporum*. Dyes Pigments 75:550–555

Nielsen J, Olsson L (2002) An expanded role for microbial physiology in metabolic engineering and functional genomics: moving towards systems biology. FEMS Yeast Res 2:175–181

Orak T, Caglar O, Ortucu S, Ozkan H, Taskin M (2018) Chicken feather peptone: a new alternative nitrogen source for pigment production by *Monascus purpureus*. J Biotechnol 271:56–62

Ordenes Aenishanslins N, Anziani-Ostuni G, Vargas-Reyes M, Alarcon J, Tello A, Perez-Donoso JM (2016) Pigments from UV-resistant Antarctic bacteria as photosensitizers in dye sensitized solar cells. J Photochem Photobiol, B 162:707–714

Palágyi ZS, Ferenczy L, Vágvölgyi C (2001) Carbon-source assimilation pattern of the astaxanthin-producing yeast *Phaffia rhodozyma*. World J Microbiol Biotechnol 17:95–97

Palanivel V, Hyun H, Vellingiri B, Seralathan KK, Kui-Jae L, Sang-Myung L, Jong-Chan C, Patrick JS, Byung-Taek O (2011) Monascus pigment production by solid-state fermentation with corn cob substrate. J Biosci Bioeng 112:590–594

Paola P, Gabriella C, DI Maria Emilia, Simona S, Gianna MB (2000) Canthaxanthin supplementation alters antioxidant enzymes and iron concentration in liver of Balb/c mice. J Nutr 130:1303–1308

Park P, Kim E, Chu K (2007) Chemical disruption of yeast cells for the isolation of carotenoid pigments. Sep Purif Tech 53:148–152

Portnoy VA, Bezdan D, Zengler K (2012) Adaptive laboratory evolution: harness-ing the power of biology for metabolic engineering. Curr Opin Biotechnol 22:590–604

Pradeep BV, Pradeep FS, Angayarkanni J, Palaniswamy M (2013) Optimization and production of prodigiosin from *Serratia marcescens* MBB05 using various natural substrates. Asian J Pharma Clin Res 6:34–41

Raina S, Murphy T, Vizio D, Reffatti P, Keshavarz T (2011) Novel strategies for over-production of microbial products. Chem Eng Trans 24:847–852

Rashmi D, Padmavathi T (2014) Statistical optimization of pigment production by Monascus sanguineus under stress condition. Prep Biochem Biotechnol 44:68–79
Reeba P (2014) Bioutilization of kinnow waste for the production of biopigments using submerged fermentation. Int J Food Nutr Sci 3:9–13
Saini DK, Pabbi S, Shukla P (2018) Cyanobacterial pigments: Perspectives and biotechnological approaches. Food Chem Toxicol. https://doi.org/10.1016/j.fct.2018.08.002
Sajad A, Habibi A, Taavoni S, Varmira K (2017) Beta-carotene production from soap stock by loofa-immobilized *Rhodotorula rubra* in an airlift photobioreactor. Process Biochem 54:9–19
Santhanalakshmi K, Natarajan E, Muthukumar B, Dhanasekaran D, Archunan G (2017) Fermentative production of melanin pigment from *Streptomyces griseorubens* DKR4 from agro waste products. Int J Appl Res 3:284–288
Sasidharan A, Sasidharan NK, Amma DB, Vasu RK, Nataraja AV, Bhaskaran K (2015) Antifungal activity of violacein purified from a novel strain of *Chromobacterium sp.* NIIST (MTCC 5522). J Microbiol 53:694–701
Serap DV, İsmail HB, Osman Ş, Tuncay G (2013) Optimizing a submerged Monascus cultivation for production of red pigment with bug damaged wheat using artificial neural networks. Food Sci Biotechnol 22:1639–1648
Shahitha S, Poornima K (2012) Enhanced production of prodigiosin production in *Serratia marcescens*. J Appl Pharma Sci 2:138–140
Silvana T, Silveira DJ, Daroit AB (2008) Pigment production by *Monascus purpureus* in grape waste using factorial design. LWT 41:170–174
Srianta I, Novita Y, Kusumawati N (2012) Production of monascus pigments on durian seed: effect of supplementation of carbon source. J Pure Appl Microbiol 6:59–63
Subhasree RS, Babu P, Vidyalakshmi R, Chandra V (2011) Effect of carbon and nitrogen sources on stimulation of pigment production by *Monascus purpureus* on Jackfruit seeds. In. J Microbiol Res 2:184–187
Sumathy B (2009) Microbial pigments. In: Singh-Nee Nigam P, Pandey A (eds) Biotechnology for agro-industrial residues utilisation. Springer, Netherlands
Sumathy B, Carlos RS, Ashok P (2007) Solid-state fermentation for the production of Monascus pigments from jackfruit seed. Biores Technol 98:1554–1560
Taskin M, Sisman T, Erdal S, Kurbanoglu EB (2011) Use of waste chicken feathers as peptone for production of carotenoids in submerged culture of *Rhodotorula glutinis* MT-5. Eur Food Res Technol 233:657–665
Tinoia JN, Rakariyathama RLD (2005) Simplex optimization of carotenoid production by Rhodotorula glutinis using hydrolyzed mung bean waste flour as substrate. Process Biochem 40:2551–2557
Ungureanu C, Ferdes M, Chirvase AA (2012) Torularhodin biosynthesis and extraction by yeast cells of *Rhodotorula rubra*. Rev Chim 63:316–318
Veerwaal R, Wang J, Meijnen J-P, Visser H, Sandmann G, van den Berg JA, van Ooyen A (2007) High-level production of beta-carotene in *Saccharomyces cereviseae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 73:4342–4350
Wang X, Tao J, Wei D, Shen Y, Tong W (2004) Development of an adsorption procedure for the direct separation and purification of prodigiosin from culture broth. Biotechnol Appl Biochem 40:277–280
Ye VM, Bhatia SK (2012) Pathway engineering strategies for production of beneficial carotenoids in microbial hosts. Biotech Lett 34:1405–1414
Yi-Hsuan H, Wen-Jen Y, Chih-Yu C, Huang-Mo S, Shuen-Fuh L (2017) Bostrycin production by agro-industrial residues and its potential for food processing. Food Sci Biotechnol. https://doi.org/10.1007/s10068-017-0082-6
Zheng L, Cai Y, Zhou L, Huang Ren X, Zuo A (2017) Benzoquinone from Fusarium pigment inhibits the proliferation of estrogen receptor-positive MCF-7 cells through the NF-kB pathway via estrogen receptor signaling. Int J Mol Med 39:39–46

Chapter 14
Digestive Enzymes: Industrial Applications in Food Products

Luciana Porto de Souza Vandenberghe, Susan Grace Karp, Maria Giovana Binder Pagnoncelli, Cristine Rodrigues, Adriane Bianchi Pedroni Medeiros and Carlos Ricardo Soccol

Abstract Digestion is a very complex process involving many different enzymes expressed by the human cells and by the microbial community in the digestive tract. Digestive problems such as lactose intolerance and poor digestion of vegetable oligosaccharides affect a great part of the human population, causing discomforts due to their fermentation by gas-producing microorganisms. Although the treatment may involve the supplementation with digestive enzymes, such as lactase (beta-galactosidase) and alpha-galactosidase, respectively, the industrial processing of food products is another alternative. Gluten intolerant and celiac individuals could potentially be benefited by the administration of peptidases or the consumption of peptidase-treated food, however, this is not yet considered a treatment option that substitutes the complete avoidance of gluten. Enzymes have been applied in food processing for various purposes including the removal of undesired components. Besides the galactosidases that remove specific saccharides, another example is the use of L-asparaginase to avoid the formation of acrylamide, a possible carcinogen. In this chapter, the application of digestive enzymes in food bioprocessing will be reviewed, from traditional applications of alpha- and beta-galactosidases, to potential applications of proteases and lipases. Examples of commercial products and of the most recent and relevant patents in this area will be included.

Keywords Alpha-galactosidase · Beta-galactosidase · Peptidase L-asparaginase

L. P. de Souza Vandenberghe · S. G. Karp · C. Rodrigues · A. B. P. Medeiros
C. R. Soccol (✉)
Federal University of Parana, 81-531-980, Curitiba-PR, Brazil
e-mail: soccol@ufpr.br

M. G. B. Pagnoncelli
Federal Technological University of Parana, 85-660-000, Dois Vizinhos-PR, Brazil

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_14

14.1 Introduction

Digestive disorders and poor digestion are health problems that affect a great part of the human population, their prevalence is difficult to estimate. Some of them are of genetic origin and most of them are affected by the lifestyle, especially by the diet and eating habits. Lactose intolerance as a result of congenital lactase deficiency is rare, however, the reduced ability to digest lactose is a problem that affects around 65% of the human adult population. Also, the consumption of non-digestible oligosaccharides present in legumes such as soybean may cause discomforts due to their fermentation by gas-producing microorganisms. The use of digestive enzymes such as beta-galactosidases to hydrolyze lactose and alpha-galactosidases to break the alpha-1,6 bonds of stachyose, raffinose and melibiose, the non-digestible oligosaccharides of vegetable origin, either as supplements or in the processing of food products, is the most effective strategy to avoid digestion problems associated to the consumption of dairy products and some vegetables such as soybean. Some processes are well established industrially for such purposes.

The celiac disease (CD), a chronic inflammatory intestinal disease triggered by the ingestion of gluten, affects around 70 million people globally. It is the most common disease in the digestive system of genetic origin, characterized by the abnormal response of the body's immune system to a group of proteins. The main amino acids present in gluten composition (proline and glutamine, called the prolamins) are responsible for the immune response in CD and gluten intolerance because the high proline content makes these proteins resistant to digestive enzymes. As a result, oligopeptides can reach the small intestine where they elicit an autoimmune response in susceptible individuals. A gluten-free diet is the only effective treatment for CD available, however, this problem could possibly be alleviated by using gluten-specific peptidases for gluten protein degradation into small or nonimmunogenic peptide fragments before they transit across the small intestinal mucosa.

In this chapter, the application of digestive enzymes in food bioprocessing will be reviewed, from traditional applications of alpha- and beta-galactosidases, to potential applications of peptidases, lipases, and L-asparaginases. The examples of commercial products and of the most recent and relevant patents in this area will be included.

14.2 Alpha-Galactosidases

Alpha-galactosidases are enzymes that catalyze the hydrolysis of simple and complex oligosaccharides and polysaccharides. Some food or feed products, e.g., soy-based products, are composed of galacto oligosaccharides and pectic polysaccharides, which are associated with some indigestibility problems (Kien 2008). Soy-based foods present some health benefits such as hypolipidemic,

anticholesterolemic, and antiatherogenic properties, and reduced allergenicity (Trindade et al. 2001), mainly because of their composition that includes lipids, vitamins, minerals, free sugars, isoflavones, flavonoids, saponins, and peptides of therapeutic value (Donkor et al. 2005; Sanjukta and Rai 2016). Besides, soy milk appears as an alternative to dairy products for lactose-intolerant people. Soybeans are abundant and less expensive than bovine milk and do not contain cholesterol (Hati et al. 2014). However, the beany flavor and the high levels of raffinose and stachyose which may cause gastrointestinal discomfort problems to consumers restrict the consumption of soybean and its products (Tsangalis and Shah 2004). The absence of alpha-galactosidase in the human intestinal mucosa makes the consumption of raffinose and stachyose, from soybean products, very difficult. Non-hydrolyzed oligosaccharides pass directly into the lower intestine and are then metabolized by alpha-galactosidase-producing bacteria, resulting in the production of gases (Tsangalis and Shah 2004). The use of alpha-galactosidase, or an organism that possesses high alpha-galactosidase activity, can minimize flatulence that is caused by some products composed by these oligosaccharides (Scalabrini et al. 1998). This fact could improve the nutritional quality of soy-based foods. In this way, there is great interest in the use of alpha-galactosidase pre-treated products, which may diminish gastric distress that is caused by carbohydrates fermentation in the large intestine. A promising solution for the elimination of these oligosaccharides in soy milk is the employment of alpha-galactosidases (Carević et al. 2016; LeBlanc et al. 2005).

14.2.1 Alpha-Galactosidase Classification and Action

Alpha-galactosidase, also called α-D-galactosidegalactohydrolase (E.C. 3.2.1.22), melibiase or alpha-D-galactopyranoside galacto hydrolase (IUBMB 2018) catalyzes the hydrolysis of α-(1,6)-galactosidic bonds causing the release of α-D-galactose (Anisha and Prema 2007; Du et al. 2013; Naumov 2004). Alpha-galactosidases are classified according to their substrate specificity or amino acid sequence similarity. According to Garman (2007), alpha-galactosidases can be divided into Group I and Group II. Group I is composed of enzymes that act on oligosaccharides such as the raffinose family oligosaccharides (RFOs). Group II includes enzymes that act on polysaccharides such as galacto(gluco)mannans. Alpha-galactosidases have been classified into more than 100 glycoside hydrolase (GH) families, including GH 4, 27, 36, 57, 97, and 110, which belong to the CAZy database (http://www.cazy.org/). The GH family 27 consists of the eukaryotic alpha-galactosidases. GH family 36 includes predominately bacterial alpha-galactosidases.

Some oligosaccharides, such as stachyose, melibiose, and raffinose present α-(1,6)-galactosidic links (Gote et al. 2004; Naumov 2004). They are also found in branched polysaccharides such as galactomannans and galacto (gluco) mannans (Naumov 2004; Ademark et al. 2001). For this reason, the enzyme hydrolyzes galactosides, including galactose oligosaccharides, galactomannans, and

galactolipids (IUBMB 2018). Alpha-galactosidases act on stachyose, releasing one molecule of galactose and raffinose, and on raffinose releasing galactose and sucrose, which in turn can be hydrolyzed by invertase for different industrial applications (Anisha and Prema 2007; Du et al. 2013; LeBlanc et al. 2005; Naumov 2004).

14.2.2 Microbial Production of Alpha-Galactosidases

Different sources and substrates were reported for alpha-galactosidase production. Animals, plants, bacteria, and fungi were described as alpha-galactosidase producers. However, microbial production is the most employed. Extracellular alpha-galactosidases are generally produced by *Aspergillus* (Liu et al. 2007), *Trichoderma* (Savel'ev et al. 1997) and *Penicillium* (Shibuya et al. 1995) species. In an industrial point of view, bacteria and fungi are potentially more economic alpha-galactosidases producers (Jin et al. 2001) (Table 14.1). Lower amounts of the enzyme are produced by plants and animals which represent higher levels of separation and purification.

Table 14.1 Alpha-galactosidase production from different microbial sources and conditions

Microbial source	Fermentation	Medium	Optimum activity	Source
Lactobacillus fermenti NRRL B-585	SmF	MRS broth	1.1 U/mL or 4.3 U/mg	Mudgettt and Mahoney (1985)
Bifidobacterium breve 203	SmF	PYF broth (peptone/ yeast extract/Fildes solution)	Glucose-grown: 61 U/g Raffinose-grown: 2000 U/g	Xiao et al. (2000)
Bacillus stearothermophilus NCIM—5146	SmF	–	2.0 U/mL	Gote et al. (2004)
Lactobacillus fermentum	SmF	MRS	5 U/mL	LeBlanc et al. (2004)
Lactobacillus agilis LPB 56	SmF	Vinasse	11.07 U/mL	Sanada et al. (2009)
Aspergillus sojae ATCC11906	SmF	Modified YpSs medium	10.4 U/mL	Gurkok et al. (2011)
Humicola sp. NCIM 1252	SSF	Soya flour	44.6 U/g	Kotwal et al. (1998)

(continued)

Table 14.1 (continued)

Microbial source	Fermentation	Medium	Optimum activity	Source
Aspergillus foetidus ZU-G1	SmF	Soybean meal, 2% wheat bran, 0.1% KH_2PO_4 , and 0.05% $FeSO_4 \cdot 7H_2O$	64.75 U/mL	Liu et al. (2007)
Humicola sp. NCIM 1252	SmF	Soya flour	13.9 U/g	Kotwal et al. (1998)
Aspergillus oryzae	SSF	RGPW—red gram plant waste	3.4 U/g	Shankar and Mulimani (2007)
Aspergillus oryzae	SSF	WB—wheat bran	2.7 U/g	Shankar and Mulimani (2007)
rAgas2 from Hermetia illucens (Escherichia coli)	SmF		Specific activity 128.37 U/mg	Lee et al. (2018)
Debaryomyces hansenii UFV-1	SmF	Lactose-based medium 0.62 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 5.0 g/L yeast extract	1.10 U/mL	Baffa Júnior et al. (2018)
Thielavia terrestris (designated TtGal27A) *Pichia pastoris*	SmF	See Invitrogen's manual	402.1 U/mL	Liu et al. (2018)
Aspergillus foetidus NRRL 341	SmF	1.4 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4,0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L $CaCl_2 \cdot 2H_2O$, 0.3 g/L urea, 1.0 g/L proteose peptone, 0.2 g/L Tween 80, 20 g/L soybean hull, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.0016 g/L $MnSO_4 \cdot H_2O$, 0.0014 g/L $ZnSO_4 \cdot 7H_2O$, and 0.002 g/L $CoCl_2 \cdot 2H_2O$	5.22 U/mL	Li et al. (2018)

SmF Submerged fermentation; *SSF* Solid-state fermentation

Bacterial alpha-galactosidases are generally produced by submerged fermentation (SmF), using specific conditions of pH, temperature, and agitation according to the applied strain. Alpha-galactosidases activities range from 1.11 U/mL, by *Bifidobacterium bifidum* MB239 (Mudgettt and Mahoney 1985), to 11.07 U/mL, by *Lactobacillus agilis* LPB 56 (Sanada et al. 2009). Solid-state fermentation (SSF) or submerged fermentation (SmF) are usually employed for fungal alpha-galactosidases. In SSF activities range from 2.7 to 44.6 U/g, which are normally higher than in SmF.

14.2.3 Applications of Alpha-Galactosidases

The most important industrial applications of alpha-galactosidases occur in sugar beet and soybean food processing industries and as therapeutic use for humans (Gote et al. 2007). In the first case, raffinose is the major impurity in sugarbeet, accounting for about 1% (w/w), and has a strong effect on both morphology and growth rate of sucrose crystals. The raffinose content is unstable and declines during storage. So, the use of alpha-galactosidase to remove raffinose is carried out to avoid this problem (Martin et al. 2001; Nakata et al. 2012). In the soybean industry, alpha-galactosidases have been used to reduce the oligosaccharide content in soybean meal, soy protein concentrate, soybean milk, and other soybean-based foods (Ju et al. 2018).

Concerning therapeutic use, alpha-galactosidases are administered orally to enhance the digestion of legumes such as peas, beans, especially soybeans, that have a high content of oligosaccharides. In soybean, there is a great amount of raffinose and stachyose that are carbohydrates of low molecular weight, non-metabolizable in the human intestine by the lack of α-galactosidase, thus causing flatulence. Raffinose ingestion also suppresses serum IgE level that is associated with some allergic diseases such as atopic dermatitis, allergic rhinitis, and asthma (Nagura et al. 2002; Nakata et al. 2012). Another application of alpha-galactosidase is for Fabry's disease treatment, which is related to α-galactosidase A's deficiency (Francesco et al. 2013) which causes immune system irregularities (Castaneda et al. 2008). Immune cells from affected patients display a constitutive proinflammatory pattern of cytokine expression (Francesco et al. 2013). Male patients, with little or no alpha-galactosidase activity, exhibit the "classic form" of Fabry disease (Togawa et al. 2012), which causes pain in the peripheral extremities, hypohydrosis, angiokeratomas, corneal opacities, and renal, cardiac and cerebrovascular involvement. For this problem, an enzyme replacement therapy with recombinant alpha-galactosidase is recommended (Schiffmann et al. 2006; Weidemann et al. 2009).

14.2.4 Commercial Products Containing Alpha-Galactosidases

Some commercial dietary supplements containing alpha-galactosidase are presented in Table 14.2. The cost of capsules can be \$0.08–\$0.58/capsule or \$0.13–\$0.22/g. They are employed as digestive supplements or for pancreatic problems.

14.2.5 Patents on Alpha-Galactosidases

Alpha-galactosidases were used in food products to manufacture a soy protein product having modified sugar profile (high sucrose and monosaccharide content and low indigestible oligosaccharides), rich in isoflavones and no galactinol content, resulting in a product with improved flavor and functional properties. The product was prepared from defatted soybean material, by the treatment with enzyme, removing the fiber before or after this process to achieve the requisite protein content, and inactivating the enzyme (Monagle, WO200215712-A2).

Tzortzis et al. (WO2007071987-A2) claimed a new alpha-galactosidase DNA and amino acid sequences with transgalactosylating activity isolated from

Table 14.2 Commercial supplements containing alpha-galactosidase

Name	Brand	Enzymes and complements	References
Alpha-galactosidase	Nutriteck® Canada/ United States	Alpha-galactosidase	Nutriteck (2018)
Alpha-galactosidase from *Aspergillus niger*	Bean-Zyme® United States	Alpha-galactosidase	Bean-Zyme® (2018)
Alpha-galactosidase —enzyme tablets	Beano® United States	Alpha-galactosidase	Beano® (2018)
Digestive enzymes	Baseline Nutritionals® United States	Alpha-galactosidase	Baseline Nutritionals (2018)
Digestive health supplement	Elle Belle UK® England	Digestive enzymes blend (amylase, cellulase, caseine protease, invertase, phytase, lipase, penthosanase (hemi cellulose), alpha-galactosidase)	ElleBelleUK® (2018)
Gas enzyme alpha-galactosidase	Vitacost® United States	Alpha-galactosidase	Vitacost® (2018)
Pancreatic enzymes and alpha-galactosidase	AOR Zymes® Canada	Alpha-galactosidase	Supplete (2018)

Bifidobacterium bifidum. The enzyme is useful for converting melibiose to alpha-galactobiose disaccharides that can be incorporated into food and feed products that promote the growth of bifidobacteria in the gut, thus improving the health.

Alpha-galactosidase was also used to increase the yield of sucrose from beet molasses by reducing the content of raffinose. The enzyme was added to beet juice with a concentration of 10–60° Brix and withdrawn before the sugar-boiling and centrifugal separation process, thereby hydrolyzing raffinose into sucrose and galactose (Suzuki et al. US3992260-A).

A patent with 28 citations describes an invertase-free alpha-galactosidase preparation by culturing *Saccharomyces cerevisiae* NRRL-Y-12533 in a medium containing enzyme-inducing substance (e.g., glucose, melibiose, or galactose), and recovering the enzyme; this preparation can be used to remove raffinose from soybean meal or sugar beet. A method for degrading oligosaccharides (stachyose and raffinose) in soybean milk comprises contacting the milk with cells of *S. cerevisiae* NRRL-Y-12533, with extracts of the cells, or with alpha-galactosidase purified from the extracts. The enzyme can also be used to hydrolyze raffinose in sugar beets without hydrolyzing sucrose (Olivieri et al. EP81262-A).

Alpha- and beta-galactosidases can also be added as components in food products. Zanarotti et al. (US2016303206-A1) patented a pharmaceutical or nutritional composition containing alpha-galactosidase and beta-galactosidase and excipient or carrier. The composition is beneficial to individuals with gastroesophageal reflux disease and allows digestion of food rich in both lactose and oligosaccharide.

14.3 Beta-Galactosidases—Lactases

Beta-galactosidases, also called lactases, are one of the most important enzyme groups used in food processing (Mahdian et al. 2016). They are also employed in technological and environmental applications. Milk and dairy products have protein, calcium, phosphorous, magnesium, and other micro- and macronutrients in their composition. Almost 70% of the world adult population is discouraged in consuming milk due to lactase deficiency (Mattar et al. 2012; Lomer et al. 2008). In this way, the consumption of dairy products by this group of people became possible with the use of lactases for lactose hydrolysis into glucose and galactose (Husain 2010). Besides, additional characteristics of the products could be ameliorated such as sweetness. Additionally, de-lactosed milk or whey products amplify the value and possibilities of using whey, which is a highly polluting waste (Ansari and Satar 2012; Panesar et al. 2016).

The industrial-scale production of microbial beta-galactosidases is established, even though there are some obstacles in large-scale production that are still discussed. Some points of investigation are related to the discovery of novel microbial sources of beta-galactosidases with different characteristics and applications.

Recombinant beta-galactosidases of bacterial, fungal, and yeast origin were also reported (Ansari and Satar 2012; Anisha 2017).

14.3.1 Beta-Galactosidases' Classification and Action

Beta-galactosidases (EC 3.2.1.23) catalyze the hydrolysis of lactose (β-D-galacto-pyranosyl-(1-4)-D-glucopyranose) releasing D-glucose and D-galactose as an end product. Lactases are also involved in transgalactosylation reactions producing galactooligosaccharides (GOS) (Gosling et al. 2010; Mussatto and Mancilha 2007), which are consumed by bifidobacteria as growth-promoting substrates in human intestine (Kamran et al. 2016; Ansari and Satar 2012).

14.3.2 Microbial Production of Beta-Galactosidases

Beta-galactosidases or lactases can be produced in high yields by bacteria, fungi, yeasts. They also occur in plants (almonds, peaches, apricots, and apples) as well as in animal tissues (Husain 2010; Mahdian et al. 2016). Some examples of microbial sources and substrates for beta-galactosidases are presented in Table 14.3.

Table 14.3 Beta-galactosidases produced from different microbial sources and substrates

Microorganism	Medium	Activity	Reference
Bacillus sp.	Lactose (1%) and $MgCl_2$	50 U/mL	Kamran et al. (2016)
Bacillus sp. MPTK	Lactose and $MgCl_2$	~65 U/mL	Kumar et al. (2012)
Bacillus safensis	Lactose and glucose (3:1–39:1)	~0.14 U	Nath et al. (2014)
Streptococcus thermophilus	Acid whey	7.76 U/mL	Princely et al. (2013)
Kluyveromyces lactis NRRL Y8279	Lactose medium	14.10 U/mL	Dagbagli and Goksungur (2008)
Kluyveromyces marxianus	Lactose medium	31 U/mL	Cortes et al. (2005)
*Kluyveromyces marxianus*CCT 7082	Lactose medium	8.5 U/mL	Manera et al. (2008)
Aspergillus tubengensis GR1	Czapeck Dox Lactose	169 U/mL	Raol et al. (2015)
Trichoderma acidotherma AIU BGA-1	Lactose (2%)	54.6 U	Yamada et al. (2016)

14.3.3 Applications of Beta-Galactosidases

Beta-galactosidases are mainly employed in food and beverages, dietary supplements, and pharmaceuticals. Dairy and pharmaceutical industries are the major users of lactases. In the case of condensed milk and frozen dairy products, for example, they can be used to reduce the effect of lactose crystallization. The enzyme is also employed in the hydrolysis of whey components from the cheese processing industry, to decrease water pollution (Kamran et al. 2016; Husain 2010). The main application of beta-galactosidases is in the food industry. The use of lactases in whey hydrolysis, for example, can release sugar substitutes for cooking, confectionery, and nonalcoholic beverages (Panesar et al. 2016), which is another alternative for reducing the environmental impact of the sub-product (Marwaha and Kennedy 1988; Mahdian et al. 2016). Beta-galactosidases are also employed in transgalactosylation reactions to produce GOS (Gosling et al. 2010; Mussatto and Mancilha 2007) that are non-digestible oligosaccharides. They can be consumed as prebiotic food ingredients to improve the growth of bifidobacteria population, which is benefic for health as these bacteria reduce cholesterol level, producing different essential vitamins and also having anticarcinogenic properties (Grosová et al. 2008; Kamran et al. 2016).

14.3.4 Commercial Products Containing Beta-Galactosidases

The world's lactase market is continuously growing at 3.7% from 2017 to 2025 reaching around US$1,235 Mn in 2017 and it is expected to rise to over US$1,647 Mn by the end of 2025 (Persistence Market Research 2018). *Kluyveromyces lactis, Kluyveromyces fragilis, Kluyveromyces marxianus, Candida kefyr, Saccharomyces cerevisiae,* and the fungi *Aspergillus niger* and *Aspergillus oryzae* are the main lactase producers (Ansari and Satar 2012). Novozymes, Merck KGaA (Sigma-Aldrich), Sternenzyme, Amano Enzyme Inc., Calza Clemente, Senson, Natural Factors Inc., Specialty Enzymes and Biotechnologies, Nature's Way Products, LLC, Chr. Hansen A/S, Advanced Enzyme Technologies Limited, DuPont de Nemours and Company, DSM Chemicals and others are the main lactase industrial manufacturers. Liquid and solid enzymatic commercial formulations can be found (Table 14.4).

Table 14.4 Beta-galactosidases producers and suppliers

Enzyme source	Product name	Supplier
Bacteria		
Bacillus sp.	Novozym 231	Novozymes A/S, Bagsvaerd, Denmark
Escherichia coli	β-Galactosidase	Sigma Aldrich, UK
Yeast		
Candida pseudotropicallis	Neutral lactase	Pfizer, Milwaukee, USA
Kluyveromyces sp.	Lactase NL	Enzyme Development Corporation
Kluyveromyces lactis	Maxilact	DSM Food Specialties, Delf, The Netherlands
	β-Galactosidase	SNAM Projetti, Italy
	Lactase	Sigma Aldrich, UK
Kluyveromyces marxianus	Lactozyme	Novozymes A/S, Bagsvaerd, Denmark
Saccharomyces fragilis	β-Galactosidase	Sigma Aldrich, UK
Fungi		
Aspergillus niger	Sumylact	Sumitomo Chemical, Japan
	Lactase	Vallio Laboratory, Finland
	Aspergillus galactosidase	AmanoEnzyme Inc., Europe
Aspergillus oryzae	Astrolac	Calza Clemente, Italy
	Fungal lactase	
	Biolacatase	
	Lactase 2214C β-Galactosidase	

Source Modified from Panesar et al. (2010)

14.3.5 Patents on Beta-Galactosidases

The most recent patents on the use of lactase in food products are related to methods for lactose hydrolysis in milk products. These processes usually involved lactase hydrolysis and filtration procedures.

Within these processes, Doering et al. (EP3251515-A1) developed a method for preparing lactose-free dairy products by ultrafiltering the starting milk (lactose content of 3–5 wt%), performing reverse osmosis of the first permeate, mixing the first retentate with the second permeate and milk minerals in order to obtain a standardized milk product, and hydrolyzing the dairy product with lactase, so that the remaining amount of lactose still contained in the product (0.45–0.55 of the initial lactose value) was completely cleaved into glucose and galactose.

Knights (US2013287892-A1) invented a process for making low-lactose milk protein concentrate useful to provide protein, calcium, and other nutrients to food

compositions. It comprised heating a liquid milk composition of initial solids including whey protein, caseins, and lactose (9–25 wt%), so that the whey protein adhered to the caseins producing aggregates; cooling; membrane filtering; retaining protein aggregates, and adding lactase to reduce the concentration of lactose to less than 2 wt% relative to the total protein content. This method reduced gel formation in a milk product during storage, avoiding the whey protein crosslinking. The milk protein concentrate had a total protein content of 70 wt% of the retained solids, and the whey protein was present at 15–25 wt% of the solids.

A patent written by Stevens (US4853246-A) had 39 citations, it described the production of sweetened, high protein, low fat, and reduced lactose milk obtained by ultraheat-treating low-fat milk and adding lactase. The content of total milk solids was increased in an initial low-fat milk. The product contained 10–35% wt. total milk solids, less than ca. 2% wt. milk fat and less than ca. 1 wt% lactose.

Choi and Lee (KR2004103818-A) described the production of low-lactose milk by hydrolyzing milk with lactase and concentrating the hydrolyzed lactose milk by nanofiltration to partially remove glucose and galactose. Water was added to remove the sweetness.

Low-lactose dried milk powders of high lysine content were prepared by mixing separately dried protein retentate and lactase hydrolyzed permeates obtained by ultrafiltration or diafiltration, as described by Uiterwaal (EP108838-A). The milk protein was separated as a retentate by ultrafiltration or diafiltration, while the lactose was separated in the permeate, which was treated with lactase. These two fractions were dried and mixed. Lysin content was preserved by the prevention of Maillard reaction through the separate drying of protein and reducing sugars.

Shi et al. (CN101317599-A) claimed a method for producing lactose-free whole milk preparation by heating raw milk and degreasing by centrifugal separation, cooling skimmed milk, applying ultrafiltration, applying nanofiltration to the permeated filtrate of the ultrafiltration treatment, mixing, and homogenizing the fractions of interest (cream powder, permeated filtrate of nanofiltration and trapped fluid of ultrafiltration), pasteurizing, applying lactase enzymolysis, and finally ultrahigh temperature sterilization.

Lange (WO200045643-A1) claimed a method to produce lactose-free milk by hydrolyzing lactose to glucose and galactose, without altering the taste, i.e., without conferring a sweet taste to the product. Ultrafiltration and diafiltration were used to adjust protein and lactose concentrations to the ratio of about 1:1. The reduction of the lactose ratio was a key feature that allows its conversion into monosaccharides with an unnoticeable change in sweetness.

Finally, a method for the processing of acid whey to generate a product (acid whey solution, acid whey powder or texturized acid whey) with at least 40% less lactose was developed by Onwulata et al. (US2015150275-A1). The process involved treating the acid whey solution with alpha-galactosidase and beta-galactosidase, filtering the acid whey solution to form a retentate containing proteins and a permeate containing lactose and residual proteins, recovering lactose from the permeate and optionally drying the retentate to form acid whey powder.

Other processes to reduce the lactose content involving alternative procedures, novel lactases, and immobilization were developed. A process to produce lactose-free sour milk product, e.g., yogurt was developed by Silfverberg et al. (FI118115-B1). It involved performing lactase pre-hydrolysis on milk raw material, inactivating the enzyme by heating, and hydrolyzing the residual lactose simultaneously with the souring of the product. The resulting milk product contained less than 0.01% to less than 1% lactose.

Havlik et al. (US2016143305-A1) patented a yogurt composed by cultured milk containing milk solids and non-fat or solute chosen from glucose, galactose, lactic acid derived from milk solids from milk and lactase. The enzyme was added to hydrolyze the lactose in the milk during the culturing step performed by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

Ernst et al. (WO2009071539-A1) developed a method for the treatment of milk to reduce the content of lactose to very low levels using the lactase from *Bifidobacterium bifidum*. The enzyme was active at high temperatures (52 °C) and improved the quality of the milk. Hydrolysis occurs during fermentation and in the final dairy product.

Finally, the problem of enzyme cost could be handled by immobilizing lactase for reutilization during hydrolysis. In this sense, Ottofricke et al. (DE3310430-A) developed a process for lactose hydrolysis in whey catalyzed by lactase immobilized on carrier particles in solid bed periodically cleaned with aqueous solution accompanied by mechanical fluidization. The whey was passed through a solid bed of enzyme immobilized on carrier particles, and an aqueous solution was periodically passed in countercurrent to clean the system. The operation could be carried out continuously for long periods (around 3 months) without additional clarification of the whey, and the degree of hydrolysis remained high (80–185%) at the end of the operation.

14.4 Peptidases

Peptidases are enzymes that degrade proteins by the hydrolysis of peptide bonds (Barrett and McDonald 1986), and many of them are applicable in the food industry. Hydrolyzed proteins could enhance the protein digestibility, decrease protein allergy, and alter the sensory quality due to modifications in the matrix. In some cases, bioactive peptides may be liberated by enzyme action (Stover and Mehta 2017; Tavano et al. 2018). Although various proteases are currently under investigation for the processing of food, this topic refers only to digestive peptidases and their influence in human health and life quality.

Proteins found in grains (wheat, rye, barley, and oats) are related to a chronic inflammatory intestinal disease, called celiac disease (CD). These proteins are generally called gluten, a component with unique viscoelastic properties and widely used in different food products. The CD triggered by the ingestion of gluten affects around 70 million people globally. It is the most common disease of the digestive

system of genetic origin, characterized by the abnormal response of the body's immune system to a group of proteins (Palabiyik et al. 2016; Ribeiro et al. 2018).

The main amino acids present in gluten composition (proline and glutamine, called the prolamins) are responsible for the immune response in CD and gluten intolerance, because the high proline content makes these proteins resistant to digestive enzymes action in the gastrointestinal tract. Proline is the only amino acid whose side-chain links to the backbone, thus avoiding the protein cleavage by most proteases. In susceptible individuals, the oligopeptides elicit an autoimmune response in the small intestine and an inflammatory injury of the intestinal mucosa (Colgrave et al. 2017; Ribeiro et al. 2018). This inflammatory process leads to mal-absorption of different nutrients and the clinical manifestations vary according to the patient, including malnutrition, diarrhea, growth retardation, anemia, fatigue, and other diseases (e.g., adenocarcinoma, neurological, and hormonal disorders) (Ribeiro et al. 2018; Vaquero et al. 2018).

A gluten-free diet is the only effective treatment for CD yet, however, this very restrictive diet is not easy and it is costly. The patients usually give up the diet due to the difficulties in changing the lifestyle and avoiding any food containing gluten. For all these reasons, various alternatives of nondietary and dietary therapeutic procedures have been investigated (Jnawali et al. 2016; Rey et al. 2016; Ribeiro et al. 2018; Vaquero et al. 2018). Various dietary therapies (e.g., wheat genetic engineering and probiotic treatments) are currently under investigation to avoid the CD complications. This problem could be alleviated avoiding the gluten transit across the small intestinal mucosa, by a previous gluten protein degradation into a small or nonimmunogenic peptide. The gluten-specific peptidases are important enzymes that could be used for this purpose.

14.4.1 Peptidase' Classification and Action

Peptidases catalyze the hydrolysis of peptide bonds and show preference for small peptides as substrates. Some peptidases attack the peptide bonds in different positions, while others are selective by attacking the bonds between specific amino acids. These diverse specificities determine their classification and possible uses. Considering the position that the enzymes attack, endopeptidases are those that act in the middle of the polypeptide chain and exopeptidases are those that cleave at the end of the chain (Barrett and McDonald 1986; Tavano et al. 2018).

14.4.2 Microbial Production of Peptidases

Peptidase enzymes produced by various microorganisms (*Flavobacterium meningosepticum*, *Sphingomonas capsulate*, *Myxococcus xanthus,* and *Aspergillus niger*) are prolyl endopeptidases (PEP). The PEP is capable of degrading

proline-containing peptides (Shan et al. 2004; Stepniak et al. 2006). However, most of these enzymes are irreversibly inactivated in the stomach by pepsin and acidic pH, thus failing to degrade gluten before it reaches the small intestine. Encapsulation of these PEP was proposed in order to protect them from the acid and enzymes present in the gastrointestinal tract (Caputo et al. 2010).

An endopeptidase from *Aspergillus niger* showed efficiency in the digestion of gluten proteins and could be administered orally for the treatment of CD. It was active at the pH of the stomach and of the duodenum and retained enzymatic activity in the small intestine of rats. *Aspergillus niger* is generally recognized as a safe microorganism (GRAS) and is adequate for industrial-scale use. This PEP might be suitable for oral administration to degrade gluten proteins in the gastrointestinal tract (Mitea et al. 2008).

14.4.3 Applications of Peptidases

The high content of prolamins in the primary structure of gluten proteins is the main obstacle to their total hydrolysis in the gastrointestinal tract. Then, for the complete degradation of gluten and to avoid the immune response, gluten-specific peptidases with high specificity or selectivity are necessary. In vitro experiments showed good results of gluten degradation using a PEP obtained from *Aspergillus niger* (Stepniak et al. 2006). The PEP addition into the gastrointestinal tract is being considered as a therapeutic strategy since the digestive enzymes could degrade and prevent the accumulation of these glutamine and proline-rich peptides (Vaquero et al. 2018). The oral administration of PEP has some limitations, whereas the enzymes and bile acids present in the gastrointestinal tract could degrade them. For this purpose, the enzymes must be resistant and active under acidic conditions and be specific to gluten proteins (Lähdeaho et al. 2014; Rey et al. 2016; Ribeiro et al. 2018). The oral administration of an enzyme has the advantage of avoiding the loss of the original and unique viscoelasticity of the gluten network. In the case of enzyme addition to a food product, the hydrolytic mechanisms and the ingredients composition would have to be carefully studied and optimized.

14.5 Lipases

Obesity and overweight have serious health consequences and affect about 1.9 billion adults worldwide. It is estimated that 3 billion people will be overweight in 2030 (WHO 2015). The consumption of high-calorie foods and sedentary lifestyle are the main causes of increased obesity (Tchernof and Despres 2013; Kushner 2014). Facing this problem and the growing public awareness of the negative effects of fat intake on health, a variety of functional less-caloric foods has appeared on the market (Dupont et al. 2018). Designing new food structures and

using carbohydrate or fat substitutes are some of the strategies to reduce calories in food formulations. In another perspective, novel foods and additives capable of modulating the metabolism of nutrients in the gastrointestinal tract may be included. Concerning specifically the digestion of the fats, some studies have looked for compounds that interfere in the activity of the digestive enzymes (Tan and Chang 2017).

14.5.1 Lipases Classification

Triacylglycerol lipase (E.C. 3.1.1.3) or triacylglycerol ester hydrolases are a group of enzymes that catalyze the hydrolysis of fats in aqueous media. The hydrolysis of triacylglycerols to glycerol, diacylglycerols, monoglycerols, and free fatty acids occur in an oil–water interface(Treichel et al. 2010). In addition, lipases are also involved in the interesterification, alcoholysis, acidolysis, and esterification (Yu et al. 2016). Considering their specificities, lipases are classified into three categories: (i) substrate-specific, (ii) regioselective and (iii) enantioselective (Sarmah et al. 2018).

14.5.2 Microbial Production of Lipases

Lipases can be produced by yeasts, fungi, bacteria, animals, and plants. Recent findings also relate the enzymes from marine sources (Navvabi et al. 2018). Microbial lipases find wide applicability in many industries due to properties such as high stability, selectivity, and vast substrate specificity (Treichel et al. 2010). They are extensively used in food, dairy, flavor, pharmaceutical, biofuels, leather, cosmetic, detergent, and chemical industries. The microbial lipases represent the most widely used class of enzymes in biotechnological applications and organic chemistry. Microbial enzymes may catalyze the reaction by different mechanisms even when they belong to the same class. Nonspecific lipases act independently of the position, catalyzing the complete hydrolysis of triacylglycerols into fatty acids and glycerol in a random way (e.g., lipases produced by *Candida cylindracea*, *Staphylococcus aureus*, and *Pseudomonas* spp.). Specific 1, 3 microbial lipases catalyze reactions only at the outer positions of the glycerol backbone (e.g., lipases from *Aspergillus niger*, *Rhizopus delemar*, *Rhizopus oryzae*, *Candida lipolytica*, and *Penicillium roquefortii*) (Sharma and Kanwar 2014).

14.5.3 Applications of Lipases

Lipases are very versatile catalysts. In the food industry, they are related to the production of esters and specialty fats, improvement of food texture, flavor modification, and in the taste of butter, cheeses, and margarine. They have been applied to decrease the time of cheeses ripening, in the fermentation of vegetables, in the curing of meat products, in the processing of fish, in the modification of soybean milk, and in flavor improvement of alcoholic beverages. They can also promote changes in the lipids of flour used in the manufacture of bread and act in the synthesis of structured lipids for infant foods. In addition, lipases are useful in increasing the titer of polyunsaturated fatty acids in vegetable oils and improving the digestibility of natural lipids (Aravindan et al. 2007; Ferreira-Dias et al. 2013). Prospects for the use of lipases are great not only in food processing but also in pharmaceutical products. A variety of lipases from diverse sources such as animal gastric, microbial and plant lipases have been tested for replacement therapy (Fieker et al. 2011). Lipase activators and inhibitors have been either derived from natural sources (plants/animals/microbes) or have been artificially synthesized (Jawed et al. 2018). Colantuono (2018) showed different classes of polyphenols with different inhibitory capacities for lipase, α-amylase, and α-glucosidase and that these differences were linked to characteristic chemical structures of the enzyme. Microbial lipases of fungal or bacterial origin are of potential interest because of their acid and protease-stable properties and their activity at pH 3 to 10 (Borowitz et al. 2006; Aloulou et al. 2007).

In order to be used in industrial processing or as food additives, lipases need to be stable against proteolytic action, against thermal processing and also against oxidative compounds and detergent ingredients. Even with the aid of genetic engineering tools, specific and desired characteristics for lipases can still be developed. The development of lipases with application in novel foods, food additives or therapeutic supplements is an area with a prospect of expansion, given the projections of increased cases resulting from diseases related to inadequate diets and the population’s concern for life quality.

14.6 Enzymes Used to Remove Undesirable Compounds

Several enzymes are used for the elimination of compounds harmful to health in the different industries. In the processing of food products, L-asparaginase stands out presenting important industrial applications.

14.6.1 L-Asparaginase

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) is a type of hydrolytic enzyme that catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia under physiological conditions.

L-asparaginase is an agent commonly used as an alternative for the treatment of different cancers such as a malignant disease of the lymphoid system named ALL (acute lymphoblastic leukemia) and Hodgkin's lymphomas. L-asparagine can be synthesized in the human body, and lymphoblasts depend on the free L-asparagine available in the bloodstream for survival. Thus the addition of L-asparaginase into the blood performed with intravenous injection can lead tolymphoblasts' death (Meena et al. 2015; Horvat et al. 2016). L-asparaginase can be obtained from *Escherichia coli* and *Erwinia chrysanthemi* (Ebrahiminezhad et al. 2014; Shrivastava et al. 2016; Prabhu et al. 2017).

In the food industry, L-asparaginase can be used in the pre-treatment of baked or fried starchy foods, to reduce the formation of acrylamide by depleting the precursor asparagine (Hendriksen et al. 2009). Acrylamide (2-propenamide, C_3H_5NO) has long been considered a neurotoxin classified as potentially carcinogenic, discovered in foods in 2002. It is present in many food products, especially in baked and fried ones because of the reaction at high temperature between reducing sugars, formed by a process called Maillard reaction, and asparagine. Several potential strategies have been proposed to reduce acrylamide levels in foods. The use of the L-asparaginases has been reported, resulting in a decrease of acrylamide level of 30–97% (Mihooliya et al. 2017).

Different microorganisms have been reported as L-asparaginase producers including *Erwinia carotovora*, *Streptomyces*, *Escherichia coli*, *Aerobacter* spp., *Photobacterium* spp., *Serratia* spp., *Xanthomonas* spp., *Pseudomonas aeruginosa*, *Vibrio* spp., *Aspergillus tamari*, *Aspergillus niger*, *Aspergillus oryzae*, *Cladosporium* sp., *Beauveria bassiana*, *Bacillus* spp. (Qeshmi et al. 2018). Specifically for use in the reduction of acrylamide in foods, L-asparaginases from *Aspergillus oryzae*, *Thermococcus zilligii*, *Cladosporium* sp. and *Bacillus licheniformis* and recombinant strains of *Aspergillus oryzae* and *Aspergillus niger* have been studied (Shi et al. 2017; Dias et al. 2017).

Only recently L-asparaginase products have been made available to the food industry, thus their use is very expensive. These enzymes need to be stable to different forms of the degradation to which they are exposed under the conditions of food processing, be produced on a large scale and must not induce allergic effects. In this sense, additional studies are needed aiming at the economical enzyme production and efficient utilization. To enable enzyme reutilization, immobilization studies have been carried out with the use of several natural or synthetic carriers such as silica gel, dextran, alginate, gelatin, chitosan, and others (Agrawal et al. 2018).

Another strategy for L-asparaginase production is the substitution of submerged fermentation used industrially by the more economical solid-state fermentation with

agroindustrial residues as substrate. Soybean meal, red gram husk, coconut oil cake, rice bran are examples of substrates that have been used (Doriya and Kumar 2018). Nevertheless, factors as substrate source, control of pH, salinity, and temperature into the process significantly affect L-asparaginase production and need to be taken into account so that the process can be economically viable.

14.6.2 Beta-Glucanases

Beta-glucanases are hydrolytic enzymes, also called 1,3-1,4-β-glucanases (1,3-1,4-β-D-glucan-4-glucanohydrolase; GH family 17; EC 3.2.1.73), that are capable of releasing oligosaccharides by hydrolyzing high molecular weight glucans. Beta-glucans are linear polysaccharides consisting of cello oligosaccharide blocks united by β-1,3 linkages, normally present in the cell walls of many cereals like rice, oats, and wheat. The β-glucan content is an important parameter of quality in various food industries, for example, the barley β-glucans may result in higher viscosity in beer during the mashing process (Furtado et al. 2011). The use of 1,3-1,4-β-glucanases in brewing improves the process because they act in the reduction of viscosity and turbidity, which increases the rate of filtration and yield, producing a malt of the highest quality (Chaari et al. 2014).

Beta-glucanases are fiber-digesting enzymes that could be used to increase nutrients absorption. Beta-glucooligosaccharides (β-GOS) are produced by the cleavage of the β-(1 → 4) glycosidic linkage of glucans with the use of an endo-1,3-1,4-β-D-glucan-4-glucanohydrolase (EC 3.2.1.73). Beta-GOS as well as β-glucan promote different health benefits because they exhibit anti-oxidant and antibacterial activity, can reduce serum cholesterol and, for some probiotic bacteria, provide a selective substrate. Thus, this β-glucanase has shown promising applications as an additive in food, especially in the development of probiotic bacteria (Cho et al. 2018).

14.6.3 Others

Crosslinking enzymes can be used in the bioprocessing of dairy products in order to alter milk proteins' allergenicity and digestibility. The formation of molecular bonds between enzymes and proteins in combination with a mediator decreases the rate of digestion, which can result in elimination of allergens. In this sense, studies suggest a decrease in the potential for allergenicity because in the enzymatic crosslinking of β-casein using transglutaminase, tyrosinase, and laccase, also in combination with caffeic acid, highly polymerized caseins with increased digestion stability and high potential for IgE binding inhibition were obtained (Stanic et al. 2010).

References

Ademark P, Larsson M, Tjerneld F, Stålbrand H (2001) Multiple alpha-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificities. Enzyme Microbial Technol 29:441–448

Agrawal S, Sharma I, Prajapati BP, Suryawanshi RK, Kango N (2018) Catalytic characteristics and application of L-asparaginase immobilized on aluminum oxide pellets. Int J Biol Macromol 114:504–511

Aloulou A, Puccinelli D, De Caro A, Leblond Y, Carrière F (2007) A comparative study on two fungal lipases from *Thermomyces lanuginosus* and *Yarrowia lipolytica* shows the combined effects of detergents and pH on lipase adsorption and activity. Biochimica et Biophysica Acta 1771:1446–1456

Anisha GS, Prema P (2007) Production of α-galactosidase by a novel actinomycete *Streptomyces griseoloalbus* and its application in soymilk hydrolysis. World J Microbiol Biotechnol 23 (6):859–864

Anisha GS (2017) β-Galactosidases. In: Pandey A, Negi S, Soccol CR (eds) Current developments in biotechnology and bioengineering: production, isolation and purification of industrial products, pp 395–421 (2017)

Ansari SA, Satar R (2012) Recombinant α-galactosidases—past, present and future: a mini review. J Mol Catal B Enzym 81:1–6

Aravindan R, Anbumathi P, Viruthagiri T (2007) Lipase applications in food industry. Indian J Biotechnol 6:141–158

Baffa Júnior JC, Viana PA, Rezende ST, OSares NFF, Guimarães VM (2018) Immobilization of alpha-galactosidase from *Debaryomyces hansenni* UFV-1 in cellulose film and its application in oligosaccharides hydrolysis. Food Bioprod Process 111:30–36

Barrett AJ, McDonald JK (1986) Nomenclature: protease, proteinase and peptidase. Biochem J 237:935

Baseline_Nutritionals®. Digestive enzymes. http://www.baselinenutritionals.com/products/digestive-enzymes.php. Last accessed 15 Jun 2018

Beano®. Alpha-galactosidase—enzyme tablets. Retrieved July 30, 2014, from http://www.beanogas.com/. Last accessed 15 Jun 2018

Bean-Zyme®. Alpha galactosidase from *Aspergillus niger*. http://www.bean-zyme.com/. Last accessed 15 Jun 2018

Borowitz D, Goss CH, Limauro S (2006) Study of a novel pancreatic enzyme replacement therapy in pancreatic insufficient subjects with cystic fibrosis. J Pediatric 149:658–662

Caputo I, Lepretti M, Martucciello S, Esposito C (2010) Enzymatic strategies to detoxify gluten: implications for celiac disease. Enzyme Res 2010:1–9

Carević M, Banjanac K, Ćorović M, Jakovetić S, Milivojević A, Vukašinović-Sekulić M, Bezbradica D (2016) Selection of lactic acid bacteria strain for simultaneous production of α- and *β*-galactosidases. Zaštita Materijala 57(2):265–273

Castaneda JA, Lim MJ, Cooper JD, Pearce DA (2008) Immune system irregularities in lysosomal storage disorders. Acta Neuropathol 115:159–174

CAZy Homepage. http://www.cazy.org/. Last accessed 15 Jun 2018

Chaari F, Belghith-Fendri L, Blibech M, Driss D, Ellouzi SZ, Sameh M et al (2014) Biochemical characterization of a lichenase from *Penicillium occitanis* Pol6 and its potential application in the brewing industry. Process Biochem 49(6):1040–1046

Cho HJ, Jang WJ, Moon SY, Lee JM, Kim J, Han H, Kim K, Lee B, Kong I (2018) Immobilization of *β*-1,3-1,4-glucanase from *Bacillus* sp. on porous silica for production of *β*-glucooligosaccharides. Enzyme Microbial Technol 110:30–37

Colantuono A (2018) Vegetable by-products as a resource for the development of functional foods. In: Reference module in food science. Available online at https://doi.org/10.1016/B978-0-08-100596-5.22142-2

Colgrave ML, Byrne K, Howitt CA (2017) Food for thought: selecting the right enzyme for the digestion of gluten. Food Chem 234:389–397

Cortes G, Trujillo-Roldán MA, Ramírez OT, Galindo E (2005) Production of β-galactosidase by *Kluyveromyces marxianus* under oscillating dissolved oxygen tension. Process Biochem 40 (2):773–778

Dagbagli S, Goksungur Y (2008) Optimization of β-galactosidase production using *Kluyveromyces lactis* NRRL Y-8279 by response surface methodology. Electron J Biotechnol 11(4). Available on line at http://www.ejbiotechnology.info/content/vol11/issue4/full/12/

Dias FFG, Junior SB, Hantao LW, Augusto F, Sato HH (2017) Acrylamide mitigation in French fries using native L-asparaginase from *Aspergillus oryzae* CCT 3940. LWT—Food Sci Technol 76:222–229

Donkor ON, Henriksson A, Vasiljevic T, Shah NP (2005) Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. J Food Sci 70:375–381

Doriya K, Kumar DS (2018) Optimization of solid substrate mixture and process parameters for the production of L-asparaginase and scale-up using tray bioreactor. Biocatal Agric Biotechnol 13:244–250

Du F, Zhu M, Wang H, Ng T (2013) Plant physiology and biochemistry purification and characterization of an α-galactosidase from Phaseolus coccineus seeds showing degrading capability on raffinose family oligosaccharides. Plant Physiol Biochem 69:49–53

Dupont D, Le Feunteun S, Marze S, Souchon I (2018) Structuring food to control its disintegration in the gastrointestinal tract and optimize nutrient bioavailability. Innov Food Sci Emerg Technol 46:83–90

Ebrahiminezhad A, Rasoul-Amini S, Ghoshoon MB, Ghasemi Y (2014) *Chlorellavulgaris*, a novel microalgalsourcefor L-asparaginaseproduction. Biocatal Agric Biotechnol 3(2):214–217

ElleBelleUK®. Digestive health supplement. http://www.ellebelleuk.com/womens-health-supplements/digestive-health-supplement?cPath=63_128. Last accessed 14 Jun 2018

Ferreira-Dias S, Sandoval G, Plou F, Valero F (2013) The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries. Electron J Biotechnol 16:1–38. Available on line at http://dx.doi.org/10.2225/vol16-issue3-fulltext-5

Fieker A, Philpott J, Armand M (2011) Enzyme replacement therapy for pancreatic insufficiency: present and future. Clin Exp Gastroenterol 4:55–73. Available online at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3132852/

Francesco PNF, Mucci JM, Ceci R, Fossati CA, Rozenfeld PA (2013) Fabry disease peripheral blood immune cells release inflammatory cytokines: role of globotriaosylceramide. Mol Genet Metab 109(1):93–99

Furtado GP, Ribeiro LF, Santos CR, Tonoli CC, de Souza AR, Oliveira RR et al (2011) Biochemical and structural characterization of a β-1,3-1,4- glucanase from *Bacillus subtilis* 168. Process Biochem 46(5):1202–1206

Garman SC (2007) Structure-function relationships in alpha-galactosidase A. Acta Paediatrica, Int J Paediatr 6–16 (2007)

Gosling A, Stevens GW, Barber AR, Kentish SE, Gras SL (2010) Recent advances refining galacto oligosaccharide production from lactose. Food Chem 121:307–318

Gote M, Umalkar H, Khan I, Khire J (2004) Thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) and its application in the removal of flatulence causing factors from soymilk. Process Biochem 39(11):1723–1729

Gote MM, Khan MI, Khire JM (2007) Active site directed chemical modification of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146): involvement of lysine, tryptophan and carboxylate residues in catalytic site. Enzyme Microbial Technol 40(5):1312–1320

Gurkok S, Cekmecelioglu D, Ogel ZB (2011) Optimization of culture conditions for *Aspergillus sojae* expressing an *Aspergillus fumigatus* α-galactosidase. Biores Technol 102(7):4925–4929

Grosová Z, Rosenberg M, Rebroš M (2008) Perspectives and applications of immobilised β-galactosidase in food industry—a review. Czech J Food Sci 26:1–14
Hati S, Vij S, Mandal S, Malik RK, Kumari V, Khetra Y (2014) α-Galactosidase activity and oligosaccharides utilization by lactobacilli during fermentation of soy milk. J Food Process Preserv 38(3):1065–1071
Hendriksen HV, Kornbrust BA, Ostergaard PR, Stringer MA (2009) Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from *Aspergillus oryzae*. J Agric Food Chem 57(10):4168–4176
Horvat TZ, Pecoraro JJ, Daley RJ, Buie LW, King AC, Rampal RK, Tallman MS, Park JH, Douer D (2016) The use of *Erwinia* asparaginase for adult patients with acute lymphoblastic leukemia after pegaspargase intolerance. Leuk Res 50:17–20
Husain Q (2010) Beta galactosidases and their potential applications: a review. Crit Rev Biotechnol 30:41–62
IUBMB Enzyme Nomenclature. International Union of Biochemistry and Molecular Biology. http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/2/1/22.html. Last accessed 15 Jun 2018
Jawed A, Singh G, Kohli S, Sumera A, Haque S, Prasad R, Paul D (2018) Therapeutic role of lipases and lipase inhibitors derived from natural resources for remedies against metabolic disorders and lifestyle diseases. South Afr J Botany. Available online at https://doi.org/10.1016/j.sajb.2018.04.004
Jin FX, Li Y, Zhang CZ, Yu HS (2001) Thermostable alpha-amylase and alpha-galactosidase production from the thermophilic and aerobic *Bacillus* sp. JF Strain. Process Biochem 36(6):559–564
Jnawali P, Kumar V, Tanwar B (2016) Celiac disease: overview and considerations for development of gluten-free foods. Food Sci Human Wellness 5:169–176
Ju L-K, Loman AA, Islam SMM (2018) Alpha-galactosidase and its applications in food processing. Encyclopedia. https://ac.els-cdn.com/B9780081005965216430/3-s2.0-B9780081005965216430-main.pdf?_tid=23bb6fd4-d927-41dc-842ea739c399dda8&acdnat=1534707937_ece7287357bf3e93a8013f4fcbbc3e09. Last accessed 19 Aug 2018
Kamran A, Bibi Z, Aman A, Qader SAU (2016) Lactose hydrolysis approach: Isolation and production of *β*-galactosidase from newly isolated *Bacillus* strain B-2. Biocatal Agric Biotechnol 5:99–103
Kien LL (2008) Digestible and indigestible carbohydrates. In: Koletzko B (ed) Pediatric Nutr Pract, pp 42–46 (2008)
Kotwal SM, Gote SR, Sainkar SR, Khan MI, Khire JM (1998) Production of alpha-galactosidase by thermophilic fungus *Humicola* sp. in solid-state fermentation and its application in soyamilk hydrolysis. Process Biochem 33(3):337–343
Kumar DJ, Sudha M, Devika S, Balakumaran MD, Kumar MR, Kalaichelvan PT (2012) Production and optimization of *β*-galactosidase by *Bacillus* sp. MPTK 121, isolated from dairy plant soil. Annals Biol Res 3(4):1712–1718
Kushner RF (2014) Weight loss strategies for treatment of obesity. Prog Cardiovasc Dis 56:465–471
Lähdeaho ML, Kaukinen K, Laurila K, Vuotikka P, Koivurova OP, Kärjä-Lahdensuu T, Marcantonio A, Adelman DC, Mäki M (2014) Glutenase ALV003 attenuates gluten-induced mucosal injury in patients with celiac disease. Gastroenterology 146:1649–1658
LeBlanc JG, Garro MS, Savoy de Giori G (2004) Effect of pH on *Lactobacillus fermentum* growth, raffinose removal, α-galactosidase activity and fermentation products. Appl Microbiol Biotechnol 65(1):119–123
Lee C-M, Kim S-Y, Song J, Lee Y-S, Sim J-S, Hahn B-S (2018) Isolation and characterization of a halotolerant and protease-resistant α-galactosidase from the gut metagenome of *Hermetiaillucens*. J Biotechnol 279:47–54
Li Q, Loman AA, Callow NV, Islam SMM, Ju L-K (2018) Leveraging pH profiles to direct enzyme production (cellulase, xylanase, polygalacturonase, pectinase,-galactosidase, and invertase) by *Aspergillus foetidus*. Biochem Eng J 137:247–254

Liu C, Ruan H, Shen H, Chen Q, Zhou B, Li Y, He G (2007) Optimization of the fermentation medium for alpha-galactosidase production from *Aspergillus foetidus* ZU-G1 using response surface methodology. J Food Sci 72(4):M120–M125
Liu Y, Yang S, Liu J, Jiang Z (2018) High-level expression of a novel protease-resistant α-galactosidase from *Thielavia terrestres*. Process Biochem 71:82–91
Lomer MC, Parkes GC, Sanderson JD (2008) Review article: lactose intolerance in clinical practice–myths and realities. Aliment Pharmacol Ther 27:93–103
Mahdian SMA, Karimi E, Tanipour MH, Parizadeh SMR, Ghayour-Mobarhan M, Bazaz BM (2016) Expression of a functional cold active β-galactosidase from *Planococcus* sp-L4 in *Pichia pastoris*. Protein Expr Purif 125:19–25
Manera AP, Ores JC, Ribeiro VA, Burkert CAV, Kalil SJ (2008) Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. Food Technol Biotechnol 46(1):66–72
Martin SS, Narum JA, Chambers KH (2001) Sugarbeet biochemical changes during pile storage. Part I Sugars. J Sugar Beet Res 38:35–53
Mattar R, Mazo DFC, Carrilho FJ (2012) Lactose intolerance: diagnosis, genetic, and clinical factors. Clin Exp Gastroenterol 5:113–121
Marwaha SS, Kennedy JF (1988) Whey–pollution problem and potential utilization. Int J Food Sci Technol 23:323–336
Meena B, Anburajan L, Dheenan PS, Begum M, Vinithkumar NV, Dharani G, Kirubagaran R (2015) Novel glutaminase free L-asparaginase from *Nocardiopsis alba* NIOT-VKMA08: production, optimization, functional and molecular characterization. Bioprocess Biosyst Eng 38(2):373–388
Mihooliya KN, Nandal J, Swami L, Verma H, Chopra L, Sahoo DK (2017) A new pH indicator dye-based method for rapid and efficient screening of L-asparaginase producing microorganisms. Enzyme Microbial Technol 107:72–81
Mitea C, Havenaar R, WouterDrijfhout J, Edens L, Dekking L, Koning F, Dekking EHA (2008) Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut 57:25–32
Mudgettt RE, Mahoney RR (1985) Kinetic properties of D-galactosidase from *Lactobacillus fermenti*. Enzyme Microbial Technol 7:207–211
Mussatto SI, Mancilha IM (2007) Non-digestible oligosaccharides: a review. Carbohyd Polym 68:587–597
Nagura T, Hachimura S, Hashiguchi M, Ueda Y, Kanno T, Kikuchi H, Kaminogawa S (2002) Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. British J Nutr 88 (4):421–426
Nakata H, Sakurai H, Nagura T, Tanaka M, Sone T, Asano K (2012) Raffinose production using alpha-galactosidase from *Paraphaeosphaeria* sp. Mycoscience 54(4):247–251
Nath A, Datta S, Chowdhury R, Bhattacharjee C (2014) Fermentative production of intracellular β-galactosidase by *Bacillus safensis* (JUCHE 1) growing on lactose and glucose–Modeling and experimental. Biocatal Agric Biotechnol 3:246–258
Naumov DG (2004) Phylogenetic analysis of alpha-galactosidases of the GH27 family. Mol Biol 38(3):463–476
Navvabi A, Razzaghi M, Fernandes P, Karami L, Homaei A (2018) Novel lipases discovery specifically from marine organisms for industrial production and practical applications. Process Biochem 70:61–70
Nutriteck®. Alpha-galactosidase. http://www.nutriteck.com/galactosidase.html. Last accessed 15 Jun 2018
Palabiyik I, Yildiz O, Toker OS, Cavus M, Ceylanm MM, Yurt B (2016) Investigating the addition of enzymes in gluten-free flours. Effect Pasting Textural Prop 69:633–641
Panesar PS, Kumari S, Panesar R (2010) Potential applications of immobilized beta-galactosidase in food processing industries. Enzyme Res, ID 473137, 16 p

Persistence Market Research (2018) Global market study on lactase: growing consumption of lactase in pharmaceuticals to provide market players with lucrative prospects. https://www.prnewswire.com/news-releases/global-lactase-market-to-reach-us-1647-mn-by-2025—persistence-market-research-676226763.html. Last accessed 15 Jun 2018

Prabhu RH, Bhise KS, Patravale VB (2017) Chapter one—marine enzymes in cancer: a new paradigm. In: Kim S-K, Toldrá F (eds) Advances in Food and Nutrition Research, vol 80. Academic Press, pp 1–14

Princely S, Basha NS, Kirubakaran JJ (2013) Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from *Streptococcus thermophilus* grown in whey. Eur J Exp Biol 3(2):242–251

Qeshmi FI, Homaei A, Fernandes P, Javadpour S (2018) Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. Microbiol Res 208:99–112

Raol GG, Raol BV, Prajapati VS, Bhavsar NH (2015) Utilization of agro-industrial waste for β-galactosidase production under solid state fermentation using halotolerant *Aspergillus tubingensis* GR1 isolate. Biotech 5(4):411–421

Rey M, Yang M, Lee L, Zhang Y, Sheff JG, Sensen CW, Mrazek H, Halada P, Man P, McCarville JL, Verdu EF, Schriemer DC (2016) Addressing proteolytic efficiency in enzymatic degradation therapy for celiac disease. Sci Rep 6:1–13

Ribeiro M, Nunes FM, Rodriguez-Quijano M, Carrillo JM, Branlard G, Igrejas G (2018) Next-generation therapies for celiac disease: the gluten-targeted approaches. Trends Food Sci Technol 75:56–71

Sanada CTN, Karp SG, Spier MR, Portella AC, Gouvêa PM, Yamaguishi CT, Soccol CR (2009) Utilization of soybean vinasse for α-galactosidase production. Food Res Int 42(4):476–483

Sanjukta S, Rai AK (2016) Production of bioactive peptides during soybean fermentation and their potential health benefits. Trends Food Sci Technol 50:1–10

Sarmah N, Revathi D, Sheelu G, Rani KY, Sridhar S, Mehtab V, Sumana C (2018) Recent advances on sources and industrial applications of lipases. Biotechnol Prog 34:5–28

Savel'ev AN, Eneyskaya EV, Isaeva-Ivanova LS, Shabalin KA, Golubev AM, Neustroev KN (1997) The carbohydrate moiety of alpha-galactosidase from *Trichoderma reesei*. Glycoconjugate J 14(8):897–905

Scalabrini P, Rossi M, Spettoli P, Matteuzzi D (1998) Characterization of *Bifidobacterium* strains for use in soymilk fermentation. Int J Food Microbiol 39(3):213–219

Schiffmann R, Ries M, Timmons M, Flaherty JT, Brady RO (2006) Long-term therapy with agalsidase alfa for Fabry disease: safety and effects on renal function in a home infusion setting. Nephrol Dial Transplant: Official Publ Eur Dial Transplant Assoc—Eur Renal Assoc 21(2):345–354

Shan L, Marti T, Sollid LM, Gray GM, Khosla C (2004) Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. Biochem J 383:311–318

Shankar SK, Mulimani VH (2007) Alpha-galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Biores Technol 98(4):958–961

Sharma S, Kanwar SS (2014) Organic solvent tolerant lipases and applications. Sci World J. Available online at http://dx.doi.org/10.1155/2014/625258

Shi R, Liu Y, Mu Q, Jiang Z, Yang S (2017) Biochemical characterization of a novel L-asparaginase from *Paenibacillus barengoltzii* being suitable for acrylamide reduction in potato chips and mooncakes. Int J Biol Macromol 96:93–99

Shibuya H, Kobayashi H, Park G, Komatsu Y, Sato T, Kaneko R, Nagasaki H, Yoshida S, Kasamo K, Kusakabe I (1995) Purification and some properties of alpha-galactosidase from *Penicillium purpurogenum*. Biosci Biotechnol Biochem 59(12):2333–2335

Shrivastava A, Khan AA, Khurshid M, Kalam MA, Jain SK, Singhal PK (2016) Recent developments in L-asparaginase discovery and its potential as anticancer agent. Crit Rev Oncol Hematol 100:1–10

Stanic D, Monogioudi E, Dilek E, Radosavljevic J, Atanaskovic-Markovic M, Vuckovic O et al (2010) Digestibility and allergenicity assessment of enzymatically crosslinked β-casein. Mol Nutr Food Res 54:1273–1284

Stepniak D, Spaenij-Dekking L, Mitea C, Moester M, de Ru A, Baak-Pablo R, van Veelen P, Edens L, Koning F (2006) Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am J Physiol Gastrointest Liver Physiol 291: G621–G629

Stover PJ, Mehta S (2017) Editorial overview: food biotechnology. Curr Opin Biotechnol 44:v–vi

Supplete. AOR zymes—pancreatic enzymes and alpha-galactosidase. http://www.supplete.com/product-p/aor04109.htm. Last accessed 15 Jun 2018

Tan Y, Chang SKC (2017) Digestive enzyme inhibition activity of the phenolic substances in selected fruits, vegetables and tea as compared to black legumes. J Funct Foods 38:644–655

Tavano OL, Berenguer-Murcia A, Secundo F, Fernandez-Lafuente R (2018) Biotechnological applications of proteases in food technology. Compr Rev Food Sci Food Saf 17:412–436

Tchernof A, Despres JP (2013) Pathophysiology of human visceral obesity: an update. Physiol Rev 93:359–404

Togawa T, Tsukimura T, Kodama T, Tanaka T, Kawashima I, Saito S, Sakuraba H (2012) Fabry disease: biochemical, pathological and structural studies of the α-galactosidase A with E66Q amino acid substitution. Mol Genet Metab 105(4):615–620

Treichel H, Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV (2010) A review on microbial lipases production. Food Bioprocess Technol 3:182–196

Trindade CS, Terzi SC, Trugo LC, Della Modesta RC, Couri S (2001) Development and sensory evaluation of soy milk-based yoghurt. Arch Latinoam Nutr 51(1):100–104

Tsangalis D, Shah NP (2004) Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria. Int J Food Sci Technol 39(5):541–554

Vaquero L, Rodríguez-Martín L, León F, Jorquera F, Vivas S (2018) New coeliac disease treatments and their complications. Gastroenterología y Hepatología 41(3):191–204

Vitacost®. Gas enzyme—alpha galactosidase. http://www.vitacost.com/vitacost-gas-enzyme-alpha-galactosidase-300-galu-per-serving-120-chewable-tablets. Last accessed 15 Jun 2018

Weidemann F, Niemann M, Breunig F, Herrmann S, Beer M, Störk S, Strotmann J (2009) Long-term effects of enzyme replacement therapy on fabry cardiomyopathy: evidence for a better outcome with early treatment. Circulation 119(4):524–529

WHO Technical Report: Obesity and overweight fact sheet N°311. Updated January 2015. Available online at http://www.who.int/mediacentre/factsheets/fs311/en/. Last accessed 01 Jun 2018

Xiao M, Tanaka K, Qian XM, Yamamoto K, Kumagai H (2000) High-yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose. Biotech Lett 22:747–751

Yamada M, Chiba S, Endo Y, Isobe K (2016) New alkalophilic β-galactosidase with high activity in alkaline pH region from Teratosphaeria acidotherma AIU BGA-1. J Biosci Bioeng 123 (1):15–19

Yu X-W, Xu Y, Xiao R (2016) Lipases from the genus *Rhizopus*: characteristics, expression, protein engineering and application. Prog Lipid Res 64:57–68

Chapter 15
Industrial Enzymes as Feed Supplements—Advantages to Nutrition and Global Environment

Sharrel Rebello, Divya Balakrishnan, A. N. Anoopkumar, Raveendran Sindhu, Parameswaran Binod, Ashok Pandey and Embalil Mathachan Aneesh

Abstract The relevance of enzyme fortification in animal feeds has been well established and exploited to generate a high income generating sector of industrial enzymes. Apart from aiding the better nutritional uptake and utility of the food constituents from the animal feed, the economic benefits gained by the production of better meat yield from livestock has prompted the acceptability of these enzymes in the feedstock. The current review outlines the various types of enzymes supplemented in animal feeds, their functional role and advantages of feed enzymes on animal growth and productivity. The effectiveness of feed enzymes in reducing the release of unused residual metabolites into the environment and the contributory role of these enzymes in diminishing the aftermaths of global warming are also discussed. The various strategies adopted in the individual and combinatorial generation of feed enzymes, their substrates and the characteristics of such feed enzymes are also presented in a concise manner. Finally, the advancements in the industrial production of feed enzymes and its supplementation are reviewed.

Keywords Feed enzymes · Applications · Advantages · Phytase

S. Rebello (✉) · A. N. Anoopkumar · E. M. Aneesh
Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Kerala, India
e-mail: sharrel.rebello@gmail.com

D. Balakrishnan
Cashew Exporters Promotion Council of India (CEPCI) Laboratory and Research Institute, Kollam 691001, India

R. Sindhu · P. Binod
Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum 695019, India

A. Pandey
CSIR-Indian Institute of Toxicology Research (CSIR-IITR), 31 MG Marg, Lucknow 226001, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_15

15.1 Introduction

Enzyme supplementation in feed has gathered great momentum in the modern world due to the increased interest for improved nutritional quality of food. Research and relevance of feed enzymes primarily took its origin in the United States, emphasizing their nutritive benefits in barley supported food products (Jensen et al. 1957; Willingham et al. 1961). Early studies on phytases, for example, augmented the phosphorus availability on poultry diets which enhanced the growth performance and facilitated digestion (Singh 2008). Irrespective of the wide utility of enzymes in a wide array of products, research on feed enzymes is still at a state of infancy. The development of target specific enzymes is however, gaining momentum in the last 3 decades. More research carried out in fermentation and microbiological technologies resulted new opportunity to produce feed enzymes in more cost-effective and in commercial formulations (Ravindran 2013).

Enzymes otherwise nicknamed as the biological catalysts are proteins with a compact three-dimensional molecular structure and substrate specificity, capable of accelerating chemical reactions and released by biological cells (Aehle 2007). Apart from its common role of adding nutritive value to food; enzymes differ in their specific mechanisms of action, molecular characteristics, stability (demanding or disregarding protective layers), site of action and efficiency of catalytic role. Thus different recipes of enzyme preparations are utilized in different food products to meet the nutritive demand of the food consumer as specified by the nutritionist.

15.2 Types of Feed Enzymes

Poultry feed, as well as most of the food, occurs as a mixture of proteins, fats, carbohydrates and proteins; demanding the use of enzyme cocktails to enable the breakdown of its nutritive molecules and better absorption by consumers (Selle and Ravindran 2007). Thus generally feed enzymes can be basically classified as cellulase (ß-glucanases), phytases, proteases, lipases and galactosidases as enlisted in Table 15.1. Sometimes they are merely grouped as carbohydrases (lysing any carbohydrate), proteases (breaking proteins) and phytases (lysing phytate content) based on the target molecules they act on. Enzyme addition to feed may have a varied effect on different animals influencing the rates of nutrient utilization and digestive ability (Juanpere et al. 2005). The combinatorial use of multienzymes along with phytase augmented the nutrient utility and performance of broiler chickens compared to unsupplemented (Attia et al. 2012; Choct 2006; Rani et al. 2003). However, generalization of enzyme recipes for entire animals does not seem to be effective; as different organisms respond differently to enzyme supplementation. In rainbow trout, for instance, phytase supplemented with protease showed a reverse impact on the growth performance; whereas an enzyme mix of pectinase, phytase, cellulase or enzyme mix had an anti-nutritive effect on nutrient utilization (Yigit and Keser 2016; Yigit et al. 2018) (Table 15.2).

Table 15.1 List of feed enzymes and their targeted feedstuff

Enzyme	Target substrate	Target feedstuff
Phytases	Phytic acid	All plant-derived ingredients
β-Glucanases	β-Glucan	Barley, oats, and rye
Xylanases	Arabinoxylans	Wheat, rye, triticale, barley, fibrous plant materials
α-Galactosidases	Oligosaccharides	Soybean meal, grain legumes
Proteases	Proteins	All plant protein sources
Amylase	Starch	Cereal grains, grain legumes
Lipases	Lipids	Lipids in feed ingredients
Mannanases, cellulases, hemi cellulases pectinases	Cell wall matrix (fiber components)	Plant-derived ingredients, fibrous plant materials

Table 15.2 A list of some commercially available feed enzyme with their characteristics

Trade name of enzyme	Type of enzyme	Company	Characteristics
Ronozyme Hi Phos	Phytase	Ronozyme	Increases phosphorus availability
QUANTUM® Blue	Phytase	ROAL. AB Vista	release phytate bound nutrients
HiZyme P-5000	Phytase	Vetline India	Aid phosphorus use in poultry feeds
FINASE® EC	*E. coli* derived phytase	ROAL. AB Vista	Used in pig and poultry feeds
RONOZYME® ProAct	Protease	Ronozyme	Improve protein digestion
RONOZYME® RumiStar™	Amylase	Ronozyme	Improves corn utilization in dairy diets
ECONASE® GT	Beta-glucanase	ROAL. AB Vista	Used in barley- and oat-based diets for piglets and broilers
ECONASE® XT	Beta 1-4, endo-xylanase	ROAL. AB Vista	Maximise energy utilization in pig and poultry feeds
HiZyme (Multi-Enzyme)	Blend of enzymes	Vetline India	Aids better-feed utilization, increased growth rate and production in broilers
CBT XL	Combination of non-starch polysaccharide (NSP) Enzymes with Probiotics	V. Excel International	Combination of heat tolerant enzymes targeting feed with high oil and fat content, supplemented with probiotics
AveMix® enzyme	Phytases, protease and non non-starch polysaccharide (NSP) hydrolase	Avevebiochem	Aid in polysaccharide, protein and phytate digestion

Carbohydrases include NSP (Non-starch polysaccharides) degrading enzyme that degrade fiber or NSP found on the plant cell wall, constituents large feed enzyme segment. Certain enzymes included in the segment is xylanases, beta-glucanases, xyloglucanses, galactomannanases, pectinases and debranching enzymes such as arabinofuranosidases and ferulic acid esterases. Among these enzymes, most widely used and important enzyme classes are xylanases and arabinoxylans that constituents a major part of NSP as feed ingredients (Le et al. 2013). These enzymes reduce the anti-nutritional factors of NSP by degrading the soluble fiber to reduce gut viscosity and enhance the nutrient absorption. Moreover, the degradation of polysaccharides yields oligosaccharides that act as prebiotics which benefits the gut microflora. Another class of carbohydrases widely used in the poultry industry is amylase, used in fast-growing broilers to improve starch digestibility level. Moreover, amylase supplemented with cellulase results in the enhancement of milk yield and back fat thickness. Commercially available enzymes are derived from *Bacillus* of different species viz, *halmapalus*, *licheniformis* and *stearothermophilus* (Gessesse et al. 2011). One of the basic problems with starch is that is it harder to absorb in the gut in form of pelleted feed as opposed to crushed feed and starch digestion seems to be less effective in fast-growing modern boiler breeds than low growing breeds; necessitating amylase in their diet (Svihus and Hetland 2001).

The other important class of enzyme is protease, included in feed with the purpose of increasing protein hydrolysis and thus improving the nitrogen utilization (Oxenboll et al. 2011). Protein digestion enables the better amino acid availability and enhanced absorption of other valuable nutrients. Another advantage of protein digestion is the reduced release of undigested protein in manure thereby reducing the drastic environmental effects such as eutrophication and acidification.

Phytases are enzymes supplemented to break down the anti-nutrient factor phytate present commonly in animal feed; thereby enabling the better functional utility of biomolecules (Rebello et al. 2018). Such phytate breakdown will enable the mobilization of various nutrient minerals from the animal feed into its body for utilization (Rebello et al. 2017).

15.3 Mode of Action and Functional Role of Enzymes

The use of enzymes in animal feed is of great commercial importance. Consistent increase in the price of feed ingredients had been a major limitation in most of the developing countries. (http://www.enzymesinc.com/vegetarian-vs.-animal-derived-enzymes.html). The ultimate aim of adding enzymes is to improve growth and profitability through enhanced digestion of dietary components (protein, amino acids, starch, lipids, and energy) in ingredients. However, the acceptance of feed enzymes could also be due to the necessity of varied feed formulations (in the presence of different feedstuffs), development of homogenous nutritive content even in different batches of production, reduced manure output, lowering of water

content in excreta, better digestibility and absorption of nutrients, upliftment of gut immunity of animals and development of uniformly healthy animals (Bedford and Cowieson 2012; Collett 2012; Jaroni et al. 1999).

Different feed enzymes in the industry have different modes of action due to substrate specificity, yet their exact mechanism is still unclear. This would involve sometimes the degradation of anti-nutrient factors or undigestible bonds in feed or even the breaking of physical cell walls aiding nutrient release (Bedford and Partridge 2001). In such an environment, it normally results in an altered pattern of digestion and the necessity of endogenous proteins for digestion is greatly reduced (Cowieson et al. 2009). The subsequent reduction in intestinal weight, alterations in intestinal microflora has a direct effect on its constituent enzymes and it often enhances the digestion rates (Svihus 2010).

15.4 Benefits on Nutrition

Various animal experiments bring significant data indicating the limited digestibility of nutrient elements in the small intestine of poultry (Low and Longland 1990; Pettersson and Åman 1989). Therefore, sufficient enzyme production might be renowned as the major factor responsible for the aforesaid state. The feed enzymes used in poultry might change the nutritional profile of feed elements to increase the efficiency of egg and meat production (Bedford 2000). Hence, appropriate use of feed enzymes in poultry might enrich the nutrient content and dietary energy together with the economic benefits for the public (Pettersson and Åman 1989).

Recently, considerable interest has been shown in the use of phytase as a feed additive, as it not only increases the availability of phosphate in plants but also reduces environmental pollution. The type and amount of cereals included in the diet, the level of anti-nutritive factor in the cereal, the concentration of enzymes used, the type and age of the animal (younger animals respond better to enzymes than older animals), type of gut micro-flora in digestive track and finally the physiology of the bird (Khattak et al. 2006). Although the majority of research trials of feed enzyme were conducted on broilers, the responses of egg-laying hens to enzyme-supplemented feeds are also well-documented. The use of an enzyme complex containing carbohydrases and phytase improves the growth potential of broilers, egg-laying hens, ducks, and Japanese quail (Yang et al. 2010). Certain enzymes, such as cellulase, xylanase, phytase, etc., required for the digestion of NSPs and phytates are not produced by birds. Hence, supplementation of NSP degrading enzymes and phytase not only reduce the anti-nutritive effects of NSPs and phytates but releases some nutrients to be utilized by the birds (Attia 2012).

15.5 Economic Advantages

The extensive characteristic feature of the poultry sector has allowed the faster uptake of advanced technologies including the use of feed enzymes. The extended use of these feed enzymes in animal nutrition is becoming a most widely used practice to overcome the harmful impacts of anti-nutritional elements (Ravindran 2013). The global market of enzymes is rapidly raised up to 7% during 2015. Based on the regional perception, North America was documented as the prevalent consumer of enzyme products in the regional markets of Western Europe. Similarly, the demand for feed enzymes in Asia/Pacific region including Japan, China, and India have increased rapidly (Adrio and Demain 2014; Li et al. 2012). In addition to this, the feed enzyme consumption in China was promptly growing at an annual rate of 7.5% in 2013 (Li et al. 2012).

The current research on farming systems receives much more attention towards feed enzymes for animal nutrition (Choct 2006). The economic advantages of most of all the enzymes used for poultry nutrition are associated with the reduction of feeding cost. The supplementation of different kinds of enzymes such as proteases, carbohydrases, lipases, and phytases on diet might upsurge the production efficiency and quality of poultry (Bedford and Partridge 2001).

One of the major economic advantages of supplementing feed enzymes into poultry is the enhanced production performances (Chen et al. 2002). Previous studies have reported that 10% of the total production performance of broilers can be improved by using the aforesaid feed enzymes (Cowieson and Ravindran 2008). However, the performance of feed enzymes on animal nutrition absolutely depends on the quality and quantity of feedstuff used together with the environmental conditions (Acamovic 2001). Perić et al. in 2011 reported that the supplementation of multi-enzyme complex of feed enzymes in animal nutrition improved economic advantages of poultry including reduced production cost (Perić et al. 2011).

15.6 Environmental Benefits

The production of poultry over the world has been rapidly increased during the sixties; the livestock population is found to be nearly 4 billion animals, and 500 million tons of manure has been produced in every year (Oxenboll et al. 2011). The overproduction of poultry is considered as one of the major drivers for enhanced production of nitrogen fertilizers and the emission of the aforesaid nitrogen-containing compounds might generate adverse impacts over the environment by atmospheric and water pollution (Crutzen et al. 2016). In order to increase the utilization of nutrients and reduce environmental pollution caused by poultry waste, several factors in the animal feeding aspects must be documented.

Nitrogen-containing compounds from the poultry waste are considered as one of the major perspectives for air and water pollution (Kendall et al. 1999). A number

of environmental benefits have been offered when protease is used as an additive in animal nutrition. Oxenboll et al. in 2011 reported that the use of protease as an additive in animal feed could contribute predominantly to reduce the quantity of nitrous compounds enter into the aquatic environment together with the reduction of acidification and eutrophication (Carpenter et al. 1998; Erisman et al. 2007; Oxenboll et al. 2011). The addition of feed enzymes to broiler diet offered better protein digestibility together with the drastic reduction of Nitrogen output from the broiler waste (Charlton and Pugh 1995). Manipulation of specific feeding enzymes to animal nutrition has greatly affected excretion of Nitrogen in urine and feces (Parsons et al. 1992).

The emission of ammonia can be reduced by employing low protein diet and specific nutrition techniques in animal feeding (Backus et al. 1997). Abdel and Tahir in 2015 reported that the supplementation of phytase enzyme in broilers has significantly reduced the phosphorus pollution from poultry waste, (Abdel-Megeed and Tahir 2015). The administration of phytase enzyme in broiler chicken has made substantial effects on pH and phosphorous content of excreta. They also discussed that the feed enzyme supplementation might decrease feed cost for phosphorous supplementation and the importance of inorganic phosphorus as a non-renewable element for the sustainable farming (Abdel-Megeed and Tahir 2015) (Fig. 15.1).

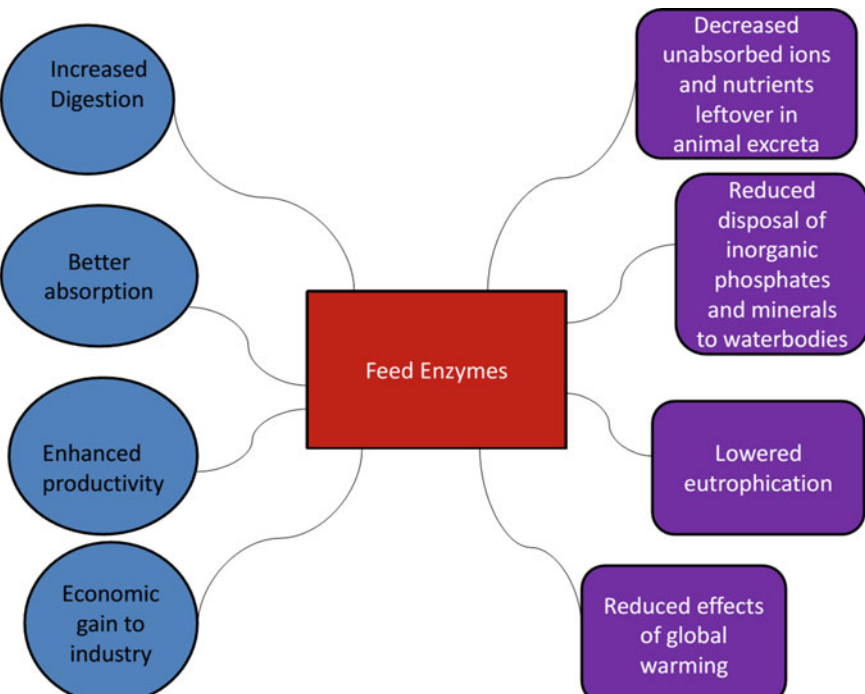


Fig. 15.1 Impacts of feed enzyme supplementation an overview

15.7 Industrial Production of Feed Enzymes

The industrial production of enzymes plays a significant role in modern biotechnology since the enzymes have been used in various biological systems including animal nutrition (Headon and Walsh 1994). The advanced developments in the biotechnology including genetic engineering have now enabled the industrial production of enzymes easy (Singhania et al. 2010). Hence the field of enzymology has been reflected as one of the most established branches of biotechnology. The discovery of novel enzymes together with the understanding of formerly reported enzymes and their functions offers several innovative applications for the industrial production and utilization of the aforesaid enzymes (Headon and Walsh 1994; Kirk et al. 2002; Volesky et al. 1984).

The amylases of plant and microbial (fungal and bacterial amylases) origin have played a major role in animal nutrition as feed enzymes since the industrial production of amylases offers low cost, consistency, and less time-consuming processes (Burhan et al. 2003). The solid-state fermentation (SSF) and submerged fermentation (SmF) is considered as the principal techniques for the rapid production of amylases at low cost (Hamliton et al. 1999; Haq et al. 1997; Pandey 2008). Several *Bacillus* bacterial species such as *Bacillus subtilis*, *B. licheniformis*, and *Bacillus stearothermophilus* are widely employed for the industrial production of α-amylases (Janeček 2002). The industrial sectors predominantly use *B. subtilis* as major producers for the large-scale production of proteases (Deviram et al. 2015). In addition to this, the microbial organisms from the genus *Aspergillus* have been also renowned as a good producer for the industrial production of α-amylases and proteases (Ward et al. 2009; Vihinen and Mantsiila 1989).

The Solid-state fermentation (SSF) technique has gained much more importance for the industrial production of α-Galactosidase due to its economic benefits such as improved enzyme yield, increased product stability, and lower cost (Hölker et al. 2004). Manipulation of several parameters such as pH, temperature, carbon sources, nitrogen sources, moisture, metal ions, and surfactants has been required for the large-scale production of feed enzymes (Ashraf et al. 2005; Burhan et al. 2003; Chandra et al. 1980; Ramesh and Lonsane 1990).

The fore-stomach tissue of lambs, pancreatic tissues of pigs, and plants can be used as an excellent source for the production of lipases. However, microbial lipases are presently receiving much more attention than others because of its economic benefits and technical advantages together with the drawbacks of using animal lipases (Vakhlu 2006). The bacteria, yeast, fungi, and actinomycetes are renowned as admirable sources lipases (Ertuğrul et al. 2007). Submerged culture, solid-state fermentation methods, and immobilized cell culture (used in rare cases) have been used for the large-scale production of lipases (Chen et al. 1999; Hemachander et al. 2001). Several previous studies have been conducted to understand the optimal nutritional requirements for the large-scale production of lipases using submerged culture since the production processes is strongly

influenced by several factors such as culture pH, dissolved oxygen concentration, temperature followed by the concentration of nitrogen and carbon sources (Elibol and Ozer 2000).

15.8 Conclusion

The practise of enzyme supplementation in feeds becomes very essential in the viewpoint of increased nutrition, better productivity, and yield of livestock. Apart from the nutritional benefits, the reduction in cost associated with waste management of poultry can be greatly reduced by enzyme addition in animal feeds. Moreover, the problems associated with increased environmental pollution and global warming can be addressed effectively by the practise of enzyme uptake by inhabitant animals grown in farms.

Acknowledgements Sharrel Rebello acknowledges SERB for National Post Doctoral Fellowship (File no. PDF/2015/000472). One of the authors Raveendran Sindhu acknowledges Department of Science and Technology for sanctioning a project under WOS-B scheme.

References

Abdel-Megeed A, Tahir A (2015) Reduction of phosphorus pollution from broilers waste through supplementation of wheat based broilers feed with phytase. J Chem (2015)

Acamovic T (2001) Commercial application of enzyme technology for poultry production. World's Poult Sci J 57:225–242

Adrio JL, Demain AL (2014) Microbial enzymes: tools for biotechnological processes. Biomolecules 4:117–139

Aehle W (2007) Enzymes in industry: production and applications. Wiley, New York

Ashraf H, Qadeer M, Iqbal J (2005) Pearl millet, a source of alpha amylase production by Bacillus licheniformis. Bioresour Technol 96:1201–1204

Attia YA, El-Tahawy WS, Abd El-Hamid AE-HE, Hassan SS, Nizza A, El-Kelaway MI (2012) Effect of phytase with or without multienzyme supplementation on performance and nutrient digestibility of young broiler chicks fed mash or crumble diets. Ital J Anim Sci 11:e56

Backus G et al (1997) Comparative study of four housing systems for non-lactating sows. In: Proceedings of 5th International Symposium Livestock Environment, Bloomington, Minnesota, pp 273–279

Bedford MR (2000) Exogenous enzymes in monogastric nutrition—their current value and future benefits. Anim Feed Sci Technol 86:1–13

Bedford M, Cowieson A (2012) Exogenous enzymes and their effects on intestinal microbiology. Anim Feed Sci Technol 173:76–85

Bedford MR, Partridge GG (2001) Enzymes in farm animal nutrition. CABI

Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. Process Biochem 38:1397–1403

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl 8:559–568

Chandra A, Medda S, Bhadra A (1980) Production of extracellular thermostable α-amylase by *Bacillus licheniformis*. J Ferment Technol 58:1–10

Charlton P, Pugh R (1995) Expanding enzyme applications: higher amino acid and energy values for vegetable proteins. Feed Compounder (United Kingdom)

Chen JY, Wen CM, Chen TL (1999) Effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*. Biotechnol Bioeng 62:311–316

Chen X, Zhang B, Fischer JA (2002) A specific protein substrate for a deubiquitinating enzyme: liquid facets is the substrate of Fat facets. Genes Dev 16:289–294

Choct M (2006) Enzymes for the feed industry: past, present and future. World's Poult Sci J 62:5–16

Collett SR (2012) Nutrition and wet litter problems in poultry. Anim Feed Sci Technol 173:65–75

Cowieson A, Ravindran V (2008) Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. Br Poult Sci 49:37–44

Cowieson A, Bedford M, Selle P, Ravindran V (2009) Phytate and microbial phytase: implications for endogenous nitrogen losses and nutrient availability. World's Poult Sci J 65:401–418

Crutzen PJ, Mosier AR, Smith KA, Winiwarter W (2016) N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. In: Crutzen PJ (ed) A pioneer on atmospheric chemistry and climate change in the anthropocene. Springer, Berlin, pp 227–238

Deviram G et al (2015) Production, optimization and partial purification of protease from Bacillus subtilis

Elibol M, Ozer D (2000) Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. Process Biochem 36:325–329

Erisman J, Bleeker A, Galloway J, Sutton M (2007) Reduced nitrogen in ecology and the environment. Environ Pollut 150:140–149

Ertuğrul S, Dönmez G, Takaç S (2007) Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. J Hazard Mater 149:720–724

Gessesse A, Mulaa F, Lyantagaye S, Nyina-Wamwiza L, Mattiasson B, Pandey A (2011) Industrial enzymes for sustainable bio-economy: large scale production and application in industry, environment, and agriculture in Eastern Africa

Hamliton L, Fogarty W, Kelly C (1999) Puri fication and properties of the raw starch degrading α amylase of Bacillus sp. IMD 434. Biotechnol Lett 21:111–115

Haq I, Ashraf H, Ali S, Qadeer M (1997) Submerged fermentation of alpha amylase by Bacillus licheniformis GCB-36. Biologia (Bratislava) 43:39–45

Headon D, Walsh G (1994) The industrial production of enzymes. Biotechnol Adv 12:635–646

Hemachander C, Bose N, Puvanakrishnan R (2001) Whole cell immobilization of *Ralstonia pickettii* for lipase production. Process Biochem 36:629–633

Hölker U, Höfer M, Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl Microbiol Biotechnol 64:175–186

Janeček Š (2002) How many conserved sequence regions are there in the α-amylase family. Biologia 57:29–41

Jaroni D, Scheideler SE, Beck M, Wyatt C (1999) The effect of dietary wheat middlings and enzyme supplementation. 1. Late egg production efficiency, egg yields, and egg composition in two strains of Leghorn hens. Poult Sci 78:841–847

Jensen LS, Fry RE, Allred JB, McGinnis J (1957) Improvement in the nutritional value of barley for chicks by enzyme supplementation. Poult Sci 36:919–921

Juanpere J, Perez-Vendrell A, Angulo E, Brufau J (2005) Assessment of potential interactions between phytase and glycosidase enzyme supplementation on nutrient digestibility in broilers. Poult Sci 84:571–580

Kendall D et al (1999) Effects of fiber addition (10% soybean hulls) to a reduced crude protein diet supplemented with synthetic amino acids versus a standard commercial diet on pig performance, pit composition, odor and ammonia levels in swine buildings. J Anim Sci 77:176
Khattak F, Pasha T, Hayat Z, Mahmud A (2006) Enzymes in poultry nutrition. J Anim Plant Sci 16:1–7
Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351
Le DM, Fojan P, Azem E, Pettersson D, Pedersen NR (2013) Visualization of the anticaging effect of Ronozyme WX xylanase on wheat substrates. Cereal Chem 90:439–444
Li S, Yang X, Yang S, Zhu M, Wang X (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J 2:e201209017
Low A, Longland A (1990) Carbohydrate and dietary fibre digestion in the pig and the possible influence of feed enzymes. Feed Compd 10:37–42
Oxenboll K, Pontoppidan K, Fru-Nji F (2011) Use of a protease in poultry feed offers promising environmental benefits. Int J Poult Sci 10:842–848
Pandey B (2008) Biotechnology. APH Publishing
Parsons C, Hashimoto K, Wedekind K, Han Y, Baker D (1992) Effect of overprocessing on availability of amino acids and energy in soybean meal. Poult Sci 71:133–140
Perić L, Sartowska K, Milošević N, Đukić-Stojčić M, Bjedov S, Nikolova N (2011) The effect of enzymes on the economics of poultry meat and egg production. Maced J Anim Sci 1:113–117
Pettersson D, Åman P (1989) Enzyme supplementation of a poultry diet containing rye and wheat. Br J Nutr 62:139–149
Ramesh M, Lonsane B (1990) Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system. Appl Microbiol Biotechnol 33:501–505
Rani BK, Desai J, Reddy D, Radhakrishna P (2003) Effect of supplementation of enzymes for non starch polysaccharides in corn-soya diet in broilers. Indian J Anim Nutr 20:63–69
Ravindran V (2013) Feed enzymes: the science, practice, and metabolic realities. J Appl Poult Res 22:628–636
Rebello S, Jose L, Sindhu R, Aneesh EM (2017) Molecular advancements in the development of thermostable phytases. Appl Microbiol Biotechnol 101:2677–2689
Rebello S, Anoopkumar A, Puthur S, Sindhu R, Binod P, Pandey A, Aneesh EM (2018) Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour Technol Rep
Selle PH, Ravindran V (2007). Microbial phytase in poultry nutrition. Anim Feed Sci Technol 2007 May 15;135(1-2):1-41
Singh P (2008) Significance of phytic acid and supplemental phytase in chicken nutrition: a review. World's Poult Sci J 64:553–580
Singhania RR, Patel AK, Pandey A (2010) The industrial production of enzymes. Industrial biotechnology: sustainable growth and economic success, pp 207–226
Svihus B (2010) Effect of digestive tract conditions, feed processing and ingredients on response to NSP enzymes. Enzym Farm Anim Nutr 129
Svihus B, Hetland H (2001) Ileal starch digestibility in growing broiler chickens fed on a wheat-based diet is improved by mash feeding, dilution with cellulose or whole wheat inclusion. Br Poult Sci 42:633–637
Vakhlu J (2006) Yeast lipases: enzyme purification, biochemical properties and gene cloning. Electron J Biotechnol 9
Vihinen M, Mantsiila P (1989) Microbial amylolytic enzyme. Crit Rev Biochem Mol Biol 24:329–418
Volesky B, Luong JH, Aunstrup K (1984) Microbial enzymes: production, purification, and isolation. Crit Rev Biotechnol 2:119–146

Ward OP, Rao MB, Kulkarni A (2009). Proteases, Production. Appl Microbiol Indust 495–511
Willingham HE, Fry RE, Leong KC, Jensen LS, McGinnis J (1961) Studies on stability of enzyme supplements to pelleting, long storage, and other treatments. Poult Sci 40:854–857
Yang J et al (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42:565
Yigit N, Keser E (2016) Effect of cellulase, phytase and pectinase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) fry fed diets containing canola meal. J Appl Ichthyol 32:938–942
Yigit NO, Bahadir Koca S, Didinen BI, Diler I (2018) Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (Oncorhynchus mykiss, Walbaum) fed soybean meal-based diets. J Appl Anim Res 46:29–32

Chapter 16
Intriguing Disposition of Marine Algae-Derived Enzymes in Food Biotechnology

Ravichandran Rathna, Ekambaram Nakkeeran, Sunita Varjani and Bethu Madhumitha

Abstract Marine algae are bestowed with the surfeit of bioactive molecules that play an important role in the field of food and pharmaceuticals for its idiosyncratic properties and prodigious applications. The bioactive compounds have a wide application in food and nutraceuticals as preservatives, additives, functional supplements, and so on. Marine algae are specially recognized for its unprecedented enzymes that are comparatively unique from terrestrial-derived enzymes. This chapter deals with the marine algae-derived enzymes, its biotechnological applications, interaction with human metabolism and innovations in enzyme technology. Further, manifestation and biomanufacturing of marine algae-derived enzymes are also discussed. This chapter also highlights the rapid advancement in marine algae-derived enzymes and the need for collaborative research for the better assessment of marine environment.

Keywords Enzyme market · Marine algae-derived enzymes · Applications Food industry · Challenges

16.1 Introduction

The ocean harbors a pageant of the unexplored reservoir of biodiversity, from paltry microbes to substantial fishes and corals. Microbial communities present in the marine ecosystem are considered as a prolific source of potential bioactive compounds that play a vital role in various industries. In addition, the growth rate, metabolic activity, and bioactive compounds isolated from marine organisms are

R. Rathna · E. Nakkeeran (✉) · B. Madhumitha
Research Laboratory, Department of Biotechnology,
Sri Venkateswara College of Engineering (Autonomous),
Sriperumbudur 602 117, Tamil Nadu, India
e-mail: nakkeeran@svce.ac.in

S. Varjani
Gujarat Pollution Control Board, Sector-10A, Gandhinagar 382010, Gujarat, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_16

unique than the terrestrial sources (Shahidi and Ambigaipalan 2015). Furthermore, the microalgae are known to accord 25% of global photosynthetic productivity. Over the past decades, researchers and industrial practitioners have gained interest in marine algae since it is bestowed with a surfeit of bioactive molecules that play an important role in the field of food and pharmaceuticals for its idiosyncratic properties and prodigious applications. Figure 16.1 depicts the application of marine algae-derived bioactive compounds. In recent years, consumers resurgence toward the untapped potential of products derived from natural sources is increasing substantially. Moreover, the social consciousness about the association and importance of nutrition and health is also considerably enhancing.

Enzymes are biocatalysts produced by the living organisms for its growth and metabolic process. The unique properties of enzymes have impetus its demand in industrial processes. Enzymes have its diverse array of applications in food and pharmaceutical industries, environment management, wastewater treatment, textile industries, biotechnology industries, production of fine chemicals and biodiesel. Increasing modernization and advancements in scientific and technological developments have further accelerated the demand for industrial enzymes on the global spectrum. Globally, amylase, lipase, protease, ligase, phytase, cellulase, and xylanase are enzymes of prime importance (Sarrouh et al. 2012). In the case of industrial enzymes—a global market overview, a recent report published by Research and Markets states the global market for industrial enzymes is forecasted

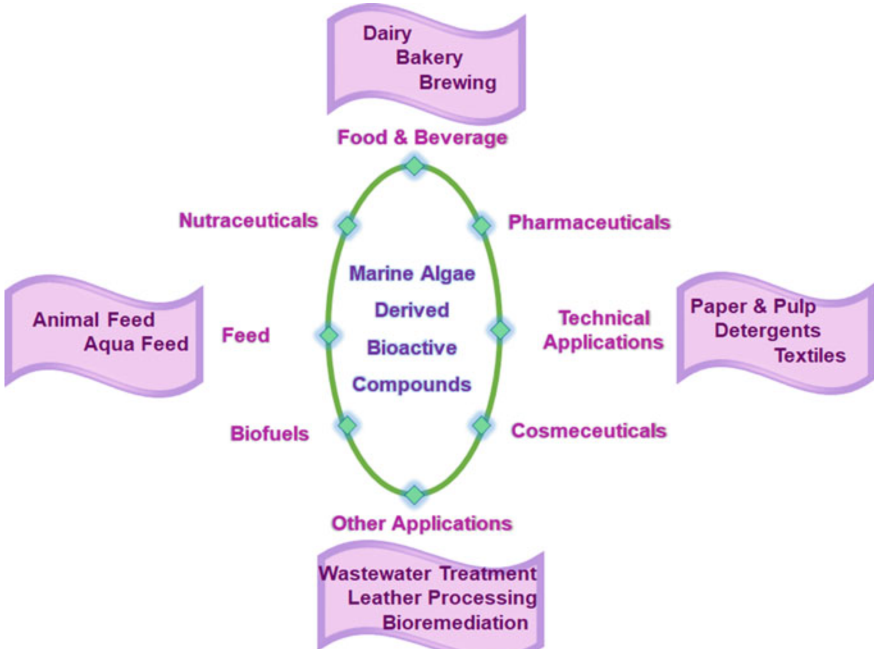


Fig. 16.1 Schematic representation of various applications of marine algae

to reach US$5.6 billion in 2018. The Freedonia Group, a division of Market Research reported that global demand for industrial enzymes is expected to reach $5.0 billion in 2021 with an annual growth rate of 4%. The largest market for industrial enzymes is food and beverage sectors with an estimated share of 26% followed by biofuels with 18% and the detergent sector with 14%. Further, in 2024, industrial enzymes in the biofuel sector are forecast to grow with a compound annual growth rate of 7.3%. Table 16.1 illustrates the major companies involved in the enzyme market.

Table 16.1 The major players in enzyme market

Company Name	Country	Established year	Sector
AB Enzymes GmbH	Germany	1907	Food, animal feed, technical applications
Adisseo France SAS	France	1939	Animal, poultry feed
Advanced Enzyme Technologies Limited	India	1957	Human and animal nutrition, food processing enzymes
Ecozymes	South Africa	2006	Animal feed, eco-chemicals, bio-enzymes
Amano Enzyme Inc.	Japan	1899	Food processing enzymes
American Biosystems, Inc.	USA	1979	Enzymes, direct-fed microbial products
Aum enzymes	India	2004	Food, feed, botanical grade enzymes
Biocatalysts Limited	United Kingdom	1986	Enzyme development for food industry
BioResource International, Inc.	United States	1999	Animal feed additives
Buckman Laboratories International, Inc.	United States	1945	Poultry feed
Cargill, Incorporation	United States	1865	Poultry feed, animal nutrition
Chr. Hansen Holding A/S	Denmark	1874	Food processing enzymes
Codexis, Inc.	United States	2002	Therapeutics, food, molecular diagnostics
Creative Enzymes	United States	2005	Diagnostic, industrial enzymes
DIREVO Industrial Biotechnology GmbH	Germany	2008	Food processing enzymes
Dyadic International, Inc.	United States	2002	Therapeutic, animal health, enzyme catalysts
Enzyme India Pvt. Ltd.	India	1995	Food, beverages
Hayashibara Co. Limited	Japan	1883	Health food ingredients
Iogen Corporation	Canada	1975	Feed stocks

(continued)

Table 16.1 (continued)

Company Name	Country	Established year	Sector
Maps Enzymes Limited	India	1975	Animal feed, brewing, starch
Novozymes A/S	Denmark	1925	Bioenergy, food, and beverages
Novus International, Inc.	United States	1991	Health products, animal feed
Royal DSM N.V.	The Netherlands	1902	Food, baking, beverages
Specialty Enzymes & Biotechnologies Co.	United States	1985	Digestive enzymes and probiotic blends
Sunson Industry Group	China	1996	Animal feed, food, and beverage

Marine algae are a photoautotrophic microorganism that forms the basic source of food for marine animals such as sea urchins and fishes. Broadly, marine algae are classified into two types, namely, microalgae and macroalgae. Microalgae (phytoplankton) such as chlorella, diatoms are unicellular eukaryotic photoautotrophic while macroalgae are multicellular photoautotrophic organisms that are generally classified based on their color into three types namely, Chlorophyta-green algae, Rhodophyta-red algae, and Phaeophyceae-brown algae. Since 1950, microalgae are commercially exploited as a food source, further, few products developed from algae are affirmed as generally recognized as safe (GRAS) by the United State Food and Drug Administration (FDA). For instance, TerraVia Algae Butter recently received GRAS status from FDA. Moreover, the European Union and Australia New Zealand Food Standards codes regulate the algal-based food products through Novel Food legislation. In India, according to Food Safety and Standards (Food Products Standards and Food Additives) Regulations 2011, algal oils are permitted in foods for infant nutrition.

Traditionally, algae are used as a protein source that has several advantages over the terrestrial plants in terms of nutritional value, cost, and productivity. Proteins extracted from algal sources like macroalgae (seaweed) and microalgae (duckweed) are categorized under aquatic protein. European countries use aquatic protein that has the potential nutritive feed for poultry and swine. Microalgae are a potential cost-effective source for biodiesel production, however, besides oil and secondary metabolites, these strains contain a significant amount of proteins (Becker 2007). The yield per hectare of seaweed, microalgae, and duckweed in Europe of 25, 15–30, 30–45 tons/ha/year, respectively, is higher than soybean (1.5–3 tons/ha/year), rapeseed (3 tons/ha/year), and legumes (4–6 tons/ha/year), making algal cultivation more attractive for farmers (Van Krimpen et al. 2013). However, conventional methods used for the extraction of proteins/enzymes from the algal source are enzyme hydrolysis, mechanical method, and chemical treatment. Recently, enzyme-assisted method, microwave-assisted method, ultrasound-assisted method, supercritical carbon dioxide extraction system, aqueous phase extraction, and membrane technology are used for the extraction of protein from marine algal

source (Grosso et al. 2015). *Chlorella* spp., *Aphanizomenon flos aquae*, *Arthrospira/Spirullina* spp., *Dunaliella salina*, *Haematococcus pluvialis*, *Cryptothecodinium cohenii* are few commercially exploited algae. This chapter deals with the marine algae-derived enzymes, its biotechnological applications, interaction with human metabolism and innovations in enzyme technology. Further, the manifestation and biomanufacturing of marine algae-derived enzymes are discussed. This chapter also highlights the rapid advancement in marine algae-derived enzymes and the need for a collaborative research for the better assessment of the marine environment.

16.2 Enzymes in Food Industries

The advent of natural science has expeditiously changed the food manufacturing sector from art to sophisticated and specialized technology. Enzymes play a predominant role in food processing and fermentation industries. Increasing awareness regarding the potential virtue of enzymes in food products has dramatically enhanced the demand, thereby urging market penetration of industrial enzymes. According to Global Market Insights, Inc. report, global food enzymes would transcend US$ 3.6 billion by 2024. Baking, dairy, juice, and brewing industries have escalated the demand for food enzymes (commercial enzymes). Enzyme specificity and activity are the important criteria to be considered while using enzymes, particularly in food industries since unwanted side reactions cause adverse effects to the system and even causes undesirable changes to product taste and texture. Five hundred years ago, Mexican Indians wrapped meat in papaya leaves (papain cysteine proteases) during cooking. Enzymes like amylases, hemicelluloses, and oxidases catalyzes the bread making process. Further, the use of bacterial collagenases during cooking and introduction of exogenous enzymes like Cathepsin B/D and calcium-activated factor mechanically recovered meat with 15–40% of bone weight used in soups and gravies (bacterial alcalase and neutrase) or animal feeds. Hydrolases are the most commonly used enzyme in food processing. In the dairy industry, proteinase (coagulating and debittering), catalase (prevents food oxidization), β-galactosidase (hydrolysis of lactose), lipase (flavoring and ripening of cheese), transglutaminase (cross-linking of protein molecules) and aminopeptidase (debittesing and improving the functional properties) are the most commonly used enzymes. In baking industries, amylase (softness), maltogenic α-amylase (enhances shelf-life), lipase (strengthens the dough), glucose oxidase (strengthens the dough), xylanase (dough conditioning), and protease (elastic nature) are used for improving the properties of bread. In juice industries, enzymes such as pectinase, cellulase, β-glucanase, glucosidases, etc., are used for extraction, clarification, flavor, color, stabilization, and improving the shelf life. Table 16.2 summarizes the applications of enzymes in a variety of food products.

Table 16.2 Food industrial applications of microbial enzymes

Enzyme	Year of discovery	Commercial name	Function	Applications
Bromelin	1891	Activin-R	Breaks down fibrin	Food ingredients (meat tenderizer)
Lactase	1980	Gasovia	Digestion of whole milk	Bakery and confectionary
Lipase	1848	Zypanc	hydrolysis of fats (lipids)	Dairy and bakery industry
Cellulase	1948	Digex	Break down cellulose	Food industry and clarification of fruit juices
Ficin	–	Debricin	Metabolization of protein into amino acids	Food industry
Pectinase	1950	BioPec	Breakdown pectin	Food and winemaking industry
Chymosin	–	CHY-MAX	Coagulation of milk	Food industry (cheese powder production)
Tannase	1786	Tannase	Degradation of tannins	Beverage and food processing
Glucoamylase	1833	–	Enzymatic determination of starch	Food industry
Glucose isomerase	1857	Glucose isomerase	Catalyzes the reversible isomerization of glucose into fructose	High-fructose corn syrup
Rennet	5000 thousand years ago	Maxiren	Hydrolysis of a specific peptide linkage	Bakery and confectionary
Pepsin	1800	Pepsin IP	Degradation of peptide molecules	Food processing (enzyme production)
Protease	1968	Batezyme	Degradation of peptide bonds	Food industry (confectionery)
Pentosenase	1930	BioPro – ACDH	Break down non-starch polysaccharides	Baking industry
Pullulanase	1961	Promozyme	Hydrolyze α-1,6 glycosidic linkages.	Bakery and confectionery
Urease	1868	Calbiochem	Break down urea into carbon dioxide and ammonia	Food production
α-amylase	1833	Gastrinex	Hydrolyses alpha bonds of starch and glycogen	Food industry (food additives)
Xylanase	1850	Xylanase PC	Breakdown hemicellulose	Baking industry
β-amylase	1833	1,4-α-D-glucan maltohydrolase	Hydrolysis of starch into sugars	Food industry (food additives)

16.3 Sources of Food Enzymes

The biologically active enzymes used in food industries are derived from a wide range of sources like plants, animals, and microbes. More than 80% of enzymes used commercially are derived from microbial sources such as fungi, yeast, and bacteria and the rest from animal and plant sources. Generally, enzymes derived from microbial sources are preferred industrially over the enzymes derived from animal/plant sources since it is more reliable and economical. Industrially important enzymes and their sources are shown in Table 16.3.

16.3.1 Enzymes Derived from Animals

Animal-based enzymes are derived from the pancreas of the animal. About 50 years ago, enzymes derived from the animal pancreas are the only source. Spleen, pancreas, liver, and stomach are the important parts accumulated with serval enzymes. Enzymes derived from animal source tend to be very narrowly focused, for instance, pancreatic enzymes are enteric coated and shuttled to the duodenum where digestion continues since they cannot survive in the acid environment of the stomach (pH 3). The main advantage of enzyme derived from animal sources is the

Table 16.3 Industrially important enzymes and their sources

Species	Enzymes	Enzyme activity
Animals		
Stomach of young calves	Rennet	–
Stomach mucosa of pig	Pepsin	>2000 U/mg protein
Chicken (egg white)	Lysozyme	>20 kU/mg
Porcine liver	Esterase	≥ 15 U/mg solid
Calf stomach	Chymosin	≥ 200 U/g
Calf abomasum	Chymosin	40 mg/l
Bovine abomasum	Pepsin	10,000 FCC U/mg
Abomasum of weaned calves	Rennet	≥ 20 U/mg protein
Plants		
Latex of unripe papaya fruit	Papain	≥ 10 U/mg protein
Pineapple juice and stem	Bromelain	2 mAnson-U/mg
Trunk of a tree (*Ficus insipida*)	Ficin	>200 Bapa-U/g
Microorganisms		
Aspergillus oryzae	Taka diastase	≥ 80 U/mg
Aspergillus ficuum	Tannase	≥ 150 U/g
Saccharomyces cerevisiae	Esterase	≥ 12 U/mg
Trichoderma viride	Xylanases	≥ 300 U/g

law of similarity and basis of homeopathy. According to the theory, though the enzymes are from different sources, it is recognized as similar by the host system and therefore, functions effectively. Trypsin and pepsin are the important animal-derived enzymes that have significant application in industries.

16.3.2 Enzymes Derived from Plants

The two important plant-based enzymes are bromelain from pineapple and papain from papaya. They function at a wide range of pH and not affected by temperature, thereby making it ideal as digestive enzymes. However, these enzymes cannot be used as systemic enzymes and contain harmful substances that cause gastrointestinal symptoms.

16.3.3 Enzymes Derived from Bacteria

There are many complex enzymes produced by bacteria. Several commercial enzymes such as amylases, invertase, glucose oxidases, and proteases are produced by bacteria. About a hundred enzymes, one-third of enzymes are obtained from bacterial sources.

16.3.4 Enzymes Derived from Fungi

For hundreds of enzymes produced industrially, over half of the enzymes are from fungi and yeast. Fungal enzyme blends can be custom formulated to suit the intended applications. This is possible because fungal sources not only include protease, amylase, and lipase activities but also offer a variety of other enzymes such as tilactase and cellulase. Overall, fungal enzymes are superior to pancreatin with regard to pH range, acid stability, activity, variety, and safety. In addition to the above advantages, fungal enzymes are animal-friendly and hence, suitable for a vegetarian diet. Most of the enzymes are produced by fungi or yeast. Some may consider this as plant-based but fungi are actually not plants. This is because the study of fungi has historically been a branch of botany.

16.3.5 Enzymes Derived from Yeast

Yeast is found to have fermentative properties. Yeast enzymes include sucrase, zymase, maltase, lactase, hexosephosphatase, reductase, carboxylase, melibiase,

and endotryptase. Different species of yeast provides various kinds of enzymes. A particular yeast may ferment one sugar but not another. At present, three of the aldohexose sugars (dextrose, D-mannose, and D-galactose) and a keto-hexose (laevulos) are fermented by yeast. Most yeast can invert and ferment the cane sugar because the enzyme sucrase is a common occurrence in the yeasts.

16.3.6 Enzymes Derived from Algae

Researchers, academicians, and industrialists are gaining interest toward algal source as a potential producer of industrial enzymes (Brasil et al. 2017). In food industries, algal source is the potential source of dyes and food colorants. Ansarifard et al. (2018) reported that the protease, lipase, and amylase enzyme activity of *Arthrospir platensis* improved the health and condition of koi fish. Spirulina and chlorella are used as protein source in salad dressings, beverages, baked goods (Wells et al. 2017). Algal enzymes are still in its infancy and further investigation on bioavailability, bio-accessibility, and bioactivity is needed.

16.4 Marine Algae-Derived Enzymes

Marine algae-derived enzymes have several advantages than other microbial sources. In general, enzymes produced by the marine sources evinces idiosyncratic propensities such as hyperthermostable (Bhosale et al. 2016), halotolerance (Huo et al. 2017), psychrophilic (Zhang et al. 2011), and distinct chemical property (Chakraborty et al. 2009). Therefore, bio-prospecting of the marine environment is efficacious in identifying novel bioactive compounds for several industrial applications. There are many studies stating the importance of marine algae as a source for many value-added products and its applications in biotechnology. Only few literature discuss the major importance of marine algae as a potential source of enormous industrially important enzymes. Marine algae served as a major breakthrough for new sources of the microbial enzyme. Algae were used in eliminating certain organic contaminations from the environment (Chekroun et al. 2014). Diverse classes of enzymes were reported to be produced by marine algae such as hydrolytic enzymes, dehydrogenases/oxidases, and lyases.

16.4.1 Cellulase

Cellulase enzyme is capable of cell wall degradation of plants or algae. Cellulases aid in the digestion of algal cell walls mainly consisting of polysaccharide and glycoprotein matrix for obtaining biomass, drugs, biofuels, foods, feeds, etc.

Marine algae are the source of primary energy in the aquatic environment. Cellulase covers a group of enzymes that are used in the synthesis or degradation of poly-oligo-dimers saccharides. Cellulase has potential application in various industries including paper mills and pulp industries, fabrications, wine production, beer brewing, fruit and vegetable juice processing, animal feedstock production, olive extraction, carotenoid extraction, detergent industry, and in waste management (Kuhad et al. 2011). Cellulase plays a key role in the fermentation process for the production of biofuels (Kumar and Murthy 2013). Additionally, cellulose has a new dimension as being used in biomedical research (Kunamneni 2016).

16.4.2 5′-Adenylylsulfate Reductase

5′-Adenylylsulfate reductase reduces sulfate for cysteine biosynthesis. Substrate preference is adenosine-5′-phosphosulfate and 3′-phosphoadenosine-5′-phosphosulfate. Glutathione or dithiothreitol is majorly used as the source of protons. 5′-Adenylylsulfate reductase was characterized by diverse marine algae. 5′-Adenylylsulfate reductase was observed in marine algae, *Enteromorpha intestinalis* and the optimum temperature required for its growth is ~25 °C (Gao et al. 2000).

16.4.3 Amylolytic Enzymes

Amylase promotes the hydrolysis of starch, oligosaccharides, and polysaccharides. α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), glucoamylase (E.C 3.2.1.3), isoamylase (EC 3.2.1.68), and glucosidases (EC 3.2.1.20) are the well-known amylases. Levis and Gibbs (1984) reported α-amylase activity in *Chlamydomonas reinhardtii* grown photoautotrophically in 12 h/12 h light/dark cycles. Rismani-Yazdi et al. (2011) and Shang et al. (2016) studied genes that encode enzymes involved in the catabolism of starch in the *Dunaliella* genus. Glycogen phosphorylase and starch phosphorylase were found in *Dunaliella parva* and *Dunaliella tertiolecta,* respectively, for the proteolytic degradation of starch.

16.4.4 Galactosidases

α-galactosidase is an exoglycosidase that hydrolyzes α-1,6-linked galactose residues found in oligosaccharides and galactomannan polysaccharides and releases α-D-galactose (Weignerová et al. 2009). Galactosidases are a homodimeric glycoprotein used in pulp and paper processing, sugar industry, and cattle-feed manufacturers (Husain 2010). Unicellular golden-brown algae, *Poterioochromonas malhamensis* exhibited intracellular α-galactosidase activity. The main industrial

application of *P. malhamensis* is to produce lactose-free dairy products. More recently, β-galactosidases with transgalactosylation activities had the potential to the synthesis of bifidus factors or prebiotics, galacto-oligosaccharides (Oliveira et al. 2011; Rosenberg 2006).

16.4.5 Superoxide Dismutase

Superoxide (O_2^-) radical is destimulated into molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) by Superoxide dismutase (SOD). Superoxide is a by-product of oxygen metabolism causing many types of cell damage if not regulated properly. Hydrogen peroxide is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. SOD is produced commercially by marine algae especially; *Euglena* sp. Cyanide inhibits SOD and the optimum temperature is ~25 °C.

16.4.6 Proteases

Proteases catalyzes the cleavage of a peptide bond in proteins and peptides (Mótyán et al. 2013). Protease has optimal pH for specific industrial applications. Thus, proteases are classified as alkaline or acidic proteases by their optimal pH conditions (Gupta and Ayyachamy 2012). Protease finds its application in detergent industry, food industry, leather industry, biomedical industry, pharmaceutical industry producing digestive and anti-inflammatory drugs (Zhang and Kim 2010). They enhance oil recovery from seafood, meat tenderization, reduced allergenicity in food industry, Improves washing tendency in detergents, textile degumming textile industry, dehairing, bating, tanning in leather processing, bioremediation waste treatment in bioremediation, anticancer, anti-inflammatory, clot-buster agents in pharmaceuticals, silver recovery, and silk degumming (Singh et al. 2016).

16.4.7 Lipases

Lipases are water-soluble acyl hydrolase enzymes that play a vital role in fat digestion by cleaving long-chain triglycerides into polar lipids. Lipases efficiently help in esterification, transesterification, and aminolysis (Joseph et al. 2008). Recombinant halophilic lipases are enzymes that enhance oil production by cleaving algal fatty acids from the acyl carrier protein. Among the enzymes, lipases have wide industrial applications. Microbial algal lipases have its application in paper production, cosmetics, food flavoring, organic synthesis, and other industrial applications.

16.4.8 Phytases

Phytases are phosphohydrolases that increased the bioavailability of organic phosphorus in livestock diets and reduced phosphorus pollution from animal waste (Abdel-Megeed and Tahir 2015). Recently, phytases exhibited prospective application in food processing applications (Shah et al. 2009, Greiner and Konietzny 2006). There are three types of phytases, 3-phytase (EC 3.1.3.8), 4-phytase (EC 3.1.3.26), and 5-phytase (EC 3.1.3.72). Klanbut et al. (2004) reported phytase activity in four species of thermotolerant blue-green algae, namely, *Synechococcus lividus* SKP50, *S. lividus* DSK74, *S. bigranulatus* Skuja, and *Chroococcidiopsis thermalis Geitler* isolated in Thailand.

16.4.9 Laccases

Laccases (EC 1.10.3.2) are extracellular glycoproteins belonging to the blue multi-copper oxidase family that oxidizes various aromatic and nonaromatic compounds via catalysis of radical reactions (Maté et al. 2011). They are a sustainable tool in various food industries such as wine production, beer brewing, fruit and vegetable juice clarification, color enhancement in tea, textile industry for denim bleaching and finishing, and paper industries (Giardina and Sannia 2015). Otto et al. (2010) reported laccase activity in algae, *Tethacystis aeria* and *Shewanella xiamenensis.* Species of the “Moewusinia” clade, including *Chlamydomonas moewusii* and *T. aeria*, secrete putative laccases that might help algae to survive in harsh environments (Otto et al. 2015).

16.4.10 β-Lactamase

This enzyme was observed in various strains of blue-green marine algae. In some cases, this enzyme permitted algae to overcome the inhibitory effects of penicillin. Production and localization of β-lactamase were studied in a unicellular species, *Coccochloris elabens* (strain 7003), and in a filamentous, nitrogen-fixing *Anabaena* species (strain 7120). When cells are grown in a neutral medium with $NaNO_3$ as a nitrogen source, the pH increases during growth; at a pH of about 10, most of the enzyme was extracellular and the entire cell-bound enzyme was expressed equally well in intact or disrupted cells. If the pH was kept near neutrality during growth by gassing with CO_2 in N_2 or by growth under conditions of N_2 fixation, the enzyme remained cell-bound and cryptic for most of the growth phase, being measurable only after cells were disrupted. The enzymes from strains 7003 and 7120 had greater activity on benzylpenicillin and other penicillin than on cephalosporin. Some differences were observed in the “substrate profiles” of penicillinases from the two strains against different penicillin.

16.5 Interaction with Human Metabolism

The enzymes extracted from marine algae serve a handful number of purposes in humans. The seaweed, commonly called marine algae is the food source for humans and several substances extracted from algae such as alginate and carrageenan are used in cooking, baking, and medicine, thereby actively interacting with the human metabolism. Also, Green algae (Chlorophyceae), *Chlorella vulgaris, H. pluvialis, D. salina, Cyanobacteria,* and *Spirulina maxima* are used as nutritional supplements for humans. Thus, the enzymes present in these algae directly have an impact on the metabolism of humans. α-1,3-Glucosidase, glucosidase II (GANAB) gly-coenzyme (NAD^+-dependent D-lactate dehydrogenase; glycolate oxidase) is an enzyme extracted from red marine microalga, *Porphyridium* sp. (Rhodophyta) which is involved in hydrolysis of terminal (1 → 3)-alpha-D-glucosidic links in (1 → 3)-alpha-D-glucans during N-glycosylation process of post-translational modification (Moenne et al. 2016). Similarly, phenolase-oxidoreductase extracted from *Monostroma fuscum* has high levels of antioxidants in which the cuprous ions present directly interact with the transport of water molecules in higher organisms (Tocher and Meeuse 1966). Similarly, the inhibition of androgen biosynthesis in humans is eliminated by extra and intracellular enzymes extracted from species of *Cylindrotheca closterium, D. salina* and *Chaetoceros muelleri* which directly target the androgen receptors and aid in the biosynthesis process of androgen hormone (Gao and Chi 2015). Squalene synthase-like enzyme extracted from *Botryococcus braunii* is helpful in the synthesis of C30 Squalene, a precursor which is essential in the process of Vitamin D, bile acids, steroids, and lipoproteins (Thapa et al. 2016). Several enzymes such as ascorbate peroxidase, glutathione peroxidase, and catalase extracted from several marine red and green macroalgae majorly help in the inhibition of metal accumulation with the help of cuprous ions for the activation of antioxidant systems which would result in improved flow of water molecules (Moenne et al. 2016; Babu et al. 2014).

16.6 Challenges and Future Perspectives

Since ancient times there is a substantial evidence of human consuming algal food products as a source of nutritional and functional compounds. Yet, there are illustrious challenges to quantify the extent of benefits and side effects. According to National Biotechnology Development Strategy 2015–2020 in India, emphasis is on the research on the distinctness of marine ecosystem diversity for effective exploitation in food, medicine, and other industrial applications. In 2016, Ira Levine, Professor of Natural and Applied Science, University of Southern Maine, US sought to expand collaborative research and education on algae in India. Council of Scientific and Industrial Research, Department of Biotechnology, Ministry of New and Renewable Energy and Department of Science and

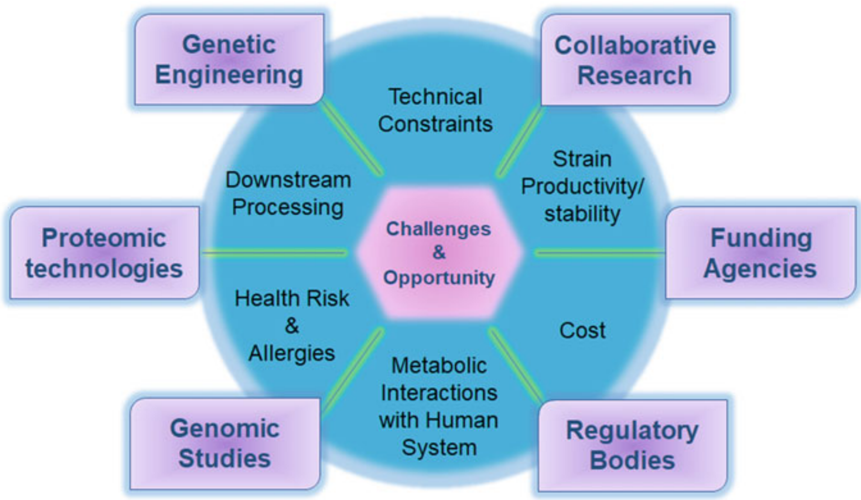


Fig. 16.2 Major challenges and opportunities on marine algae research

Technology in India is supporting research projects on the algae-based investigation. Because of the ongoing controversy on the implication of marine algae-derived in food, there is a significant regulatory framework. Regulatory bodies are more concern about product safety in case of food- and pharmaceutical-related products. Before launching marine algae-derived enzymes, the product has to be assessed for its reproducibility, pilot-scale production, capability, and specification for its effective strategy for marketing and scaling.

Figure 16.2 depicts the major challenges and opportunities faced by marine-algae-related research. Collectively, the wide range of potential enzymes derived from marine algae has the prospective to expand its health propitious attributes in food, pharmaceutical, nutraceutical, and cosmeceutical industries. The prime limitation in utilizing marine algae-derived enzymes is the lack of considerable breadth and depth of literature on the fundamental understanding of algal biology and its diversity. Currently, researches are mainly focussed on screening untapped reservoir of bioactive compounds from algal source and its applicability. The marine algae harvesting, enzyme production, storage, processing/purification techniques, and food application is also at infancy state. Investigation on optimization of cultivation conditions for the optimal productivity of desired compounds from marine algae is very limited. In addition, investigation based on the structural modification and performance of marine algae-derived enzymes by chemical or genetic methods is also required. Further, the translation research from microbiology and biochemistry into useful food system is interminable.

The advent of genomics and proteomics would assist in understanding the basic biological processes at the molecular level. Bioinformatics tools also help in studying the toxicological profile of the bioactive compounds derived from marine

algae. Therefore, the studies could be focussed on the interaction of marine-algal-derived enzymes with the human metabolism for its effective application in food industries. Consequently, the structural diversity, functional variation, accumulation, and bioavailability of the product are also given much interest by the scientist. In addition, health risks and allergies should also be assessed for product development and commercialization. Insights into the genetics and metabolic pathway are needed for quantifying metabolites and systems approaches. Therefore, for commercializing the enzyme derived from marine algae in the food sector, the accomplishment of systemic investigation on human models or clinical trials are ascertained in future.

16.7 Concluding Remarks

The increasing awareness of consumers regarding the association between health and food has raised the demand for the development of the food system for natural sources. A number of enzymes/bioactive peptides have been identified in marine algae that are is still in the development stage and further investigation is needed. The potential of bioactive compounds from marine algae in the food industries gives rise to the evolution of value-added products. Further, marine algae proteins and its derivatives have been targeted in cosmeceutical, nutraceutical, and pharmaceutical applications. For effective application of marine algal bioactive compounds, successful cultivation, harvesting, extraction, enrichment, and purification of algal protein/enzyme is essential at a commercial scale. Therefore, marine algae play an important role to facilitate a sustainable human society in near future. Hence, certain regulations and standards as to be laid that concerns the marketing and utilization of algal-derived products.

Acknowledgements The authors thank Prof. M. Sivanandham, Secretary, SVEHT and SVCE for their support and encouragement.

References

Abdel-Megeed A, Tahir A (2015) Reduction of phosphorus pollution from broilers waste through supplementation of wheat based broilers feed with phytase. J Chem

Ansarifard F, Rajabi Islami H, Shamsaie Mehrjan M, Soltani M (2018) Effects of *Arthrospir platensis* on growth, skin color and digestive enzymes of Koi, *Cyprinus carpio*. Iranian J Fish Sci 17(2):381–393

Babu MY, Palanikumar L, Nagarani N, Devi VJ, Kumar SR, Ramakritinan CM, Kumaraguru AK (2014) Cadmium and copper toxicity in three marine macroalgae: evaluation of the biochemical responses and DNA damage. Environ Sci Pollut Res 21(16):9604–9616

Becker EW (2007) Micro-algae as a source of protein. Biotechnol Adv 25(2):207–210

Bhosale H, Shaheen U, Kadam T (2016) Characterization of a hyperthermostable alkaline lipase from *Bacillus sonorensis* 4R. Enzyme Res

Brasil BDSAF, de Siqueira FG, Salum TFC, Zanette CM, Spier MR (2017) Microalgae and cyanobacteria as enzyme biofactories. Algal Res 25:76–89

Chakraborty S, Khopade A, Kokare C, Mahadik K, Chopade B (2009) Isolation and characterization of novel α-amylase from marine *Streptomyces* sp. D1. J Mol Catal B Enzymatic 58(1–4):17–23

Chekroun KB, Sánchez E, Baghour M (2014) The role of algae in bioremediation of organic pollutants. Int J Env Res Public Health 1(2):19–32

Gao J, Chi J (2015) Biodegradation of phthalate acid esters by different marine microalgal species. Mar Pollut Bull 99(1–2):70–75

Gao Y, Schofield OM, Leustek T (2000) Characterization of sulfate assimilation in marine algae focusing on the enzyme 5′-adenylylsulfate reductase. Plant Physiol 123(3):1087–1096

Giardina P, Sannia G (2015) Laccases: old enzymes with a promising future. Cell Mol Life Sci 72 (5):855

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44(2)

Grosso C, Valentão P, Ferreres F, Andrade PB (2015) Alternative and efficient extraction methods for marine-derived compounds. Marine Drugs 13(5):3182–3230

Gupta VK, Ayyachamy M (eds) (2012) Biotechnology of fungal genes. CRC Press

Huo YY, Rong Z, Jian SL, Xu CD, Li J, Xu XW (2017) A novel halotolerant thermoalkaliphilic esterase from marine bacterium *Erythrobacter seohaensis* SW-135. Front Microbiol 8:2315

Husain Q (2010) β galactosidases and their potential applications: a review. Crit Rev Biotechnol 30(1):41–62

Joseph B, Ramteke PW, Thomas G (2008) Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 26(5):457–470

Klanbut K, Peerapornpisarn Y, Khanongnuch C, Ishii M (2004) Phytase activity from thermophilic blue green algae from hot spring in Northern Thailand. Jpn J Phycol S2:57–60

Kuhad RC, Gupta R, Singh A (2011) Microbial cellulases and their industrial applications. Enzyme Res

Kumar D, Murthy GS (2013) Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. Biotechnol Biofuels 6(1):63

Kunamneni A (2016) Cellulase in biomedical research. In: New and future developments in microbial biotechnology and bioengineering, pp 267–275

Levi C, Gibbs M (1984) Starch degradation in synchronously grown Chlamydomonas reinhardtii and characterization of the amylase. Plant Physiol 74(3):459–463

Maté D, García-Ruiz E, Camarero S, Alcalde M (2011) Directed evolution of fungal laccases. Curr Genomics 12(2):113–122

Moenne A, González A, Sáez CA (2016) Mechanisms of metal tolerance in marine macroalgae, with emphasis on copper tolerance in Chlorophyta and Rhodophyta. Aquat Toxicol 176:30–37

Mótyán JA, Tóth F, Tőzsér J (2013) Research applications of proteolytic enzymes in molecular biology. Biomolecules 3(4):923–942

Oliveira C, Guimarães PM, Domingues L (2011) Recombinant microbial systems for improved β-galactosidase production and biotechnological applications. Biotechnol Adv 29(6):600–609

Otto B, Schlosser D, Reisser W (2010) First description of a laccase-like enzyme in soil algae. Arch Microbiol 192(9):759–768

Otto B, Beuchel C, Liers C, Reisser W, Harms H, Schlosser D (2015) Laccase-like enzyme activities from chlorophycean green algae with potential for bioconversion of phenolic pollutants. FEMS Microbiol Lett 362 (11)

Rismani-Yazdi H, Haznedaroglu BZ, Bibby K, Peccia J (2011) Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels. BMC Genom 12(1):148

Rosenberg ZMM (2006) Current trends of β-galactosidase application in food technology. J Food Nutr Res 45:47–54

Sarrouh B, Santos TM, Miyoshi A, Dias R, Azevedo V (2012) Up-to-date insight on industrial enzymes applications and global market. J Bioprocess Biotech S 4:002

Shah P, Bhavsar K, Soni SK, Khire JM (2009) Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. J Ind Microbiol Biotechnol 36(3):373

Shahidi F, Ambigaipalan P (2015) Novel functional food ingredients from marine sources. Current Opin Food Sci 2:123–129

Shang C, Bi G, Yuan Z, Wang Z, Alam MA, Xie J (2016) Discovery of genes for production of biofuels through transcriptome sequencing of *Dunaliella parva*. Algal Res 13:318–326

Singh R, Mittal A, Kumar M, Mehta PK (2016) Microbial protease in commercial applications. J Pharm Chem Biol Sci 4(3):365–374

Thapa HR, Naik MT, Okada S, Takada K, Molnár I, Xu Y, Devarenne TP (2016) A squalene synthase-like enzyme initiates production of tetraterpenoid hydrocarbons in *Botryococcus braunii* Race L. Nat Commun 7:11198

Tocher RD, Meeuse BJD (1966) Enzymes of marine algae: I. studies on phenolase in the green alga, *Monostroma fuscum*. Can J Bot 44(5):551–561

Van Krimpen MM, Bikker P, Van der Meer IM, Van der Peet-Schwering CMC, Vereijken JM (2013) Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products, no. 662. Wageningen UR Livestock Research

Weignerová L, Simerská P, Křen V (2009) α-Galactosidases and their applications in biotransformations. Biocatal Biotransform 27(2):79–89

Wells ML, Potin P, Craigie JS, Raven JA, Merchant SS, Helliwell KE, Smith AG, Camire ME, Brawley SH (2017) Algae as nutritional and functional food sources: revisiting our understanding. J Appl Phycol 29(2):949–982

Zhang C, Kim SK (2010) Research and application of marine microbial enzymes: status and prospects. Mar Drugs 8(6):1920–1934

Zhang SC, Sun M, Li T, Wang QH, Hao JH, Han Y, Hu XJ, Zhou M, Lin SX (2011) Structure analysis of a new psychrophilic marine protease. PLoS ONE 6(11):e26939

Chapter 17
Role of Cellulases in Food, Feed, and Beverage Industries

V. Anoop Kumar, R. Suresh Chandra Kurup, C. Snishamol and G. Nagendra Prabhu

Abstract The activities of microbial enzymes have been observed and utilized for many centuries, but it has been only in relatively recent times that the use of microbial enzymes has been commercialized. Cellulases are a group of enzymes consisting of three major components, endoglucanase, exoglucanase, and β-D-glucosidase of which endoglucanase acts on carboxymethyl cellulose causing random scission of cellulose chains yielding glucose and cello-oligosaccharides, exoglucanase acts on microcrystalline cellulose (Avicel) liberating cellobiose as the primary product β-glucosidase works on cellobiose to release glucose. All these enzymes act synergistically to release glucose as end product. Cellulase has a wide range of applications in Industrial Biotechnology and is the second most used industrial enzyme after protease. In most of the cases, they are used in combination with other enzymes like pectinase, hemicellulase, ligninase, etc. Some of the most important applications of cellulase are in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in biomass hydrolysis, agriculture, and research purposes. However, the most promising applications are in the food, feed, and beverage industries. The present review presents an overview of the role of cellulase enzyme in the food, feed, and beverage industries. Other major applications and scope for further research are also mentioned briefly.

Keywords Cellulase · Fermentation · Food · Feed · Beverage industries

V. Anoop Kumar · G. Nagendra Prabhu (✉)
Centre for Research on Aquatic Resources, S. D. College, University of Kerala, Alleppey, India
e-mail: nagendra.prabhu@gmail.com

R. Suresh Chandra Kurup
P. G. Department of Zoology, M. G. College, University of Kerala, Trivandrum, India

C. Snishamol
Department of Microbiology, S. D. College, University of Kerala, Alleppey, India

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_17

17.1 Enzymes

Enzymes are biological catalysts that accelerate chemical reactions and they are essential for almost all the cellular reactions to convert nutrients to energy. The enzymes bind to substrate and break them down which in turn increase the rate of reaction. They have been used for various purposes for several centuries. Most microbes like bacteria, fungi, actinomycetes, yeast, etc. produce enzymes having wide range of applications. About 50% of enzymes used in industries are obtained from fungi, 35% from bacteria, and only 15% from plants and animals (Saranraj and Naidu 2014).

A large number of industrial processes in the areas of agriculture, paper and pulp industry, textile, environmental, cosmetics pharmaceutical, and food biotechnology utilize enzymes (Kuhad et al. 2011). Current developments in biotechnology are yielding new applications for enzymes such as protease, cellulase, lipase, glucoamylase, pectinase, chitinase, xylanase, and papain to name a few. Cellulase is one of the most used industrial enzymes after protease and has a wide range of applications in Industrial Biotechnology.

17.2 Cellulase

Cellulase, together with other related enzymes, viz., hemicellulases and pectinases, are among the most important group of enzymes that are employed in the processing of lignocellulosic materials for the production of feed, fuel, and chemical feedstocks. Cell wall of plants is made of cellulose $(C_6H_{10}O_5)_n$, which is the most abundant organic polymer on Earth (Tomme et al. 1995). It is also produced by some animals and a few bacteria. Cellulose is found in plants as microfibrils (generally 2–20 nm diameter and 100–40,000 nm long). These form the structurally strong framework in the cell walls. Cellulose is a linear polymer of β-(1,4)-D-glucopyranose units in $^4C_1$ conformation. The fully equatorial conformation of β-linked glucopyranose residues stabilizes the chair structure, minimizing its flexibility. Cellulose is converted into smaller subunits by an enzyme called cellulase through the hydrolysis of β-1,4 linkages in cellulose chains (Henrissat 1991). The structure of cellulose is as shown in Fig. 17.1.

Fig. 17.1 Structure of cellulose

This enzyme is mostly produced by microorganisms, commonly bacteria and fungi (Bahkali 1996; Suresh Chandra Kurup et al. 2005; Snishamol et al. 2001; Suresh Chandra Kurup and Nagendra Prabhu 2001). As with all other enzymes, it was the developments in molecular biology that enhanced cellulase research in the 1980s as an academic discipline. The concept of cloning and expressing a cellulase gene in a foreign microbe was made successful during 1982–83 (Tolan and Foody 1999). Now the researchers are trying to refine the technologies to reuse enzymes which include use of carrier-free biocatalysts and magnetic biocatalysts (Margo et al. 2018).

17.2.1 Structure and Mode of Action of Cellulase

Cellulase is a complex enzyme system, comprising endo-1,4-β-D-glucanase (EC-3.2.1.4), exo-1,4-β-glucanase (exocellobiohydrolase, EC-3.2.1.91), and β-D-glucosidase (β-D-glucoside glucanohydrolase, EC-3.2.1.21). Combined action of cellulases hydrolyzes β-1,4 linkages in cellulose chains (Kuhad et al. 2011; Sukumaran et al. 2005; Juturu and Wu 2014). Compared to other glycoside hydrolases, cellulases can hydrolyze β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through acid hydrolysis mechanism. The hydrolysis products can either result in the inversion or retention of the anomeric configuration of carbon-1 at the reducing end (Birsan et al. 1998; Withers 2001).

Endoglucanases or carboxymethyl cellulases (CMCase) randomly hydrolyze the glycosidic bonds at the amorphous regions of the cellulose. This generates long-chain oligomers. These long-chain oligosaccharides are cleaved into short-chain oligosaccharides by the action of exoglucanases or cellobiohydrolases. Cellobiose or glucose is liberated through the processive action of exoglucanases on reducing or nonreducing ends (Juturu and Wu 2014). The most studied exoglucanases are cellobiohydrolases (CBH). They are produced by different microorganisms (Zhang and Zhang 2013).

Cellobiose is an inhibitor of endoglucanase and cellobiohydrolases. β-Glucosidases (BG) hydrolyze cellobiose and soluble cellodextrins to glucose. To initiate the hydrolysis BGs bind to nonreducing glucose units of cellobiose with their pocket-shaped active site. BG has least activity on insoluble celluloses (Zhang and Zhang 2013). The mechanism of enzymatic hydrolysis of cellulose is provided in Fig. 17.2.

Cellulase systems exhibit synergism, i.e., a better collective activity which is greater than the total activity of each enzyme acting individually. Synergy is necessary for complete hydrolysis of cellulosic materials. There are four forms of synergisms:

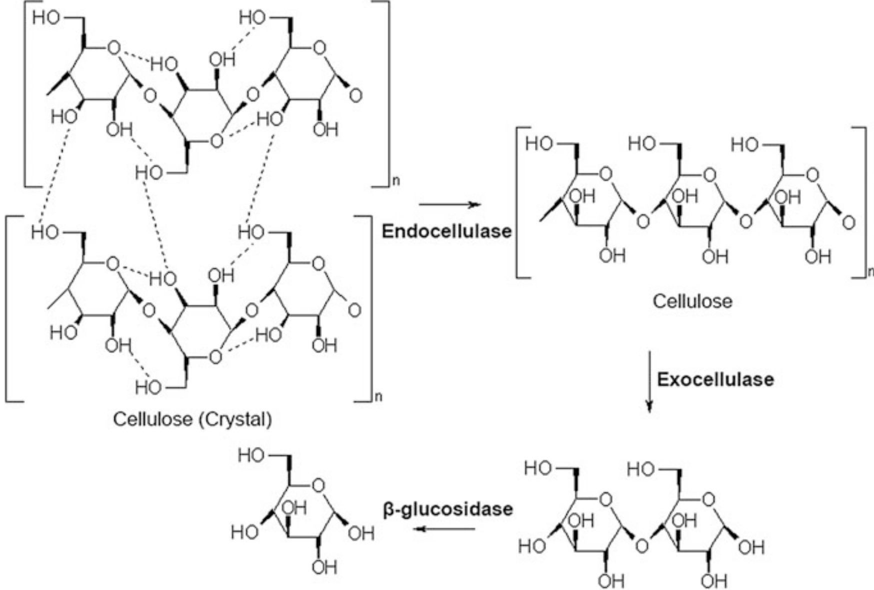


Fig. 17.2 Enzymatic hydrolysis of cellulose

(i) Endo–exo-synergy between endoglucanases and exoglucanases,
(ii) Exo–exo synergy between exoglucanases,
(iii) Synergy between exoglucanases and β-glucosidases, and
(iv) Intramolecular synergy between catalytic domains and carbohydrate-binding modules (CBMs) (Teeri 1997; Din et al. 1994).

Structure of microbial cellulases is given in Fig. 17.3.

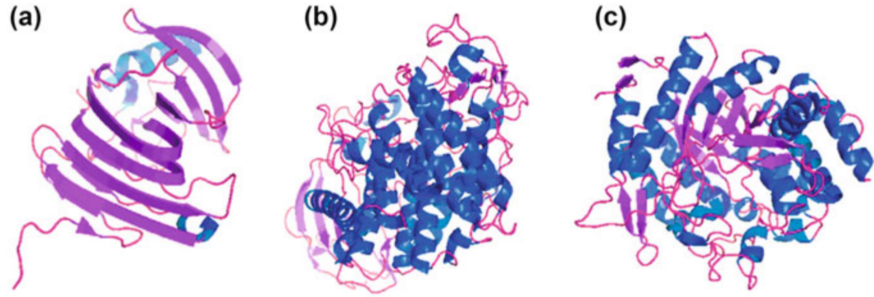


Fig. 17.3 Structure of microbial cellulasess: **a** endoglucanase, **b** cellobiohydrolase, and **c** β-glucosidase (Adapted from Liu and Kokare 2017)

17.3 Production of Cellulase

17.3.1 Microorganisms in Cellulase Production

Obtaining enzymes in a cost-effective manner is one of the key elements in industrial biotechnology. Among many economically feasible methods for the production of cellulase, submerged fermentation (SmF) and solid-state fermentation (SSF) using microorganisms are the most popular methods. A large number of microorganisms are capable of producing cellulase. The microorganisms which are the main producers of cellulase enzyme are fungi (Immanuel et al. 2006). Other microorganisms like bacteria and actinomycetes are also reported to produce cellulase (Shin et al. 2000). Fungi produce cellulase only in the presence of cellulose, whereas bacteria constitutively produce cellulase (Suto and Tomita 2001). Several methods to improve the production of enzymes are being explored, also to bring down the costs of production and to reduce time. They include optimization of parameters for fermentation, alterations in genome, new strains, isolation from different sources, etc. (Moubasher et al. 2016; Jain and Jain 2016; Xu et al. 2016; Yang et al. 2015; Tabssum et al. 2018). Further, bacteria from marine environments are also being isolated to obtain enzymes with higher salt, pressure, and temperature tolerance which are useful for their use in various industries (Nagendra Prabhu and Chandrasekharan 1996, 1997).

Fungi in Cellulase Production Fungi have long hyphae which can enter deep into cellulosic materials and this enables them to obtain more cellulose. Compared to other cellulase-producing organisms, fungi produce more amount of cellulase (Amouri and Gargouri 2006; Gaur and Tiwari 2015). *Trichoderma reesei* is the fungal strain most widely used for cellulase production and is considered as the model fungus for studying the production of enzyme (Shida et al. 2016). Other fungi such as *Humicola*, *Trichoderma*, *Penicillium,* and *Aspergillus* are also used for high yield (Jørgensen et al. 2003; Ong et al. 2004). The cellulase system of *T. reesei* (teleomorph: *Hypocrea jecorina*, initially called *Trichoderma viride*) has been the focus of research for 50 years (Reese et al. 1950; Mandeks and Reese 1957). *T. reesei* produces at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two β-glucosidases (BGLI and BGLII). Intensive efforts over several decades to enhance cellulase yields have resulted in strains that produce up to 0.33 g of protein/g of utilizable carbohydrate (Esterbauer et al. 1991). The necessity for the two exoglucanases (cellobiohydrolases) has been attributed to their particular preferences for the reducing (CBHI) and nonreducing (CBHII) ends of cellulose chains of microcrystalline cellulose (Henrissat et al. 1985; Nidetzky et al. 1994).

Moubasher et al. (2016) discovered cellulase production in extremophilic fungi from Wadi El-Natrun, a hypersaline and alkaline environment in Egypt. A newly isolated fungal strain, *Aspergillus niger* RKJP, showed enhanced cellulase production. The fungus showed higher activities of β,endo 1-4 glucanase (30.14 IU/gds), cellobiohydrolases (6.13 IU/gds), and β glucosidase (60.25 IU/gds). It was

employed for saccharification of lignocellulosic biomass into fermentable sugars with higher saccharifying efficiency of 85% (Jain and Jain 2016). On employing T-DNA insertional mutagenesis on *Humicola insolens* Y1, the mutant showed enhanced cellulase and hemicellulase activities. When compared with the wild type, activity of endoglucanase increased by 440%, cellobiohydrolases by 320%, and β-glucosidase by 41% (Xu et al. 2016).

Bacteria in Cellulase Production Several bacteria, including aerobic and anaerobic, produce cellulase enzyme. Even though fungi are mostly used for cellulase production, bacteria are also preferred in some cases, especially for recombinant enzymes. Bacteria are found in almost all habitats and have higher growth rate compared to fungi (Sadhu and Maiti 2013). Extremophiles can survive in harsh conditions which make them easier to be cultured (Maki et al. 2009). These qualities make them superior to fungi in several cases.

The cellulolytic strategies of aerobic and anaerobic bacteria are different. The anaerobes have a complex cellulase system (Bond and Stutzenberger 1989). Most of the anaerobes have their cellulases localized on their cell surface or glycocalyx matrix. Only some amount of extracellular cellulase is released into the medium. Many among the anaerobes show optimum growth characteristics when attached to the cellulosic substrate. The anaerobes convert the substrates into fermentation products such as acids, ethanol, carbon dioxide, hydrogen, etc. Therefore, the yields from anaerobes are usually lower.

Whereas the aerobic bacteria degrade cellulose by releasing a huge amount of extracellular cellulase into the culture medium (Rapp and Beerman 1991), some strains also have cellulase complexes located on the surface of their cells (Bond and Stutzenberger 1989; Wachinger et al. 1989). Because of these reasons, the bacteria need not bind to cellulose for hydrolysis. Since they produce extracellular cellulase, more yields can be expected from aerobic bacteria.

Tabssum et al. (2018) isolated a bacterium that had 98% homology with *Bacillus cereus*, from fish gut and optimized the parameters for cellulase production. Temperature of 30 °C, pH 9.0, and inoculum size of 2% (*v*/v) were most suitable for the production of exoglucanase under submerged fermentation. Maximum cellulase production was at 0.09% $MgSO_4$, 0.5% yeast extract, and 0.03% peptone. Andriani et al. (2017) studied cellulolytic and amylolytic abilities of bacterial strains *Bacillus megaterium* and *Bacillus mycoides*. The results of this study showed that they could be used as probiotics in fish and shrimp feed.

Co-culturing of microorganisms results in increased yields due to complete utilization of resources. When compared with mono-culturing, it is more efficient, eco-friendly, and cost-effective (Singh et al. 2018). But it may affect ethanol production because of the production of by-products like lactic acid and acetic acid (Herrero et al. 1985). For co-culture, microorganisms preferring similar environmental conditions must be selected. Interaction between the organisms is also a vital factor in the productivity (Kato et al. 2008, 2014). Desired bacterial strains could be designed through genetic engineering for more productivity. Table 17.1 presents a list of common fungi and bacteria used for cellulase production.

Table 17.1 Microorganisms used for cellulase production

Group	Organism	Reference
Fungi	*Aspergillus aculeatus*	Tomme et al. (1995)
	A. fumigatus	Immanuel et al. (2006)
	A. niger	Jain and Jain (2016)
	A. oryzae	Tomme et al. (1995)
	A. raperi	Jain and Jain (2016)
	Chaetomium cellulyticum	Jadhav et al. (2013)
	Fusarium moneliforme	Jadhav et al. (2013)
	Humicola insolens	Xu et al. (2016)
	Myceliophthora thermophila	Yang et al. (2015)
	Neocallimastix patriciarum	Tomme et al. (1995)
	Penicillium brasilianum	Jørgensen et al. (2003)
	P. chrysogenum	Jadhav et al. (2013)
	P. janthinellum	Tomme et al. (1995)
	Schizophyllum commune	Tomme et al. (1995)
	Trichoderma harzianum	Tomme et al. (1995)
	T. koningii	Tomme et al. (1995)
	T. longibrachiatum	Shin et al. (2000)
	T. reesei	Mandeks and Reese (1957)
	T. viride	Tomme et al. (1995)
Bacteria	*Acetobacter xylinum*	Tomme et al. (1995)
	Bacillus cereus	Tabssum et al. (2018)
	B. circulans	Tomme et al. (1995)
	B. lautus	Tomme et al. (1995)
	B. megaterium	Andriani et al. (2017)
	B. mycoides	Andriani et al. (2017)
	B. subtilis	Tomme et al. (1995)
	B. vallismortis	Gaur and Tiwari (2015)
	Caldocellum saccharolyticum	Tomme et al. (1995)
	Clostridium acetobutylium	Tomme et al. (1995)
	C. cellulolyticum	Tomme et al. (1995)
	C. straminisolvens	Kato et al. (2008)
	C. thermocellum	Herrero et al. (1985)
	Flavobacterium bolustinum	Malik et al. (2015)
	Pseudomonas fluorescens	Tomme et al. (1995)
	P. solanacearum	Tomme et al. (1995)
	Ruminococcus flavefaciens	Tomme et al. (1995)
	Streptomyces reticuli	Wachinger et al. (1989)
	S. lividans	Tomme et al. (1995)
	Thermobifida fusca	Tomme et al. (1995)
	Thermomonospora curvata	Bond and Stutzenberger (1989)

Substrates for Cellulase Production All cellulosic materials act as substrates for cellulase production. Since they have high cellulose content, the plants are usually preferred. Waste materials like coir waste (Mrudula and Murugammal 2011), sawdust (Immanuel et al. 2006), and cardboard (Szijártó et al. 2004) act as good substrates for SSF. Agricultural wastes such as paddy straw (Jadhav et al. 2013; Malik et al. 2015), sugarcane bagasse (Mandeks and Reese 1957), coffee husk

(Reese et al. 1950), wheat bran (Reese et al. 1950), and copra waste (Reese et al. 1950) are also used for cellulase production.

Aquatic weeds like *Eichhornia crassipes* and *Salvinia molesta* are also reported as novel substrates for cellulase production through SSF (Suresh Chandra Kurup et al. 2005). Water weeds are also used for the production of bioethanol (Mukhopadhyay and Chatterjee 2010). Since the weeds are available at free of cost and invade vast areas, their usage for enzyme production can bring down the cost of production. It can also result in the eradication or control of aquatic weeds through their utilization (Nagendra Prabhu 2016).

17.3.2 Fermentation Methods for Cellulase Production

Submerged Fermentation (SmF) Submerged fermentation is a method of culturing microorganisms submerged in a liquid/nutrient broth. Submerged fermentation is a low-cost method used commercially for the production of enzymes. It is preferred because of the easiness in handling and controlling the parameters like pH and temperature (Sirohi et al. 2018). It is also advantageous because of the easy purification unlike SSF. But this fermentation technique is energy-consuming and costly (Sadhu and Maiti 2013). This method of fermentation can be applied only for microorganisms such as bacteria requiring high moisture (Sadhu and Maiti 2013).

Solid-State Fermentation (SSF) Solid-state fermentation is the technique of culturing microorganisms on the surface of solid substrates in the absence of free water (Cannel and Moo-Young 1980). Because it consumes less water, lesser effluents are produced. Compared to submerged fermentation, SSF results in more enzyme yields (Mrudula and Murugammal 2011; Imran et al. 2016) and hence it is the most researched technique of enzyme production. SSF is widely used for several purposes such as production of commercial enzymes, acids, alcohols, alkaloids, antibiotics protein enriched feed, fuel, mushroom cultivation, and in solid waste decomposition (Lonsane 1994). It is a simple technique which can be performed by using bacteria, fungi, or any other microorganism that can adsorb onto the solid substrate and derive nutrients and energy necessary for its growth. It is mostly applied for enzyme production using fungi, as they prefer solid substrates over liquid media.
The results of a study at the author's laboratory clearly demonstrate the bacterial adsorption (Fig. 17.4) onto the substrate and degradation of the leaf surface due to cellulase production (Fig. 17.5) during the SSF process (Snishamol 2012). SSF can also be carried out on a wide range of solid substrates for a variety of purposes (Aidoo et al. 1982; Suresh and Nagendra Prabhu 2012). It is generally a low-energy-consuming process that requires only low-cost and low-volume equipment (Sadhu and Maiti 2013).

The applications of cellulases with special reference to food, feed, and beverage industries are presented in the following sections.

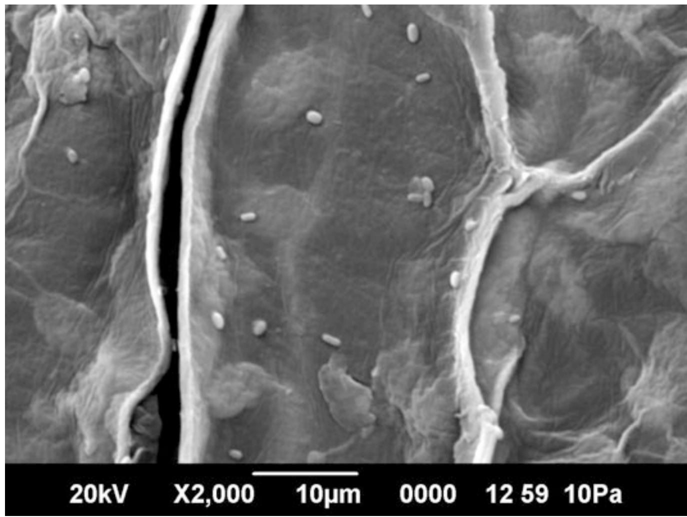


Fig. 17.4 SEM image showing bacteria attached on the lea on *Salvinia molesta* under SSF (Snishamol 2012)

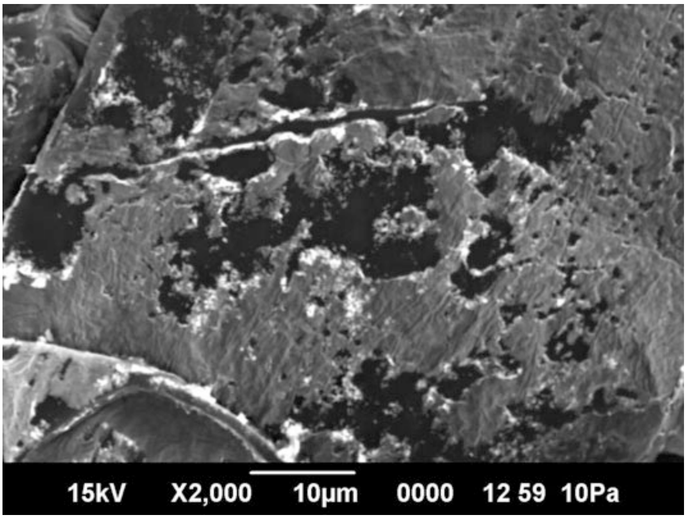


Fig. 17.5 SEM image of the leaf of *Salvinia molesta* after degradation by cellulase (Snishamol 2012)

17.4 Applications

The major applications of cellulase enzyme include textile, paper and pulp, food, animal feed, fuel, and chemical industries. Additionally, it is also used in waste management, pharmaceutical industry, protoplast fusion, and genetic engineering (Bhat 2001; Howard et al. 2003).

17.4.1 Food Industry

Food production has become a major concern for humanity today in the context of population increase, urbanization, and climate change. The increasing food produced due to technological advances in agricultural practices and biotechnology requires robust food and beverage industries for their processing, value addition, and preservation. These industries have earned the status of "major industries" and contribute significantly toward catering to the needs of people across the globe. The food industry mainly processes the food to retain or improve its nutritional qualities, eliminating human health concerns and extending the product shelf life. Other efforts include making of food with better flavor, color, texture, and to make them convenient to pack and consume (Chandrasekharan 2012).

Enzymes are playing a key role to achieve in many of the above targets in the food industry. Application of cellulase in food industry increases the nutritional properties of products. In food industries, cellulase is used in combination with pectinase and hemicellulase (Kumar 2015; Bhat 2000). The combination of these three enzymes constitutes the macerating enzymes. Macerating enzymes also have an important role in the extraction of olive oils. Their application results in increased extraction, better quality, more antioxidants, lower chance of rancidity, and less wastage (Galante et al. 1998). Fruits in their natural form tend to rot fast, and hence converting them to juice or puree can increase the shelf life. Addition of macerating enzymes during these processes yields better product and reduce browning (Sims and Bates 1994).

Cellulose, if present in its pure form, can affect baking process. Addition of cellulase reduces the size cellulose polymers to smaller glucose polymers (Tony 1996). The cellulases from *A. niger*, *T. reesi,* and *H. insolens* have been purified and used in the baking industry to break up roughage in dough (Chandrasekharan 2012).

17.4.2 Feed Industry

Animal feeds play a leading role in the global food industry, enabling economic production of animal proteins throughout the world. Feed is the largest and most

important component to ensuring safe, abundant, and affordable animal proteins. The global commercial feed manufacturing produces about 1 billion tons of feed annually and generates an estimated annual turnover of over US$400 billion. Commercial production or sale of manufactured feed products takes place in more than 130 countries and directly employs more than a quarter of a million skilled workers, technicians, managers, and professionals (http://www.ifif.org/pages/t/The+global+feed+industry; International Feed Industry Federation—Annual report 2016/17).

Economic importance of cellulase is based on its ability to convert plant materials into a consumable form. Cellulose acts as a primary food for many organisms. Cellulose-digesting ability of many mammals is very limited, whereas ruminants and hindgut fermenters are with high cellulose-digesting ability because of symbiotic bacteria. The currently available commercial feeds cannot satisfy the nutrient requirements of animals, whereas more than 50% of expenses in animal farms are spent on feed. So as to reduce the huge expenses involved, farmers include forages and agricultural by-products in feed. The authors are currently developing feed for fish and duck from aquatic weeds such as *E. crassipes*.

The lignocellulosic materials in general contain low protein, high fiber, some anti-nutritional factors, and low digestibility coefficients (Moubasher et al. 2016), which reduce meat and/or milk production. To increase digestibility, the linkage between cellulose, hemicellulose, and lignin must be broken down (Murad and Azzaz 2010). So the farmers supplement enzymes to improve the feed conversion (Liu and Kokare 2017). Enzymes are used in feedstuffs since early twentieth century. It is found that the addition of enzymes increased digestibility of feeds in cows (Yang et al. 2000). Fibrolytic enzymes also improve the ruminal enzymatic activity (Giraldo et al. 2008). Animals provided with feed containing cellulase enzyme were found to consume more food and this resulted in 5–25% increment in milk production (Stella et al. 2007). However, in most of the in vivo studies, the positive results of the in vitro trials were not reproduced. In most cases, there was no positive effect on the digestibility of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), or acid detergent fiber (ADF). It is because the effect of enzymes depends on application rate and mode, stability and source of enzyme, and type of feed (Li et al. 2015; Harrison and Tricario 2007). Table 17.2 presents the effect of cellulase supplemented feed on different animals.

17.4.3 Beverage Industry

The beverage industry is a $110 billion worth involving diverse segments representing alcoholic and non-alcoholic drinks. Examples include milk, coffee, tea, carbonated drinks, bottled water, and fruit juices. The beverage industry is the largest food processing industry, where fermentation is used to produce both plant-based and animal-based beverages (Chandrasekharan 2012).

Table 17.2 Effect of supplementation of cellulase in animal feed

Animal	Source of enzyme	Feed (%)	Effects	Reference
Cow	Promote®, Cargill, Minnetonka	Bermuda grass silage, corn silage, corn, citrus pulp, whole cottonseed, Soy Plus, soybean meal, mineral mix, Biophos (calcium phosphate)	No effect on intake of DM, NDF, or CP, did not improve milk production, decreased ruminal pH	Dean et al. (2013)
Lactating Cow	FinnFeeds Int., Marlborough, UK	Corn silage, alfalfa hay, cottonseed, corn, soybean meal, dried corn distillers grains, molasses, limestone, dicalcium phosphate, fat, sodium bicarbonate, trace-mineralized salt, MgO, Zinpro, Dairymicro premix, vitamin E Premix	Increased milk production when blend of cellulose and xylanase was used, more response during early lactation	Schingoethe et al. (1999)
Lamb	*Fomes* sp. EUM1	Corn stover, alfalfa hay	Improved digestibility of DM, NDF, and ADF with intermediate dose of enzyme, increased avg. daily weight gain up to 15%	Arce-Cervantes et al. (2013)
Pig	*Bacillus pumilus*, *B. licheniformis*, *B. subtilis*	Sorghum, wheat, soybean	Slight improvement in gain, no effect in dressing percentage, no effect on digestibility	Kim et al. (1993)
Rabbit	*Trichoderma reesei* Rut C-30	Purina, Debrecen, Hungary	Improved feed conversion ratio for 2 weeks, nonsignificantly higher weight gain	Szijártó et al. (2004)

About 90% of ethanol produced is from natural sugars and starch. In nature, sugars exist mostly as cellulose and hemicellulose. So, cellulosic biomaterials have great potential for alcohol production. The conversion of cellulosic biomass to fermentable sugars and alcohols through cellulase enzyme produced by various cellulolytic organisms has attracted worldwide attention (Ladish et al. 1983). Cellulase can be used for starch liquefaction even from lower quality grains. This in turn results in good-quality alcohol from low-quality sources (Lewis 1996). There are studies showing that application of cellulases results in good-quality alcohol with better properties. In winemaking, addition of cellulase, pectinase, and xylanase resulted in increased extraction and filtration rate. It consumed lesser energy for fermenter and also resulted in better wine stability with decreased viscosity (Galante et al. 1998; Bamforth 2009; Oksanen et al. 1985). Xue et al. (2018) reported the identification of an ethanol-tolerant endoglucanase from *A. niger* isolated from wine fermentation cellar. It was also stable in acidic pH and had good thermostability. This enzyme has great potential for simultaneous saccharification and fermentation.

There is a greater demand for fruit juices because of the rising health consciousness among people. Traditional juice extraction processes do not produce good quantity and/or quality of fruit juices due to several reasons. One among them is the presence of cellulosic polysaccharides in fruits, which constitute 30% of dry matter in cell wall. They are abundant in both primary and secondary cell walls and provides shape, strength, and rigidity to cells. But this forms a big barrier to juice manufacturers as it reduces yield. Application of cellulase can degrade cellulose fibrils which in turn causes reduction in wall strength. It also solubilizes the cell wall polysaccharides resulting in almost complete (95%) liquefaction (Grassin and Fauquembergue 1996).

Fibers in juices are another great problem faced by the industries. They get clogged in the manufacturing line, affecting productivity and causing huge loses to the manufacturers. Most of the fibers in fruit juice are cellulosic in nature and therefore, filtering of juices becomes easier on application of cellulases. Macerating enzymes are used for complete liquefaction of fruit pulp after crushing the fruit and also for better clarification and reduced viscosity of the final product (Galante et al. 1998; Grassin and Fauquembergue 1996). Enzymatic extraction of juices also results in higher yield and minimum wastage. It is employed in the production of juices, nectars, and purees from mango, banana, papaya, apricot, plum, pear, apple, etc. (Grassin and Fauquembergue 1996). When used in combination with other enzymes, cellulase is found to increase taste and aroma and to decrease bitterness in citrus fruits (Sajith et al. 2016). The results of a study conducted by Neagu et al. (2014) show that there is enhancement in the yield of carotenoids from tomatoes on using crude cellulase for extraction. The obtained carotenoids have the potential to be used as coloring agents in the food and beverage industries.

Instant coffee powder is made by solubilizing the polysaccharides that are present in roasted coffee. Industries employ an energy-consuming technique called thermal hydrolysis for this. As reported by Baraldi et al. (2016), cellulase can be used for enzymatic hydrolysis which is cheaper and uses lesser energy compared to

thermal hydrolysis. This also reduced the quantity of undesirable volatile compounds in instant coffee. In the tea industry, fresh leaves are usually preferred for green tea production. But polyphenols, the main antioxidants, are present in old tea leaves which are considered as subproducts. Han et al. (2016) found that addition of cellulase can be used for the extraction of tea polyphenols from old tea leaves. In the study, total polyphenol content (TP) was maximum at 74.45 mg GAE/g and 25.39% of polyphenol purity at enzyme concentration of 2% v/w. Results obtained were higher than that of the samples treated with enzyme at lower concentrations.

17.4.4 Hydrolysis of Cellulosic Biomass

Conversion of cellulosic biomass to industrially useful products or fuels is of vital importance in the present scenario. Most of the cellulosic biomass is either agricultural by-products or waste materials from industries, municipal wastes, etc. (Álvarez et al. 2016). Eradication of these wastes is a major concern to respective industries. With the help of cellulose-digesting enzymes, they could be converted into sugars, bioethanol, bio-methane, bio-hydrogen, etc. Enzymatic hydrolysis of cellulose is affected by different factors such as structural features of the solid substrate and enzyme-related factors like the source of enzyme, thermal sensitivity, specificity, enzyme processibility, enzyme compatibility, and product inhibition (Yang et al. 2011).

The global demand for fuels is increasing and crude oil price is exhibiting an increasing trend. Fossil fuel availability is decreasing at a rapid rate. In this scenario, production of bioethanol from lignocellulosic biomass would be an alternative, sustainable, and eco-friendly source of energy. Cellulase is employed for the bioconversion of lignocellulosic biomass to bioethanol (Demain et al. 2005; Singhania et al. 2015). Lignin is a physical barrier for the enzymes to act on celluloses in plants. Therefore, the plant materials are pretreated to remove lignin, and the hemicellulose and cellulose biomass are broken down into smaller sized sugars by the action of cellulases. These sugars are later converted into ethanol through fermentation (Murugan et al. 2013). Vaishnav et al. (2018) found that cellulase obtained from *Penicillium* is suitable for biomass conversion to bioethanol. When compared with *Trichoderma*, it has more genes encoding β-glucosidases and hemicellulases.

Solid waste management is another area that is currently being explored. Cellulase enzyme was utilized to convert solid wastes from agricultural industries into useful materials (Jadhav et al. 2013; Manfredi et al. 2018). Bio-methane and bio-hydrogen, which are renewable energy sources, can also be produced from lignocellulosic materials, especially wastes (Levin et al. 2007; Antoni et al. 2007; Saratale et al. 2014).

17.4.5 Other Applications

In addition to the abovementioned applications, cellulase is used in several other industries. Detergent cellulases were introduced in the market since late 1980s. Cellulase is used in textile industries for washing clothes to give them more finish and softness. This finishing process is called bio-polishing. Fabric treated with immobilized cellulase has lower weight loss and high tensile strength (Sankarraj and Nallathambi 2018). Cellulase is also used for stonewashing and antipilling treatment. Cotton textiles lose their color and become dull on a long-term use. Treating with cellulose removes detached fibers, thereby regaining the color. Cellulase treatment also helps in removal of soil particles which are trapped in between the cellulosic fibers (Eriksen 1996).

Another major application is in the paper and pulp industry. Cellulase in combination with hemicellulase obtained from *Trichoderma* was used for biomechanical pulping. Treatment with enzymes refines the wood chips and makes them suitable for mechanical pulping, which in turn reduces energy consumption (Pere et al. 1996). Cellulase can be used for enzymatic or bio-de-inking (Buchert et al. 1998) as well as to remove fine fibers of cellulose in drain water, thereby allowing better drainage after pulping (Bommarius and Riebel 2005; Kantelinen et al. 1995). In many cases, appropriate mediators are also used along with the enzymes (Li et al. 2001).

Gupta et al. (2015) reported that cellulase produced from a strain of *Aspergillus* spp. could be used to treat pulp for obtaining paper with better tear index, tensile index, burst index, and double fold. Paper with the above properties is generally preferred for currency or security paper. Cellulase obtained from fungi can degrade cell walls of plants (Bhat 2000), and this property is exploited in agriculture sector to control weeds and plant pathogens. Awasthi et al. (2018) found that *Bacillus thuringiensis* and *Bacillus licheniformis* could be used to degrade food wastes along with amylolytic bacterial strains. Table 17.3 presents the various applications of cellulase in different industries at a glance.

Table 17.3 Applications of cellulase in different industries

Industry	Applications
Food	Higher yield in juice and oil production, fruit juice clarification, reduction of juice viscosity, increased antioxidants in oil, increased shelf life, baking
Feed	Reduced expenses on feed, increase in digestibility coefficients, milk production, and weight gain
Beverage	Increased rate of extraction and filtration, decrease in energy requirement, instant coffee powder production, extraction of tea polyphenols
Textile	Bio-polishing, stonewashing,
Paper and pulp	Bio-de-inking, biomechanical pulping, better drainage, increased tear, tensile, and burst indices
Agriculture	Weed control, value addition of waste materials
Energy	Production of alternate energy sources like bioethanol, bio-hydrogen, and bio-methane

17.5 Conclusions

From the studies conducted so far, it is clear that enzyme-based industries are gaining importance over the conventional chemical industries. The food, feed, and beverage industries constitute the major industries which are of great relevance to humans, both in terms of quantity and quality. Enzymes play a crucial role in bringing down the problems faced by humanity as they are used in waste management, agriculture, food, feed, pharmaceutical, and several other industrial applications. Cellulase constitutes the second largest enzyme having a wide range of applications in the three industries as well as other related ones. New avenues are being discovered and exciting opportunities are in the offering. Molecular biology and biochemistry are expanding day by day with the help of sophisticated techniques and instruments. This in turn results in better techniques for production, purification, and characterization of cellulase and other enzymes that can reduce the cost of production and improve the quality, quantity, and efficiency.

There is an urgent need to discover cheaper substrates and novel microorganisms from diverse environments producing the enzyme with better properties for their use in the industries. Industrial production of cellulase mostly uses fungi. But nowadays, studies are also being carried out to utilize novel bacterial strains and substrates for the production of cellulase. Biotechnology and genetic engineering techniques can be applied to select or to design newer strains with desired qualities like thermal stability, resistance to pH variation, wider substrate choice, etc. Improvements in the above areas of research will lead toward a sustainable use of resources, making the maximum use through low-energy, cost-effective, and environment-friendly green bioprocesses.

Acknowledgements The corresponding author (G. Nagendra Prabhu) is grateful to Department of Science and Technology, Government of India, University Grants Commission, Government of India, Kerala State Council for Science, Technology & Environment and Kerala Biotechnology Commission for providing financial support to conduct the projects for cellulase production using aquatic weeds. V. Anoop Kumar thanks the University Grants Commission for providing Junior Research Fellowship.

References

Aidoo KE, Hendry R, Wood BJB (1982) Solid state fermentations. Adv Appl Microbiol 28:201–237

Álvarez C, Sosa FMR, Díez B (2016) Enzymatic hydrolysis of biomass from wood. Microb Biotechnol 9(2):149–156

Amouri B, Gargouri A (2006) Characterization of a novel β-glucosidase from a *Stachybotrys* strain. Biochem Eng J 32:191–197

Andriani Y, Rochima E, Ratu S, Rahayuningsih SR (2017) Characterization of *Bacillus megaterium* and *Bacillus mycoides* bacteria as probiotic bacteria in fish and shrimp feed. KnE Life Sci 2(6):127–135

Antoni D, Zverlov VV, Schwarz WH (2007) Biofuels from microbes. Appl Microbiol Biotechnol 77:23–25

Arce-Cervantes O, Mendoza GD, Hernandez PA, Meneses M, Torres-Salado N, Loera O (2013) The effects of a lignocellulolytic extract of *Fomes* sp. EUM1 on the intake, digestibility, feed efficiency and growth of lambs. Anim Nutr Feed Technol 13:360–372

Awasthi MK, Wong JWC, Kumar S, Awasthi SK, Wang Q (2018) Biodegradation of food waste using microbial cultures producing thermostable α-amylase and cellulase under different pH and temperature. Bioresour Technol 248:160–170

Bahkali AH (1996) Influence of various carbohydrates on xylanase production in *Verticulum tricorpus*. Bioresour Technol 57(3):265–268

Bamforth CW (2009) Current perspectives on the role of enzymes in brewing. J Cereal Sci 50 (3):353–357

Baraldi IJ, Giordano RLC, Zangirolami TC (2016) Enzymatic hydrolysis as an environmentally friendly process compared to thermal hydrolysis for instant coffee production. Br J Chem Eng 33(4):763–771

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18(5):355–383

Bhat MK (2001) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–387

Birsan C, Johnson P, Joshi M, MacLeod A, McIntosh L, Monem V, Nitz M, Rose DR, Tull D, Wakarchuck WW, Wang Q, Warren RAJ, White A, Withers SG (1998) Mechanisms of cellulases and xylanases. Biochem Soc Trans 26(2):156–160

Bommarius AS, Riebel BR (2005) Applications of enzymes as bulk actives: detergents, textiles, pulp and paper, animal feed. In: Biocatalysis. Wiley, pp 135–158

Bond K, Stutzenberger F (1989) A note on the localization of cellulosome formation in *Thermomonospora curvata*. J Appl Bacteriol 67:605–609

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: *Trichoderma* & *Gliocladium*—enzymes, biological control and commercial applications, vol 2. Taylor & Francis, London, UK, pp 343–363

Cannel E, Moo-Young M (1980) Solid state fermentation systems I. Process Biochem 15(5):2–7

Chandrasekharan M (2012) Valorization of food processing by-products. CRC-Taylor Press, New York

Dean DB, Staples CR, Littell RC, Kim S, Adesogan AT (2013) Effect of method of adding a fibrolytic enzyme to dairy cow diets on feed intake digestibility, milk production, ruminal fermentation, and blood metabolites. Anim Nutr Feed Technol 13:337–353

Demain AL, Newcomb M, Wu JHD (2005) Cellulase, *Clostridia* and ethanol. Microbiol Mol Biol Rev 69:124–154

Din N, Damude HG, Gilkes NR, Miller RC, Warren RAJ, Kilburn DG (1994) C_1-C_x revisited: intra-molecular synergism in a cellulase. Proc Natl Acad Sci (USA) 91:11383–11387

Eriksen N (1996) Detergents. In: Industrial enzymology, 2nd edn. Stockton Press, New York, pp 189–200

Esterbauer H, Driguez H, Viet C, Schülein M (1991) Production of *Trichoderma* cellulase in laboratory and pilot scale. Bioresour Technol 36(1):51–65

Galante YM, De Conti A, Monterverdi R (1998) Application of *Trichoderma* enzymes in food and feed industries. In: Trichoderma & gliocladium—enzymes, biological control and commercial. Taylor & Francis, London, UK, pp 327–342

Gaur R, Tiwari S (2015) Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC Biotechnol 15(19)

Giraldo LA, Tejido M, Ranilla MJ, Ramos S, Carro MD (2008) Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. J Anim Sci 86:1617–1623

Grassin C, Fauquembergue P (1996) Fruit juices. In: Industrial enzymology, 2nd edn. Stockton Press, New York, pp 227–264

Gupta C, Jain P, Kumar D, Dixit AK, Jain RK (2015) Production of cellulase enzyme from isolated fungus and its application as efficient refining aid for production of security paper. Int J Appl Microbiol Biotechnol Res 3:11–19

Han TC, Nam ND, Hing Anh LT, Vu TA, Man PV (2016) Enzyme assisted extraction of polyphenols from the old tea leaves. J Nutr Health Sci 3(4)

Harrison GA, Tricario JM (2007) Effects of an *Aspergillus oryzae* extract containing α-amylase activity on lactational performance in commercial dairy herds. Prof Anim Sci 23(3):291–294

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

Henrissat H, Driguez H, Viet C, Schülein M (1985) Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. Bio/Technology 36:722–726

Herrero AA, Gomez RF, Roberts MF (1985) 31P NMR studies of *Clostridium thermocellum*. Mechanism of end product inhibition by ethanol. J Biol Chem 25(260):7442–7451

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

Immanuel G, Bhagavath C, Iyappa Raj P, Esakkiraj P, Palavesam A (2006) Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigates* fermented in coir waste and sawdust. Internet J Microbiol 3(1):1–11

Imran M, Anwar Z, Irshad M, Asad MJ, Ashfaq H (2016) Cellulase production from species of fungi and bacteria from agricultural wastes and its utilization in industry: a review. Adv Enzyme Res 4:44–55

International Feed Industry Federation. http://www.ifif.org/pages/t/The+global+feed+industry. Last accessed 2018/07/08

International Feed Industry Federation—Annual report 2016/17. http://annualreport.ifif.org. Last accessed 2018/07/08

Jadhav AR, Girdhe AV, More SM, More SB, Khan S (2013) Cellulase production by utilizing agricultural wastes. Res J Agric For Sci 1(7):6–9

Jain P, Jain RK (2016) Enhanced cellulase production from isolated fungus *Aspergillus niger* RKJP and its application in lignocellulosic saccharification for bioethanol production. Biotechnol Res 2(2):61–68

Jørgensen H, Eriksson T, Börjesson J, Tjerneld F, Olsson L (2003) Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enzyme Microb Technol 32:851–861

Juturu V, Wu JC (2014) Microbial Cellulases: engineering, production and applications. Renew Sustain Energy Rev 33:188–203

Kantelinen A, Jokinen O, Sarkki ML, Pettersson C, Sundberg K, Eckerman C, Ekman R, Holmbom B (1995) Effects of enzymes on the stability of colloidal pitch. In: Proceedings of 8th international symposium on wood and pulping chemistry, vol 2, Helsinki, Finland

Kato S, Haruta S, Cui ZJ, Ishii M, Igarashi Y (2008) Network relationships of bacteria in a stable mixed culture. Microb Ecol 56(3):403–411

Kato S, Yoshida R, Yamaguchi T, Sato T, Yumoto I, Kamagata Y (2014) The effects of elevated CO_2 concentration on competitive interaction between aceticlastic and syntrophic methanogenesis in a model microbial consortium. Front Microbiol 5:575

Kim IH, Hancock JD, Hines RH, Risley CR (1993) Effects of cellulase enzymes and bacterial feed additives on the nutritional value of sorghum grain for finishing pigs. Asian-Australas J Anim Sci 11(5):538–544

Kuhad RC, Gupta R, Singh A (2011) Microbial cellulases and their industrial applications. Enzyme Res 1–10

Kumar S (2015) Role of enzymes in fruit juice processing and its quality enhancement. Adv Appl Sci Res 6(6):114–124

Ladish MR, Lun KW, Volch M, Taso GT (1983) Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb Technol 5:82–90

Levin DB, Zhu H, Beland M, Cicek N, Holbein BE (2007) Potential for hydrogen and methane production from biomass residues in Canada. Bioresour Technol 98:654–660

Lewis SM (1996) Fermentation alcohol. In: Industrial enzymology, 2nd edn. Stockton Press, New York, pp 11–48
Li K, Nagendra Prabhu G, Cooper DA, Xu F, Elder T, Eriksson K-EL (2001) Development of new Laccase-mediators for pulp bleaching. Oxidative Delignification Chem 785:400–412
Li Q, Jiao J, Tang S, He Z, Zhou C, Han X, Wang M, Kang J, Odongo NE, Tan Z (2015) Effects of dietary cellulase and xylanase addition on digestion, rumen fermentation and methane emission in growing goats. Arch Anim Nutr
Liu X, Kokare C (2017) Microbial enzymes of use in industry. In: Biotechnology of microbial enzymes. Elsevier, pp 267–298
Lonsane BK (1994) Resurgence of interest in solid state fermentation: reasons and justifications. In: Solid state fermentation. Wiley Eastern Ltd., New Delhi, pp 11–20
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
Malik S, Sharma S, Sharma J, Mandhan RP (2015) Bioprocessing of crop residues using fibrolytic enzymes and *Flavobacterium bolustinum* for enriching animal feed. Int J Biotechnol Wellness Ind 4:12–17
Mandeks M, Reese ET (1957) Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J Bacteriol 73:269–278
Manfredi AP, Ballesteros I, Sáez F, Perotti NI, Martínez MA, Negro MJ (2018) Integral process assessment of sugarcane agricultural crop residues conversion to ethanol. Bioresour Technol 260:241–247
Margo LD, Silveira VCC, de Menezes EW, Benvenutti EV, Nicolodi S, Hertz PF, Klein MP, Rodrigues RC (2018) Magnetic biocatalysts of pectinase and cellulase: synthesis and characterization of two preparations for application in grape juice clarification. Int J Biol Macromol 115:35–44
Moubasher A-AH, Ismail MA, Hussein NA, Gouda HA (2016) Enzyme producing capabilities of some extremophilic fungal strains isolated from different habitats of Wadi El-Natrun, Egypt. Part 2: cellulase, xylanase and pectinase. Eur J Biol Res 6(2):103–111
Mrudula S, Murugammal R (2011) Production of cellulose by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. Br J Microbiol 42(3):1119–1127
Mukhopadhyay S, Chatterjee NC (2010) Bioconversion of water hyacinth hydrolysate into ethanol. Bioresources 5(2):1301–1310
Murad HA, Azzaz HH (2010) Cellulase and dairy animal feeding. Biotechnology 9(3):238–256
Murugan A, Singh M, Ranjit AJA (2013) Sugarcane. In: Valorization of food processing by-products. CRC-Taylor & Francis, New York, pp 415–454
Nagendra Prabhu G (2016) Economic impact of aquatic weeds—a third world approach. J Aquat Biol Fish 4:8–14
Nagendra Prabhu G, Chandrasekharan M (1996) L-Glutaminase production by marine *Vibrio costicola* under solid state fermentation using different substrates. J Mar Biotechnol 4:176–179
Nagendra Prabhu G, Chandrasekharan M (1997) Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* in solid state fermentation using polystyrene as an inert support. Process Biochem 32(4):285–289
Neagu D, Leopold LF, Thonart P, Destain J, Socaciu C (2014) Enzyme assisted extraction of carotenoids and phenolic derivatives from tomato. Bull UASVM Anim Sci Biotechnol 71 (1):20–26
Nidetzky B, Steiner W, Hayn M, Claeyssens M (1994) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interactions. Biochem J 298:705–710
Oksanen J, Ahvenainen J, Home S (1985) Microbial cellulase for improving filterability of wort and beer. In: Proceedings of the 20th European brewery chemistry congress, Helsinki, Finland
Ong LG, Abd-Aziz S, Noraini S, Karim MI, Hassan MA (2004) Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. Appl Biochem Biotechnol 118:73–79

Pere J, Paavilainen L, Siika-Aho M, Cheng Z, Viikari L (1996) Potential use of enzymes in drainage control of non-wood pulps. In: Proceedings of 3rd international non-wood fibre pulping and paper making conference, vol 2, Beijing

Rapp P, Beerman A (1991) Bacterial cellulases. In: Biosynthesis and biodegradation of cellulose. Marcel Dekker, Inc., New York, pp 535–595

Reese ET, Sui RGH, Levinson HS (1950) The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J Bacteriol 59:485–497

Sadhu S, Maiti TK (2013) Cellulase production by bacteria: a review. Br Microbiol Res J 3 (3):235–258

Sajith S, Priji P, Sreedevi S, Benjamin S (2016) An overview on fungal cellulases with an industrial perspective. J Nutr Food Sci 6:461

Sankarraj N, Nallathambi G (2018) Enzymatic biopolishing of cotton fabric with free/immobilized cellulase. Carbohydr Polym 191:95–102

Saranraj P, Naidu MA (2014) Microbial pectinases: a review. Global J Tradit Med Syst 3:1–9

Saratale GD, Kshirsagar SD, Sampange VT, Saratale RG, Oh SE, Govindwar SP, Oh MK (2014) Cellulolytic enzymes production by utilizing agricultural wastes under solid state fermentation and its application for bio-hydrogen production. Appl Biochem Biotechnol 174(8):2801–2817

Schingoethe DJ, Stegeman GA, Treacher RJ (1999) Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. J Dairy Sci 82:996–1003

Shida Y, Furukawa T, Ogasawara W (2016) Deciphering the molecular mechanisms behind cellulase production in *Trichoderma reesei*, the hyper-cellulolytic filamentous fungus. Biosci Biotechnol Biochem

Shin CS, Lee JP, Lee JS, Park SC (2000) Enzyme production of *Trichoderma reesei* rut C-30 on various lignocellulosic substrates. Appl Biochem Biotechnol 84(1–9):237–245

Sims CA, Bates RP (1994) Challenges to processing tropical fruit juices: banana as an example. Proc Florida State Hortic Soc 107:315–319

Singh N, Devi A, Jaryal R, Rani K (2018) An ecofriendly and efficient strategy for cost effective production of lignocellulotic enzymes. Waste Biomass Valorization 9(6):891–898

Singhania RR, Saini R, Adsul M, Saini JK, Mathur A, Tuli D (2015) An integrative process for bio-ethanol production employing SSF produced cellulase without extraction. Biochem Eng J 102:45–48

Sirohi R, Singh A, Tarafdar A, Shahi NV, Verma AV, Kushwaha A (2018) Cellulase production from pre-treated pea hulls using *Trichoderma reesei* under submerged fermentation. Waste Biomass Valorization 1–9

Snishamol C (2012) Utilization of *Salvinia molesta* for cellulase production under solid state fermentation. PhD thesis, University of Kerala, Thiruvananthapuram, Kerala

Snishamol C, Suresh Chandra Kurup R, Nagendra Prabhu G (2001) Isolation and preliminary characterisation of cellulolytic bacteria associated with common aquatic weeds of Kerala. In: Annual conference on microbial biotechnology, Association of Microbiologists of India, Gulbarga, Karnataka, India

Stella AV, Paratte R, Valnegri L, Cigalino G, Soncini G, Chevaux E, Dell'Orto V, Savoini G (2007) Effect of administration of live *Saccharomyces cerevisiae* on milk production, milk composition, blood metabolites, and faecal flora in early lactating dairy goats. Small Ruminant Res 67(1):7–13

Sukumaran RK, Singhania RR, Pandey A (2005) Microbial cellulases- production, applications and challenges. J Sci Ind Res 64:832–844

Suresh Chandra Kurup R, Nagendra Prabhu G (2001) Utilization of water hyacinth for cellulase production using native microflora under SSF. In: International conference on new horizons in biotechnology, Trivandrum, Kerala, India

Suresh Chandra Kurup R, Snishamol C, Nagendra Prabhu G (2005) Cellulase production by native bacteria using water hyacinth as substrate under solid state fermentation. Malays J Microbiol 1 (2):25–29

Suresh PV, Nagendra Prabhu G (2012) Valorization of seafood processing by-products by biological methods. In: Valoriztion of food processing by-products. CRC-Taylor Press, New York, pp 685–736
Suto M, Tomita F (2001) Induction and catabolite repression mechanisms of cellulase in fungi. J Biosci Bioeng 92(4):305–311
Szijártó N, Faigl Z, Réczey K, Mézes M, Bersényi A (2004) Cellulase fermentation on a novel substrate (waste cardboard) and subsequent utilization of home-produced cellulase and commercial amylase in a rabbit feeding trial. Ind Crops Prod 20:49–57
Tabssum F, Irfan M, Shakir HA, Qazi JI (2018) RSM based optimization of nutritional conditions for cellulase mediated saccharification by *Bacillus cereus*. J Biol Eng 12(7)
Teeri TT (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol 15:160–167
Tolan SJ, Foody B (1999) Cellulase from submerged fermentation. In: Tsao GT (eds) Recent progress in bioconversion of lignocellulosics. Springer, Berlin, pp 43–49
Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microb Physiol 37:1–81
Tony G (1996) Baking. In: Industrial enzymology, 2nd edn. Stockton Press, New York, pp 87–102
Vaishnav N, Singh N, Adsul M, Dixit P (2018) Penicillium: the next emerging champion for cellulase. Bioresour Technol Rep 2:131–140
Wachinger G, Bronnenmeier K, Staudenbauer WL, Schrempf H (1989) Identification of mycelium-associated cellulase from *Streptomyces reticuli*. Appl Environ Microbiol 55:2633–2657
Withers SG (2001) Mechanisms of glycosyl transferases and hydrolases. Carbohydr Polym 44 (4):325–337
Xu X, Li J, Shi P, Ji W, Liu B, Zhang Y, Yao B, Fan Y, Zhang W (2016) The use of T-DNA insertional mutagenesis to improve cellulase production by the thermophilic fungus *Humicola insolens* Y1. Sci Rep 6:31108
Xue D, Zeng X, Lin D, Yao S (2018) Ethanol tolerant endoglucanase from *Aspergillus niger* isolated from wine fermentation cellar. Biocatal Agric Biotechnol 15:19–24
Yang WZ, Beauchemin KA, Rode LM (2000) A comparison of methods of adding fibrolytic enzymes to lactating cow diets. J Dairy Sci 83:2512–2520
Yang B, Dai Z, Ding S-Y, Wyman CE (2011) Enzymatic hydrolysis of cellulosic biomass. Biofuels 2(4):421–450
Yang F, Gong Y, Liu G, Zhao S, Wang J (2015) Enhancing cellulase production in thermophilic fungus *Myceliophthora thermophila* ATCC42464 by RNA interference of *cre1* gene expression. J Microbiol Biotechnol 25(7):1101–1107
Zhang X-Z, Zhang Y-HP (2013) Cellulases: characteristics, sources, production, and applications. In: Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers, 1st edn. Wiley, pp 131–146

Chapter 18
Production of a Transfructosylating Enzymatic Activity Associated to Fructooligosaccharides

Abril Flores-Maltos, Solange I. Mussatto, Juan C. Contreras-Esquivel, Raul Rodríguez, Jose A. Teixeira and Cristobal N. Aguilar

Abstract Biotransformation of sucrose to fructooligosaccharides (FOS) was investigated using the catalytic action of fructosyltransferase (FFase) originated from solid-state fermentation of agro-industrial wastes (sugarcane bagasse, sotol bagasse, *Agave* fibers, and polyurethane) using four fungal strains (*Aspergillus niger* GH1, *A. niger* PSH, *Penicillium citrinum,* and *Penicillium purpurogenum*) which have demonstrated ability to produce great diversity of metabolites of industrial interest. Microorganisms and supports were selected based on transfructosylating activity and FOS production. *Agave* fibers were the best support material since permitted the highest amounts of FOS and FFase, with a FOS productivity of 10.88 g/L * h and yield of 2.70 g/g based on total substrate. Moreover, the A_t/A_h ratio of FFase was higher for cells cultivated on *Agave* fibers than those values obtained for the other wastes. Such results showed that *Agave* fibers can be successfully used as support of *A. niger* PSH strain for FOS and FTase production.

Keywords *Aspergillus* · Fructooligosaccharides · Fructosyltransferase *Penicillium* · Solid-state fermentation

---

A. Flores-Maltos · J. C. Contreras-Esquivel · R. Rodríguez · C. N. Aguilar (✉)
Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico
e-mail: cristobal.aguilar@uadec.edu.mx

S. I. Mussatto
Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800 Kongens Lyngby, Denmark

J. A. Teixeira
Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_18

18.1 Introduction

Fructooligosaccharides (FOS) are defined as short-chain oligomers, chemically composed of fructose units linked by glycosidic bridges β-(2 → 1) and containing a single glucose molecule terminal, usually represented by the formula GF_n, mainly composed of 1-kestose (GF_2), nystose (GF_3), and 1F-β-fructofuranosylnystose (GF_4) (Sangeetha et al. 2005; Santos and Maugeri 2007; Sánchez et al. 2010; Ganaie et al. 2013). FOS are a major class of bifidogenic oligosaccharides used by the food industry as prebiotic ingredients in functional food and to improve quality of them. FOS has functional characteristics as included: regulating intestinal microflora, multiplying probiotic bacteria in the colon, relieving constipation, low calorie, non-cariogenic, and aid during absorption of calcium and magnesium. In addition, reduce blood levels of phospholipids, triglycerides, and cholesterol, enhancing immunity and performing other useful applications (Sánchez et al. 2010; Zeng et al. 2016; Flores-Maltos et al. 2014).

Currently, the FOS are obtained from sucrose by enzymatic synthesis with action of microbial enzymes with high transfructosylating activity, as the β-D-fructosyltransferases (EC 2.4.1.9, FTase) or β-fructofuranosidases (EC 3.2.1.26, FFase). There are researcher groups reporting about the production of these enzymes by different microorganisms among them such as *Penicillium* sp. (Dhake and Patil 2007), *Fusarium* sp. (Patel et al. 1994), *Aspergillus* sp. (Lateef et al. 2012), and *Aureobasidium* sp. (Yoshikawa et al. 2008), but only a few these strains have the industrial potential required for this application.

Microbial production of FOS from sucrose is more feasible than plant sources and provides a cost-effective and convenient alternative at industrial level compared to chemical synthesis (La Rotta et al. 1998), as the FOS can be synthesized by enzymes produced during fermentation and the fungi growth (Prapulla et al. 2000; Yoshikawa et al. 2007). The fungal strains isolated from Coahuila semidesert and from the Department of Food Research collection (Autonomous University of Coahuila, UAdeC) have shown ability to produce great diversity of metabolites of industrial interest; some of them are tannase (Flores-Maltos et al. 2011; Rodriguez-Duran et al. 2011), invertase (Veana et al. 2011), inulinase (Flores-Gallegos et al. 2012), and ellagitannase (Buenrostro-Figueroa et al. 2013).

In this work, we attempt to extend the frontiers in synthesis of FOS from sucrose using FTase of *Aspergillus niger* GH1, *A. niger* PSH, *Penicillium purpurogenum,* and *P. citrinum* grown on agro-industrial residues as support and source of nutrients. In this context, this study was performed using a full factorial design to evaluate two variables: FOS production and enzyme activity.

18.2 Materials and Methods

18.2.1 Supports Preparation and Physical–Chemical Characterization

Four materials were tested as supports: polyurethane foam (PUF), sugarcane bagasse (SCB), Sotol bagasse (SB), and *Agave* fibers (AF). These materials were pretreated by boiling them for 10 min, washed three times with distilled water, and then dried in an oven at 60 °C for 24 h (Orzua et al. 2009) and pulverized. All of them with particles sizes of approximately 50 µm were used in the experiments. First, supports were physical–chemical characterized regarding water absorption index (WAI) and critical humidity point (CHP). WAI was determined using the methodology reported by Orzua et al. (2009). The sample (2.5 g) was suspended in 30 mL of distilled water in a tared 60 mL centrifuge tube. The suspension was stirred with a glass rod for about 1 min at room temperature (25–27 °C) and then centrifuged at 3000 rpm for 10 min at 25 °C. The supernatant was discarded, and the WAI was calculated from weight of the remaining gel and expressed as g gel/g dry weight. CHP was determined for the different supports using the methodology mentioned by Robledo et al. (2008) which consisted in adding 1 g of sample in a thermobalance at 120 °C for 60 min or until a constant weight. Prior to use, supports were autoclaved at 121 °C for 20 min.

18.2.2 Strain and Cell Culture

Four strains were used: *A. niger* PSH, *A. niger* GH1, *P. purpurogenum,* and *P. citrinum* all belonging to the microbial collection of Food Research Department of the UAdeC. Strains were cryopreserved with glycerol–skim milk at −50 °C. For spore production, fungal strains were grown on potato dextrose agar (PDA-Bioxon®) at 30 °C for 5–8 days. A concentrated suspension of spores was then prepared by harvesting spores from PDA flasks with a 0.1% (w/v) solution of Tween 80 sterilized by autoclaving at 121 °C for 20 min, spore number in the suspension was determined by counting in a Neubauer chamber, and the volume needed to give the desired initial spore concentration in the fermentation medium was calculated.

18.2.3 Fermentation Conditions and Sampling

Fermentation experiments were made in reactors of 138 mL capacity containing 1 g of the respective material, adjusted to 70% moisture with 2.33 mL of sucrose in a

concentration of 200 g/L previously sterilized, aseptically inoculated with a spore concentration 2×10^7 spores/g material and incubated at 30 ± 1 °C for 20 h.

Samples (as the total content of each reactor) were collected after 10, 13, 16, and 20 h of fermentation. For obtaining the crude extract, 2 mL of sterile distilled water was added to the fermented material, which was subsequently filtrated through 0.22 μm membranes. The obtained crude extracts were stored at −30 °C to avoid its self-hydrolysis and until for later analysis.

In the crude extracts, FOS (1-kestose, 1-nystose, and 1-β-fructosyl nystose) and the residual concentration of other sugars (sucrose, fructose, and glucose) were determined by high-performance liquid chromatography (HPLC) until the end of fermentation.

18.3 Analyses

18.3.1 Enzyme Activity

Samples of crude extract previously filtered were utilized as extracellular enzyme source. The enzyme activity was determined according to the modified method described by Yoshikawa et al. (2006). Reaction mixture contained 100 μL of crude extract, 500 μL of sucrose (600 μmoL and 400 μL of sodium acetate buffer pH 5.0 in a total volume of 1 mL. After incubation for 20 min at 30 °C, reaction was stopped by keeping the reaction mixture in boiling water for 5 min. One unit of FTase was defined as the amount of enzyme that catalyzes the formation of 1 μmoL of kestose (FOS) per minute under the above assay conditions.

18.3.2 Ratios of Transfructosylating Activity to Hydrolyzing Activity

The reaction was carried out for 20 min using 0.5 U/g of support of FTase in the reaction mixture described above. Transfructosylating (A_t) and hydrolyzing (A_h) activities were determined by measuring concentrations of 1-kestose and glucose by HPLC, respectively.

18.3.3 FOS and Sugars Concentrations

FOS (1-kestose, 1-nystose, and 1-_-fructofuranosylnystose) and other residual sugars (sucrose, glucose, and fructose) were directly analyzed by high-performance liquid chromatography (HPLC) using an equipment Varian Pro Star System with a

Prevail™ Carbohydrate ES column (5 µm) at room temperature and refractive index detector. The response generated by the refractive index detector was recorded and integrated using the Star Chromatography Workstation Software (Varian). The mobile phase used was a mixture of acetonitrile water (70:30 v/v) and 0.04% ammonium hydroxide at a flow injection rate of 1.0 mL/min. Generated results were corrected considering the dilution caused by the addition of water to obtain the crude extract. Sugars and FOS concentrations were determined from standard curves made with known concentrations of each compound. Total yield of FOS's (Y_{FOS} in g/g) was calculated as proportion of the sum of 1-kestose (Y_{GF2}), nystose (Y_{GF3}), and 1-β-fructofuranosylnystose (Y_{GF4}) to initial sucrose concentration per unit of support. FOS productivity (Q_P) was calculated as the total FOS production (g/L) by fermentation time (h).

18.3.4 Statistical Analysis

Effect of support, sucrose, and fungal strain on enzymatic activity (fructosyltransferase) and production of FOS were evaluated under a completely randomized design. All the fermentation experiments were conducted at least in triplicate and the average values were reported. Data were analyzed using ANOVA procedure using the SAS 9.0 statistical package for Windows. When needed, mean treatments were compared using Tukey's multiple range procedure ($p \leq 0.05$).

18.4 Results

18.4.1 Physical–Chemical Characterization

The water content into substrate has been widely described and reviewed by different authors (Gervais and Molin 2003; Bellon-Maurel et al. 2003; Pérez-Guerra et al. 2003), since it is a critical factor on solid-state fermentation processes because this variable has influence on microbial growth, biosynthesis, and secretion of different metabolites (Pérez-Guerra et al. 2003; Ellaiah et al. 2002). Therefore, water content in solid-state fermentation can range from 30 to 80% depending on the support material. WAI indicates ability of the sample to absorb water and hydrophilic groups available to bind water gel molecules (Mussatto et al. 2009). Materials with high values of WAI are favorable for development and microorganism growth during solid-state fermentation. The materials used in this investigation, polyurethane foam (PUF), sugarcane bagasse (SCB), Sotol bagasse (SB), and *Agave* fibers (AF), have WAI high values, but the highest value was observed in PUF (Table 18.1). Nevertheless, WAI is not the only property to be considered when choosing a support material for solid-state fermentation; CHP is an important

Table 18.1 Water absorption index (WAI) and critical humidity point (CHP) for the different materials evaluated as support

Support	WAI (g/g dry matter)	CHP (%)
Sugarcane bagasse	9.48ab	12a
Sotol bagasse	5.08c	26b
Agave fibers	6.57bc	12b
Polyurethane	9.98a	18a

There are no significant differences among the same letters

property, too. The support materials should have low values of CHP to facilitate growth of the microorganism, as high values indicate that low proportion of water is linked to support and therefore microorganism development and growth is affected considerably. Results in this study for this parameter showed the lowest value when sugarcane bagasse and *Agave* fibers were used (Table 18.1). Based on physical–chemical tests, one can say that from the four evaluated supports, two of them (*Agave* fibers and sugarcane bagasse) have great potential to be successfully used for SSF. However, each support has particular advantages or disadvantages to promote fungal production of fructooligosaccharides and fructosyltransferase.

18.4.2 Fructooligosaccharides Production in SSF

Lignocellulosic materials can be a source of carbon and nutrients during the fermentation process, and also can be used as solid support to fix the fungal cells during the growth permitting also the comsuption of the substrate and the formation of fermentation products. Filamentous fungi are microorganisms which easily adapt to solid-state fermentation conditions since its hyphae can grow over the surface particles and even penetrate the interstitial spaces and colonize the solid support (Graminha et al. 2008).

In this work, only *A. niger* strains were able to grow on the different supports, and their growth was associated with consumption of sucrose and enzymatic activity of fructosyltransferase because of the fructooligosaccharides produced during fermentation. However, *Agave* fibers and sugarcane bagasse can be detached from the other supports, because using these materials, the highest concentrations of fructooligosaccharides during fermentation were showed. Nevertheless, when *Agave* fibers were used as support, *A. niger* GH1 and *A. niger* PSH attained the maximum FOS production in a shorter time, 13 and 16 h, respectively. This difference may be attributed to the presence of other compounds in the *Agave* fibers after the wash process, such as polysaccharides and monomeric sugars, which are preferred by the fungal strains.

The selection criterion of support and microorganism strain was the ability to convert sucrose to FOS, enzyme transfructosylating (A_t), and hydrolytic (A_h) activity, and as this last one competes with the first one, the A_t/A_h ratio become very important (Hidaka et al. 1988; Fernandez et al. 2007).

The β-fructofuranosidase has both transfructosylating and hydrolytic activities, and the ratio between them also depends on sucrose concentration. For a sucrose concentration up to 5.0 g L^{-1}, the most important products formed during the enzymatic reaction are glucose and fructose. However, FOS and glucose are the predominant products when the sucrose concentration is higher than 200 g L^{-1}.

According to the experimental design, a selection index (SI) was established to select the best strain and support for fructooligosaccharides production. The response variables according to their importance in the process solid-state fermentation were transfructosylating activity (A_t), total fructooligosaccharides (FOS), ratio A_t/A_h (R), and hydrolyzing activity (A_h).

$$(\mathrm{SI} = [3.5(A_t) + 3.5(\mathrm{FOS}) + 2.0(R) + 1.0\,(A_h)]).$$

The highest value was given to the transfructosylating activity since production or polymerization of FOS is directly dependent on this activity.

The *Penicillium* strains did not show enzymatic activities. Figure 18.1 shows the chromatogram profile obtained during fermentation for FOS production by *A. niger* PSH supported in *Agave* fibers, after 10 h fermentation. Note that sucrose and oligomers were perfectly separated and eluted on the order of its polymerization degree; sucrose was eluted at 10.20 min, 1-kestose at 12.2 min, and nystose at 17.2 min

From all tested supports, it was observed that *Agave* fibers are the most suitable support for production of the fructosyltransferase enzyme and synthesis of fructooligosaccharides under solid-state fermentation (Fig. 18.2). No significant

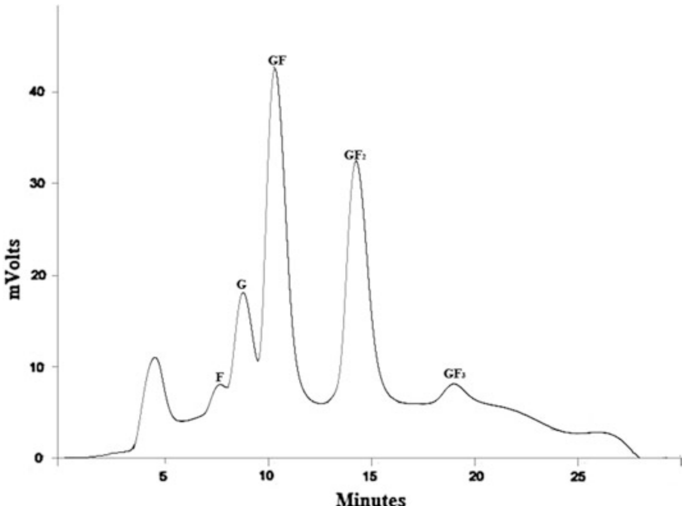


Fig. 18.1 HPLC chromatogram of FOS production by *A. niger* PSH supported in *Agave* fibers, after 10 h fermentation. F: fructose, G: glucose, GF: sucrose, GF2: 1-kestose, GF3: nystose

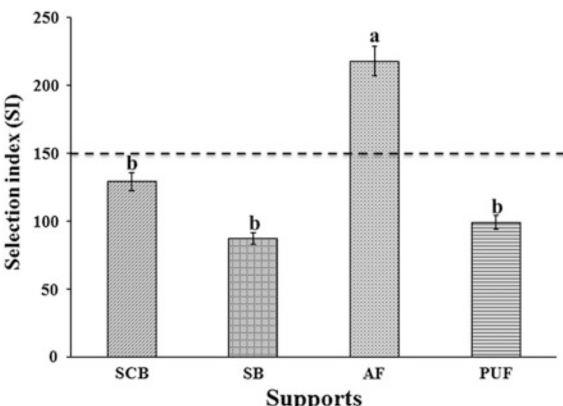


Fig. 18.2 Selection index for use of sugarcane bagasse (SCB), sotol bagasse (SB), *Agave* fibers (AF), and polyurethane (PUF) as supports in fructosyltransferase and FOS production under SSF

differences between sugarcane bagasse, sotol bagasse, and PUF were found, but there are differences between of these three supports and *Agave* fibers.

During strain selection, it was observed that *A. niger* PSH has greater trans-fructosylation activity than *Aspergillus niger* GH1 to convert sucrose into fructooligosaccharides (Fig. 18.3). *Penicillium* strains did not show enzyme activity and therefore no synthesis of FOS.

Use of *Agave* fibers as a matrix for culture media absorption and attachment for the fungal growth provides important advantages, such as higher production titers and productivity.

Associating *A. niger* PSH growth on *Agave* fibers may significantly decrease cost production, because this fungal strain has the ability to grow without any enrichment medium, and with sucrose as the sole carbon source. Using agro-industrial residues for SSF processes, it is important to take into account aspects such as cost and availability of support, environmental impact of the solids produced, as wells as process cost and product. In a next step, it will be evaluated

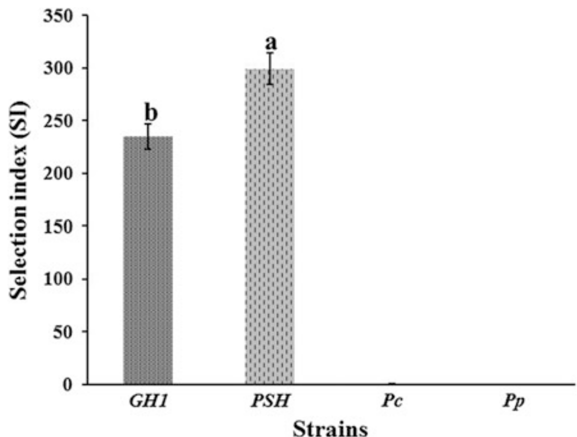


Fig. 18.3 Selection index for use of *Aspergillus niger* GH1, *A. niger* PSH, *Penicillium purpurogenum,* and *P. citrinum* as biotechnological tool for fructosyltransferase and FOS production under SSF

parameters that directly influence enzyme production as well as during fructooligosaccharides synthesis.

18.5 Discussion

According to the obtained results from the physical–chemical analysis (WAI and CHP) and selection index, *A. niger* PSH supported in *Agave* fibers may have potential for application in FOS production, because this fungus in this support has the ability to produce at the same time of FOS and enzyme fructosyltransferase during sucrose fermentation. The high productivity of fructosyltransferase from *A. niger* PSH during the enzymatic assay and U_t/U_h ratio without any optimization are very promissory and give us a positive expectation to maximize FOS production at industrial level. The development of a bioprocess for fructosyltransferase production would offer economic advantages and especially environmental friendly since it would be fully using agro-industrial waste produced by industry mescal, in fructooligosaccharides production compared to chemical methods.

Acknowledgements Authors thank Council of Science and Technology (CONACyT-Mexico) for the financial support given in the frame of the collaborative program among participating institutions.

Conflict of Interest The authors declare no financial or commercial conflict of interest

References

Bellon-Maurel V, Orliac O, Christen P (2003) Sensors and measurements in solid state fermentation: a review. Process Biochem 38(6):881–896

Buenrostro-Figueroa J, Ascacio-Valdés A, Sepúlveda L et al (2013) Potential use of different agroindustrial by-products as supports for fungal ellagitannase production under solid-state fermentation. Food Bioprod Process

Dhake AB, Patil MB (2007) Effect of substrate feeding on production of fructosyltransferase by *Penicillium purpurogenum*. Braz J Microb 38:194–199

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002) Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem 38(4):615–620

Fernandez R, Ottoni C, Silva E et al (2007) Screening of β-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. Appl Microbiol Biotechnol 75(1):87–93

Flores-Gallegos AC, Morlett-Chávez J, Aguilar CN, Rodríguez-Herrera R (2012) Inulinase production by a Mexican semi-desert Xerophylic *Penicillium citrinum* strain under submerged culture. Adv J Food Sci Technol 4(1):46–50

Flores-Maltos A, Rodríguez-Durán LV, Renovato J, Contreras JC, Rodríguez R, Aguilar CN (2011) Catalytical properties of free and immobilized *Aspergillus niger* Tannase. Enzym Res 2011:6

Flores-Maltos DA, Mussatto SI, Contreras-Esquivel JC, Rodríguez-Herrera R, Teixeira JA, Aguilar CN (2014) Biotechnological production and application of fructooligosaccharides. Crit Rev Biotechnol 1–9

Ganaie MA, Gupta US, Kango N (2013) Screening of biocatalysts for transformation of sucrose to fructooligosaccharides. J Mol Catal B Enzym 97:12–17

Gervais P, Molin P (2003) The role of water in solid-state fermentation. Biochem Eng J 13(2–3):85–101

Graminha EBN, Gonçalves AZL, Pirota RDPB, Balsalobre MAA, Da Silva R, Gomes E (2008) Enzyme production by solid-state fermentation: application to animal nutrition. Anim Feed Sci Technol 144(1–2):1–22

Hidaka H, Hirayama M, Sumi N (1988) A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611 (biological chemistry). Agric Biol Chem 52(5):1181–1187

La Rotta CE, Ospina SA, López-Munguía A (1998) Producción y Caracterización de Extractos Enzimáticos Crudos con Actividad de Fructosiltransferasa. Revista Colombiana de Ciencias Químico-Farmaceuticas 27:53–56

Lateef A, Oloke J, Gueguim-Kana E, Raimi O (2012) Production of fructosyltransferase by a local isolate of *Aspergillus niger* in both submerged and solid substrate media. Acta Aliment 41 (1):100–117

Mussatto SI, Aguilar CN, Rodrigues LR, Teixeira JA (2009) Fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. J Mol Catal B Enzym 59(1–3):76–81

Orzua MC, Mussatto SI, Contreras-Esquivel JC et al (2009) Exploitation of agro industrial wastes as immobilization carrier for solid-state fermentation. Ind Crops Prod 30(1):24–27

Patel V, Saunders G, Bucke C (1994) Production of fructooligosaccharides by *Fusarium oxysporum*. Biotech Lett 16(11):1139–1144

Pérez-Guerra N, Torrado-Agrasar A, López-Macias C, Pastrana L (2003) Main characteristics and applications of solid substrate fermentation. Electron J Environ Agricult Food Chem 2(3)

Prapulla SG, Subhaprada V, Karanth NG (2000) Microbial production of oligosaccharides: a review. Adv Appl Microbiol 47:299–343 (Academic Press)

Robledo A, Aguilera-Carbó A, Rodriguez R, Martinez J, Garza Y, Aguilar C (2008) Ellagic acid production by *Aspergillus niger* in solid state fermentation of pomegranate residues. J Ind Microbiol Biotechnol 35(6):507–513

Rodriguez-Duran LV, Contreras-Esquivel JC, Rodriguez R, Prado-Barragan LA, Aguilar CN (2011) Optimization of Tannase production by *Aspergillus niger* in solid-state packed-bed bioreactor. J Microbiol Biotechnol 21(9):960–967

Sánchez O, Rodriguez A, Silva E, Caicedo L (2010) Sucrose biotransformation to fructooligosaccharides by *Aspergillus* sp. N74 free cells. Food Bioprocess Technol 3(5):662–673

Sangeetha PT, Ramesh MN, Prapulla SG (2005) Maximization of fructooligosaccharide production by two stage continuous process and its scale up. J Food Eng 68(1):57–64

Santos AMP, Maugeri F (2007) Synthesis of Fructooligosaccharides from sucrose using inulinase from *Kluyveromyces marxianus*. Food Technol Biotechnol 45(2):181–186

Veana F, Aguilar C, Herrera RR (2011) Kinetic studies of invertase production by xerophilic *Aspergillus* and *Penicillium* strains under submerged culture. Micología Aplicada Internacional 23(2):37–45

Yoshikawa J, Amachi S, Shinoyama H, Fujii T (2006) Multiple β-fructofuranosidases by *Aureobasidium pullulans* DSM2404 and their roles in fructooligosaccharide production. FEMS Microbiol Lett 265(2):159–163

Yoshikawa J, Amachi S, Shinoyama H, Fujii T (2007) Purification and some properties of β-fructofuranosidase I formed by *Aureobasidium pullulans* DSM 2404. J Biosci Bioeng 103 (5):491–493

Yoshikawa J, Amachi S, Shinoyama H, Fujii T (2008) Production of fructooligosaccharides by crude enzyme preparations of β-fructofuranosidase from *Aureobasidium pullulans*. Biotech Lett 30(3):535–539

Zeng X-A, Zhou K, Liu D et al (2016) Preparation of fructooligosaccharides using *Aspergillus niger* 6640 whole-cell as catalyst for bio-transformation. LWT Food Sci Technol 65:1072–1079

Chapter 19
Tannase and Its Applications in Food Processing

Swaroop S. Kumar, Rakhy Sreekumar and Abdulhameed Sabu

Abstract Tannases represent a group of enzymes finding its applications in food, brewing, and pharmaceutical industries. They have a wide range of distribution and are reported form animals, plants, and microbial sources. However, tannase from microbial source is preferred over other sources for industrial uses. They act upon hydrolyzable tannins by cleaving the ester and depside bonds so as to release glucose and gallic acid. Gallic acid production is one of the most important commercial applications of tannase. Apart from that, they are extensively used in the food industry, especially in instant tea production, where it enhances the extractability and cold water solubility of key compounds. Another important application of tannase is the removal of haze formation and unflavored phenolic compounds from beer and wine. Quality of fruit juices also can be improved by tannase enzyme. Haze formation and bitterness of the fruit juices can be minimized by the application of these enzymes. Tannins are considered as anti-nutritional factors while using agro-industrial residues as animal feed. De-tannification of feed by tannase enzyme treatment can significantly improvise the quality of animal feed. Usage of tannase enzyme in the food industry with respect to its challenges is emphasized in the present chapter.

Keywords Tannase · De-tannification · Debittering · Tea processing
Flavor improvement

19.1 Introduction

Tannase or tannin acylhydrolase (EC 3.1.1.20) is an extracellular inducible enzyme, which catalyzes the hydrolysis of some tannins and gallic acid esters. These enzymes belong to serine esterase catalyzing the hydrolysis of ester bond (galloyl

S. S. Kumar · R. Sreekumar · A. Sabu (✉)
Department of Biotechnology and Microbiology, Kannur University, Thalassery Campus, Kannur 670661, Kerala, India
e-mail: drsabu@gmail.com

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_19

ester of alcohol moiety) and depside bond (galloyl ester of gallic acid) in tannins to release gallic acid (Yamada et al. 1968). Tannins are polyphenolic compounds produced by plants in order to protect themselves from invading microorganisms and herbivores (Buzzini et al. 2008). Microbial tannase production can be considered as self-defense of these microbes against tannins. Plants are rich in tannins, and gallic acid production from these plant by-products is one of the major applications of tannase enzymes (Pourrat et al. 1987). Pyrogallol derived from gallic acid has several applications such as coloring of hair, photographic plate developing agent, leathers and fur staining, antitumor agent, anti-lung cancer drug, etc. (Zeida et al. 1998). Gallic acid was produced from tannins of tara powder by microbial hydrolysis with a total yield of about 30% (with respect to the weight of raw material) suggesting the industrial potentiality of these enzymes (Pourrat et al. 1985). Tannase enzymes find their applications in cosmetic industries, food and feed industries, leather industries, and chemical industries (Aguilar and Gutierrez-Sanchez 2001). High concentrations of tannins in a beverage such as iced tea, beer, wine, fruit juices, and coffee-flavored beverages can result in the formation of precipitates due to their interaction with other molecules present in these beverages. These undesirable effects of tannins can be reduced or eliminated by a chemical or enzymatic treatment (Belmares-Cerda et al. 2003; Lekha and Lonsane 1997). Enzymatic treatment for the removal of these effects is achieved by using tannase enzymes (Boadi and Neufeld 2001). Tannases from *Aspergillus oryzae* have been in usage for various food preparations, probably for the 2000 years in Japan and other East Asian countries (Lane et al. 1997). Tannases from *Aspergillus niger* due to its Generally Regarded As Safe (GRAS) status by the US Food and Drug Administration has been approved for food applications in France (Barthomeuf et al. 1994).

19.2 Tannins—The Natural Substrate

Prior to the discussion about tannases and their applications, lets us quickly go through the details of its natural substrate, tannin. They are polyphenolic compounds representing the second most abundant naturally occurring phenol, ubiquitous in distribution among plants (Arbenz and Averous 2015). Tannins form complexes with biological macromolecules such as polysaccharides, proteins, cellulose starch, and gelatin. They have differing molecular weight depending on the molecule with which it complexes (Lekha and Lonsane 1997; Aguilar and Gutierrez-Sanchez 2001). Tannins have a wide range of biological activity such as antioxidant property, metal ion chelation, protein precipitation, etc. (Hagerman 2002). When coming to the significance of tannins in plants, they are plant's self-defense mechanism against insects, herbivores, fungi, bacteria, etc. There also find significant role during fruit ripening and usually present in blackberry, grape, apple, etc. (Swain 1977; Scalbert 1991; Aguilar et al. 2007; Lekha and Lonsane 1997).

Tannins have been classified into two major classes, hydrolyzable tannins and condensed tannins (Griffiths 1991). Another group of tannins called condensed tannins is also considered. Hydrolyzable tannins can further be categorized into gallotannins and ellagitannins (Arbenz and Averous 2015). Hydrolyzable tannins encompass a carbohydrate molecule in the central core, usually, D-glucose esterified with phenolic molecules such as gallic acid, ellagic acid, etc. (Haslem 1989). Gallotannins are esters of gallic acid with carbohydrate like glucose (Bhat et al. 1998). They hydrolyze to release gallic acid and glucose or quinic acid. They are the simplest tannins formed of polyphenolic and polyol residues (Khanbabaee and van Ree 2001). Galloyl units can partially or completely substitute the hydroxyl groups of polyol residues. Ellagitannins are esters of ellagic acid with carbohydrate like glucose. More than 500 members of this group have been identified. The basic monomer is the hexahydroxydiphenol (HHDP) (Arbenz and Averous 2015), whereas simple hydrolyzable tannins such as gallotannin or ellagitannin units form acyl bond with flavan-3-ol units like catechin to from complex tannins (Okuda and Ito 2011), e.g., Acutissimin. Condensed tannins (Proanthocyanidins) are formed of 2–50 flavonoid groups such as flavan-3-ol (catechin). They are considered to be non-hydrolyzable (Khanbabee and Van Ree 2001).

Tannins have been in practice for the tanning process for over thousand years owing to their ability to form strong interactions with proteins (Frutos et al. 2004; Aguilar et al. 2007). The word “tannin” indicates tanning substance, a plant material converting hide to leather (Arbenz and Averous 2015). The abundance of phenolic hydroxyl groups contributes their ability to complex with proteins and other macromolecules to a greater extent (Mueller-Harvey et al. 1987). Tannins have several advantages, for instance, they speed up blood coagulation, aid in wound healing, and also lower blood pressure. They also possess antimutagenic, anticancer, and antioxidant potentials. Tannins also act as immunomodulating agents and antimicrobial agent (Chung et al. 1998; Chokotho and Van Hasselt 2005).

Despite having so many advantages, tannins become undesirable under certain circumstances. Protein-binding capacity of tannins influences the nutritional quality of food. Inhibition of digestive enzymes and the removal of necessary proteins leading to indigestion make tannins an anti-nutritional factor (Kumar and Singh 1984; Lekha and Lonsane 1997). Tannins are also associated with bitterness and astringency in fruits and beverages (Aguilar et al. 2007). Tannins are also responsible for haze formation in beer and wine, cream formation in tea infusions, etc. They significantly affect the storage quality of these food products (Scharbert and Hofmann 2005; Aguilar et al. 2007; Masschelein and Batum 1981). The presence of tannins in plant materials makes them indigestible when used as animal feed (Frutos et al. 2004). Direct release of these polyphenols to the environment is a serious cause of pollution (Kumar et al. 1999). These undesired effects demand better treatment of tannin-rich foods, so that nutritional quality can be enhanced without deteriorating the key food ingredients.

19.3 Tannase Properties

The discovery of tannase was serendipitous during the experiment for the production of tannins from gallic acid where two fungal species were grown (Tieghem 1867). Later, these fungi were identified as *Penicillium glaucum* and *A. niger* (Lekha and Lonsane 1997). Tannase catalyzes the breakdown of hydrolyzable tannins (tannic acid, methyl gallate, ethyl gallate, and isoamyl gallate (Aguilar and Gutierrez 2001)). By the hydrolysis of ester bonds in tannins, these enzymes produce glucose and gallic acid. Enzymatic hydrolysis of tannic acid (nonagalloyl glucose) yields nine gallic acid molecules and one glucose molecule (Rodríguez-Durán et al. 2011). Studies on the regulation of tannase indicated that the enzyme would react with any phenolic hydroxyl group (Iibuchi et al. 1972). Tannins are thought to be the plant's defense mechanism against herbivores. Gallic acid, chebulinic acid, and hexahydroxyphenic acid are few of the plant metabolites which gets esterified with glucose to form tannins during the ripening (Lekha and Lonsane 1997). Tannase enzymes are reported from gall larva, so as to breakdown tannic acid of plant galls. Cattles also reported with low levels of tannase production (Lekha and Lonsane 1997). Microorganisms produce tannase to protect themselves from tannins (Scalbert 1991).

19.3.1 Structure of Tannase

Although these enzymes are significant industrially and used since longer time, its structural information was very little. The first crystal structure was obtained from *Lactobacillus plantarum* by Ren et al. (2013) (Fig. 19.1), followed closely by another 3D-atomic resolution model from *L. plantarum* determined by Matoba et al. (2013). The enzyme holds an α/β structure with classical serine hydrolase fold. It also carries a large cap domain protrude into the serine hydrolase fold. Active site contains Ser 163, His 451, and Asp 419 acting as a catalytic triad. Mutational studies indicated the significance of these residues in catalysis. Gallic acid binds with actives site residues to form hydrogen bonding interactions. Structural analysis gave insight toward the catalytic mechanism of tannases. Both depsidase and esterase activities were due to single galloyl-binding site and substrate binding interactions at this site (Ren et al. 2013). These enzymes thus belong to α/β-hydrolase superfamily. Glycerol binds to the galloyl binding site in a similar fashion to its substrate (Matoba et al. 2013). Secondary structural characterization of *Penicillium herquei* revealed that they constitute of α-helix (14%), antiparallel β-sheet (32.4%), β-sheet (4.8%), β-turn (18.8%), and 30% random coil (Qiu et al. 2011).

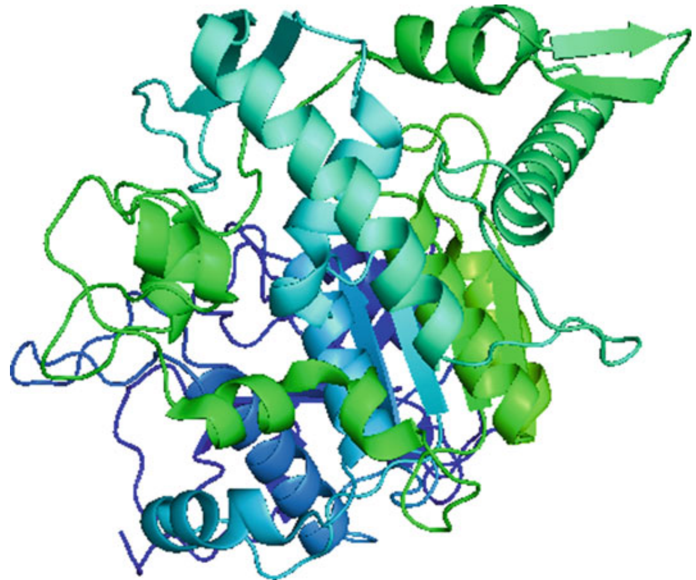


Fig. 19.1 Crystal structure of tannase from *Lactobacillus plantarum*. Three-dimensional coordinates obtained from RCSB Protein Data Bank (PDB id: 4JUI) (Ren et al. 2013) and structure were generated using PyMOL Viewer

19.3.2 Mechanism of Action

Tannases catalyze two different reactions. One is esterase activity and the other is depsidase activity. As mentioned above the single galloyl binding site was contributing both esterase and depsidase activities of the enzyme (Ren et al. 2013). Esterase activity involves the hydrolysis of ester bonds gallic acid esters such as galloyl glucose and methyl gallate. On the other hand, depsidase activity involves the cleavage of depside bonds of digallic acid, gallotannins, ellagitannins, complex tannins, etc. (Haslam and Stangroom 1966; Saxena and Saxena 2004; Sharma et al. 2000). But tannases never act on C–C bonds, and thus these enzymes are incapable of hydrolyzing condensed tannins (Haslam and Stangroom 1966).

19.4 Sources of Tannase

Although tannase is reported from plants, animals, and microorganisms, the latter represents the major source of these enzymes. These enzymes are known to have their presence in tannin-rich vegetables, fruits, leaves, and bark. Few of them are fruits of *Terminalia chebula*, leaves of *Anogeissus latifolia*, pods of *Caesalpinia coriaria,* and bark of *Cassia fistula* (Madhavakrishna et al. 1960). Also, a small

amount of tannases are reported from the small intestine and mucosal membrane of rumen in cattle (Begovic and Duzic 1976, 1977).

Microorganisms represent the largest tannase enzyme producers, with a wide range of enzymes and they are the most preferred source for commercial enzyme production. Microbial tannase enzymes have several advantages over plant and animal tannases. One of the most important features of these microbial enzymes is the remarkable stability over those from the other sources (Jana et al. 2014). Another characteristic is constant enzyme production in large magnitude by the microorganisms, and thus provides a continuous supply of enzymes for commercial use. Moreover, ease of genetic manipulation of microbial enzymes over the others is another factor preferring microorganisms (Aguilar and Gutierrez-Sanchez 2001; Purohit et al. 2006). By these genetic manipulations, improved enzymes production and also the development of tailor-made enzymes is reasonably possible. All these factors put together make microorganisms a superior choice for tannase enzyme production.

19.4.1 Tannase from Microbial Sources

Tannins are usually toxic to several microorganisms. However, few of them are resistant to tannins and capable of hydrolyzing tannins to gallic acid and pyrogallol which are industrially important molecules. Isolation of tannase enzymes was first successfully achieved by Fernbach (1900). Thereafter, numerous microorganisms were reported as tannase enzyme producers in succession. Using condensed tannins as the sole source of carbon, five bacterial strains with tannase enzyme production were isolated (Deschamps et al. 1983). Tannases are inducible enzymes and inducers like tannic acid can be used as a carbon source for expression tannase enzymes (Enemuor and Odibo 2009). Microbial tannases, in general, were either extracellular or intracellular. However, a few of them were membrane-bound also (Barthomeuf et al. 1994; Pourrat et al. 1987). Tannase producing microbiota in the gastrointestinal microenvironment of ruminants and other animals is also evidenced (Nelson et al. 1995; Goel et al. 2005).

As mentioned above, tannases are inducible enzymes. Microbial tannase production can be enhanced by phenolic compounds. Gallic acid, methyl gallate, pyrogallol, etc. are known inducers for tannase production (Bajpai and Patil 1997; Costa et al. 2008). Sugar molecules are found to regulate the tannase production. Lower glucose level induced tannase production, while higher level showed decreased production due to catabolite repression (Banerjee et al. 2001). Catabolite repression was observed under submerged cognition for tannase production from *A. niger* Aa-20, while glucose is used. However, solid-state fermentation conditions did not show the phenomenon (Aguilar et al. 2001). Regulation of enzyme production can be achieved by altering the fermentation conditions with respect to the organism.

19.4.1.1 Bacterial Tannase

First reported bacterium capable of hydrolyzing gallotannins as a sole energy source was *Achomobacter* sp. (Lewis and Starkey 1969). *Bacillus* and *Lactobacillus* genus represents the most studied group of tannase producing bacteria (Mondal et al. 2000, 2001; Sabu et al. 2006; Nishitani et al. 2004). Lactic acid bacteria have a significant role in tannin food degradation. Usually, methyl gallate is the substrate for bacterial tannases (Nishitani and Osawa 2003; Vaquero et al. 2004). Apart from this, few other bacterial tannase producers include *Citrobacter* (Kumar et al. 1999), *Corynebacterium*, *Klebsiella* (Deschamps et al. 1983), *Lonepinella* (Goel et al. 2007), *Pantonea* (Pepi et al. 2010), *Pseudomonas* (Selwal et al. 2010), *Serratia* (Belur et al. 2010), *Selenomonas* (Skene and Brooker 1995), and *Streptococcus* (Jiménez et al. 2014). The molecular weight of bacterial tannase ranges from 40 to 90 kDa (Iwamoto et al. 2008; Sharma and John 2011) and monomeric in nature. However, tannases from *Rhodococcus* sp. and *L. plantarum* have been reported with two subunits (Nadaf and Ghosh 2011). Beniwal et al. (2013) reported low molecular weight (31 kDa) of tannase from *Enterobacter cloacae*. Temperature optima of bacterial tannases were ranging from 30 to 50°C (Sabu et al. 2006), and pH optima showed was between pH 3 and 8 (Jana et al. 2013). However, few of the bacterial strains were reported to show considerable activity at higher pH. *Bacillus sphaericus* was retaining its tannase activity at pH 8 (Raghuwanshi et al. 2011), while *L. plantarum* retained 88% of tannase activity at pH 9 (Iwamoto et al. 2008). Tannases from various bacterial sources along with its properties are shown in Table 19.1.

19.4.1.2 Fungal Tannases

Fungi represent one of the most important tannase enzyme producers, and tannases from fungi are in usage perhaps since ancient times. In contrast to bacterial tannases, which usually is monomeric in nature, fungal tannases are multimeric comprising two or more subunits, occasionally bridged together by disulfide linkages (Kasieczka-Burnecka et al. 2007). The molecular weight of fungal tannases ranges from 50 and 320 kDa (Marco et al. 2009; Böer et al. 2009). Fungi are the most studied group for tannase enzyme production. *Aspergillus* is one of the most predominant tannase producers and *A. niger*, *Aspergillus phoenicis*, *Aspergillus ochraceus,* etc. are few among them (Sabu et al. 2005; Riul et al. 2013; Gonçalves et al. 2012). Tannase from *A. niger* van Tieghem was showing thermostability up to 60°C, and it also showed stability over a wide range of pH from pH 3 to 8 (Sharma et al. 1999), while *A. niger* ATTC 16620 yielded a tannase of molecular weight 168 kDa which was also stable at pH 4–8 (Sabu et al. 2005). A 45 kDa tannase enzyme was derived from *Paecilomyces variotii* which showed stability over a pH range of 4–8 and temperature ranging from 30 to 50°C (Mahendran et al. 2006). Another fungal producer was *Penicillium variable* producing a thermostable enzyme of molecular weight 158 kDa which showed considerable activity even at

Table 19.1 Tannases from bacterial sources and its characteristics

Organism	Molecular weight (kDa)	pH, temperature optima	Stabilizer	Inhibitor	Additional Properties	References
Lactobacillus plantarum CECT 748 T	–	pH 5.0, 30°C	K^{+}, Ca^{2+}, Zn^{2+}, tween 80, EDTA, DMSO and urea	Hg^{2+}	Retained 75% of the maximal activity at 50 ° C	Rodríguez et al. (2008)
Lactobacillus plantarum ATCC 1491 T (recombinant)	50	pH 8.0, 40°C	–	–	Km = 0.62 mM	Iwamoto et al. (2008)
Lactobacillus plantarum (recombinant)	50	pH 7.0, 40°C	K^{+} and Ca^{2+}	Urea, Hg^{2+} mercaptoethanol	Active at pH 6.0–8.0	Curiel et al. (2009)
Enterobacter sp.	90	pH 5.5, 40°C	–	–	Km = 3.7 mM	Sharma and John (2011)
Bacillus licheniformis KBR 6	–	pH 5.75, 60° C	–	–	–	Mondal and Pati (2000)
Bacillus cereus KBR9	–	pH 4.5, 40°C	–	–	Salt tolerant, stable up to retains 82% original activity in 3 M NaCl	Mondal et al. (2001)
Bacillus sphaericus	–	pH 5.0, 50°C	–	–	pH 3–8	Raghuwanshi et al. (2011)
Enterobacter cloacae	31	5.5, 50°C	Mg^{2+}, Zn^{2+}, Mn^{2+}	*n*-bromosuccinic acid, PMSF, Fe^{2+}, Ba^{2+}, Cu^{2+}	Km and *V*max 0.00037 M 3.401 U/ml	Beniwal et al. (2013)
Staphylococcus lugdunensis MTCC 3614 (recombinant)	66	pH 7.0, 40°C	–	Zn^{2+}, Fe^{2+}, Fe^{3+} and Mn^{2+}	–	Chaitanyakumar and Anbalagan (2016)
Enterococcus faecalis	45	pH 6.0, 40°C	–	–	–	Goel et al. (2011)

80°C. The enzyme was quite stable over a pH ranging from 4 to 8 (Sharma et al. 2008). Few other fungal genera such as *Arxula* (Boer et al. 2009), *Candida* (Aoki et al. 1976a, b), *Cryphonectria* (Farias et al. 1994), *Debaryomyces* (Deschamps et al. 1983), *Fusarium* sp. Penicillium sp. Bajpai and Patil (1996), *Hyalopus* sp. (Mahapatra and Banerjee 2009), *Verticillium* sp. (Kasieczka-Burnecka et al. 2007), *Aureobasidium* Banerjee and Pati (2007), and *Rhizopus* (Hadi et al. 1994) were also reported for tannase production. Some of the fungal tannase enzyme producers along with the properties of the enzyme are shown in Table 19.2.

19.5 Recombinant Production of Tannase Enzymes

Gene cloning and subsequent expression of enzymes have emerged as powerful for the last few decades, and it has been quite useful in the production of larger volumes of enzymes. Another advantage of recombinant techniques is that enzyme can be tailor-made to suit the industrial needs. Usually, crude preparations of *Aspergillus* species with tannase activity are used for commercial purposes (Aguilar et al. 2007). Recombinant tannase enzyme production seems challenging, owing to the complexity of tannase enzymes. Fungal enzymes are rich in disulfide bonds and often tend to assemble and form higher molecular weight molecules (Aguilar et al. 2007; Hatamoto et al. 1996; Zhong et al. 2004.) However, few studies achieved success in cloning and expression of tannases. Cloning of tannase gene from *A. oryzae* and subsequent expression indicated the presence of single polypeptide after translation and upon cleavage, it forms two subunits of 30 and 33 kDa linked together by disulfide bonds. The native enzyme is made of four pairs of these two subunits forming a hetero-octamer of 300 kDa (Hatamoto et al. 1996). Tan A, a gene encoding tannase enzyme from *Staphylococcus ludgunensis* successfully cloned and sequenced. However, as tannase producing *S. ludgunensis* is associated with colon cancer, tan A gene could be used as a potential marker for detection of these organisms (Noguchi et al. 2007). Cloning and overexpression of tannase (Ss-Tan) from *Streptomyces sviceus* with higher catalytic efficiency and better thermal stability has been successfully achieved by Wu et al. (2015). Tan 7, a gene encoding tannase from *A. niger* SH-2, was cloned and overexpressed in *A. niger* strain Bdel4 which has lesser secretory proteins, thus advantageous in large-scale enzyme production (Liu et al. 2018). A tannase-encoding gene *AotanB* from *A. oryzae* was successfully cloned and overexpressed in *Pichia pastoris* (Koseki et al. 2018).

Table 19.2 Tannases from fungal sources and its characteristics

Organism	Molecular weight (kDa)	pH, temperature optima	Stabilizer/ enhancer	Inhibitor	Additional properties	References
Aspergillus niger van Tieghem	–	pH 6.0, 60° C	–	–	Stable at pH 3.0–8.0 and temperature 30–60°C Km—0.20 mM, *V*max—5.0 µmol/min/mg	Sharma et al. (1999)
Aspergillus niger ITCC 6514.07	–	pH 6.2, 35° C	–	Zn^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, Ag^{+}	–	Srivastava and Kar (2009)
Aspergillus niger ATTC 16620	168	pH 6.0 40°C	Fe^{2+}, K^{+}	Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, $Mg2^{+}$	Stable at pH 4.0–8.0 and temperature 30–40°C	Sabu et al. (2005)
Aspergillus niger GH1	50–100	pH 6.0 60°C	Co^{2+}	Fe^{3+}, Cu^{2+}, Zn^{2+}	Stable at pH 3.0–6.0 and temperature lower than 50°C	Marco et al. (2009)
Aspergillus ochraceus	85	pH 5.0 40°C	Mn^{2+}, NH^{4+}, EDTA	Cu^{2+}, Hg^{2+}	Stable at temperatures below 60°C	Gonçalves et al. (2012)
Aspergillus phoenicis	218	pH 6.0 60°C	–	–	Stable at 4.0–7.0, and temperatures below 50°C	Riul et al. (2013)
Aspergillus aculeatus DBF 9	–	pH 5.0 60°C	–	–	The intracellular enzyme, stable at pH 4.0–6.0, and temperature 50°C Salt tolerant up to 3 M NaCl	Banerjee et al. (2001)
Aspergillus aculeatus DBF 9	–	pH 5.0 50°C	–	–	Extracellular enzyme stable at pH 4.0–6.0 Temperature 50°C Salt tolerant up to 3 M NaCl	Banerjee et al. (2001)

(continued)

Table 19.2 (continued)

Organism	Molecular weight (kDa)	pH, temperature optima	Stabilizer/ enhancer	Inhibitor	Additional properties	References
Aspergillus awamori nakazawa	–	pH 5.0, 35° C	–	–	Urea, sodium lauryl sulfate, EDTA	Mahapatra et al. (2005)
Aspergillus versicolor	–	pH 5.0 60°C	–	–	Stable at pH 7.0 and temperatures below 60°C	Batra and Saxena (2005)
Aspergillus tamarii	–	pH 5.0 30°C	–	–	Stable at pH 4.0–8.0, and temperatures lower than 45°C	Costa et al. (2008)
Arxula adeninivorans (recombinant)	320	pH 6.0 30°C	Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, EDTA	PMSF Cd^{2+}, Cu^{2+}	–	Böer et al. (2009)
Paecilomyces variotii	45	pH 6.0 40°C	–	–	Stable at pH 4.0–8.0 and temperature 30–50°C	Mahendran et al. (2006)
Penicillium variable	158	pH 5.0 50°C	–	PMSF *N*-ethylmaleimide	Stable at pH 3.0–8.0 and temperature 25–80°C	Sharma et al. (2008)
Lenzites elegans	163	pH 5.5 60°C	–	–	Stable at a pH 3.0–6.0 and temperatures 40–60°C	Ordoñez et al. (2011)
Hyalopus sp.	–	pH 6.5 60°C	–	–	Stable at pH 5.0–8.0 and temperatures below 50°C	Mahapatra and Banerjee (2009)
Emericella nidulans	302	pH 5.0 45°C	Zn^{2+}, Hg^{2+}, Co^{2+}, SDS Triton X-100	–	A heteromeric glycoprotein with 3 copies of 48 and 52 kDa subunits Stable at pH 4.0–5.0 and temperature 22–55°C	Gonçalves et al. (2011)

(continued)

Table 19.2 (continued)

Organism	Molecular weight (kDa)	pH, temperature optima	Stabilizer/ enhancer	Inhibitor	Additional properties	References
Trichoderma harzianum MTCC 10841	–	pH 5.5 40°C	–	$HgCl_2$, $ZnCl_2$, $MnCl_2$	Stable at temperature up to 40°C	Iqbal and Kapoor (2012)
Kluyveromyces marxianus	65	pH 4.5 and 8.5 35°C	–	–	Two pH optima (pH 4.5 and 8.5), Stable at temperature 30–70°C Km—0.77 mM *V*max—263.20 μmol/ml/min	Mahmoud et al. (2018)

19.6 Applications of Tannase

Tannases have a significant role in industries such as cosmetic industries, food and feed industries, leather industries, and chemical industries (Aguilar and Gutierrez-Sanchez 2001). One of the major applications of Tannase enzymes is gallic acid production from tannins. However, in this chapter, we focus mainly on its food applications. Removal of undesirable effects of tannins is the major concern of food and beverage industries and tannases come in handy during these occasions (Boadi and Neufeld 2001). Elimination of the bitter taste of fruit juices and tea infusions by enzymatic treatment enhances the quality of these drinks. Few of the patents granted on applications and production of Tannase is shown in Table 19.3.

Table 19.3 Patents granted on applications and production of tannase (those with authors information unavailable left blank)

S. No	Title/invention	Patent No.	Year granted	Reference
1	Green tea conversion using tannase and natural tea enzymes	US 3812266A	1974	Sanderson and Coggon (1974)
2	Cold water extractable tea leaf and process	US4051264A	1977	Sanderson et al. (1977)
3	Enzymatic solubilization of tea cream	US3959497A	1976	Takino (1976)
4	Enzymatic treatment of black tea leaf	EP0135222B1	1988	Tsai (1988)
5	Enzymatic synthesis of gallic acid esters	EP0137601B1	1989	Weetall (1989)
6	Process of preparing a tea product	EP0391468 B1	1993	Barmentlo et al. (1993)
7	Enzyme extraction process for tea	US 5919500A	1999	Lehmberg et al. (1999)
8	Method of enhancing color in a tea-based foodstuff	US5879730A	1999	Bouwens et al. (1999)
9	Tea concentrate prepared by enzymatic extraction and containing xanthan gum which is stable at ambient temperature	US6024991A	2000	Lehmberg and Ma (2000)
10	Black tea drink containing fruit juice	JP2000037164A	2000	Matsuyama et al. (2000)
11	Fruit or vegetable juice-containing protein beverage	JP2001340069A	2001	Hara et al. (2001)
12	Process for the production of beer having improved flavor stability	EP1122303A1	2001	Hennink et al. (2001)
13	Tooth whitening products and procedures	WO2001064175A1	2001	Huybrechts (2001)

(continued)

Table 19.3 (continued)

S. No	Title/invention	Patent No.	Year granted	Reference
14	Cold water infusing leaf tea	EP1150575B1	2003	Goodsall et al. (2003)
15	Cold water infusing leaf tea	EP1150575A1	2003	Goodsall et al. (2001)
16	Producing a tea extract	EP0777972A1	2003	Nicolas et al. (1997)
17	Process for the production of beer having improved flavor stability	EP1252285B1	2004	Hennink et al. (2004)
18	Vasodilating compound and method of use	US6706756B1	2004	Fitzpatrick (2004)
19	Process for the production of beer having improved flavor stability	US20030157217A1	2003	Schmedding et al. (2003)
20	Diagnostic agent and test method for colon cancer using tannase as index	US7090997B2	2006	Sasatsu et al. (2006)
21	Green tea formulations and methods of preparation	US7232585B2	2007	Quan and Xiong (2007)
22	Process for producing purified green tea extract	US20090081350A1	2008	Abe et al. (2009)
23	Process for preparing pomegranate extracts	EP 1967079A2	2008	López et al. (2008)
24	Compositions comprising *Lactobacillus plantarum* strains in combination with tannin and new *Lactobacillus plantarum* strains	US7507572B2	2009	Molin et al. (2009)
25	Method of enzymatically treating green tea leaves	EP2036440A1	2009	Yotsumoto (2009)
26	Green tea drink packed in container	EP2098121B1	2013	Fukuda et al. (2013)
27	Methods of reducing the inhibitory effect of a tannin of the enzymatic hydrolysis of cellulosic material	WO2009059175A2	2009	Xu (2009
28	Process for producing purified tea extract	EP2225952B1	2010	Shikata et al. (2014)
29	Grape extract, dietary supplement thereof, and processes therefore	US7767235B2	2010	Shrikhande et al. (2010)
30	Process for the preparation of theaflavin-enhanced tea products	EP2096937B1	2011	–
31	Method for producing purified tea extract	US20100184167A1	2013	Maruyama et al. (2010)
32	Tannase, gene encoding same, and process for producing same	CN102203248B	2013	–

(continued)

Table 19.3 (continued)

S. No	Title/invention	Patent No.	Year granted	Reference
33	Process for producing purified tea extract	EP2225952A1	2014	Shikata et al. (2010)
34	Method for preparing tannase	CN102796716B	2014	–
35	Preparation method of tannase	CN103436505B	2014	–
36	Method for improving green tea quality by using immobilized tannase	CN103621712B	2015	–
37	A method of preparing the isolated cells and rapid method of tannase	CN103614351B	2016	–
38	One kind of low temperature and neutral TanXZ7 tannase gene and applications	CN104004725B	2016	–
39	Method for manufacturing highly enriched and fermented gammul with colors	KR101696533B1	2017	–
40	A fixed-method from a fermentation broth directly tannase	CN104962544B	2017	–

19.6.1 Tea Industries

The most popular nonalcoholic beverage is tea (*Camellia sinensis* L.), and over two-thirds of the world population consume it. One of the major applications of tannase is instant tea preparation. When the tea is extracted with cold water, it has a limited concentration of extractable solids and the beverage obtained has pale color without the taste of tea. Upon treatment with tannase enzymes under anaerobic conditions, cold water-insoluble components will be reduced, thereby yielding a high-quality green tea beverage when extracted with cold water (Sanderson et al. 1974). Safety evaluation of tannase enzyme from *A. oryzae* was conducted and found that it was safe for its usage in tea preparation (Lane et al. 1997). *N*-nitrosodimethylamine (NDMA) inhibition by tannase-treated green tea is reported, owing to its antioxidant potential (Lu and Chen 2007). Tea infusions contain catechins such as epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (Chen et al. 2014). Catechins despite having many advantages contribute to bitterness and astringency in tea infusions (Scharbert and Hofmann 2005). With increasing concentration of catechin, the taste intensity gets increased and the taste palatability gets reduced (Zhang et al. 2016). Another disadvantage of tea catechins is their poor bioavailability (Fan et al. 2016). Tea cream formation is another attribute contributed by the tea polyphenols, which affects the

storage quality of these products. Moreover, these polyphenols are anti-nutritive by inhibiting the digestive enzymes such as amylase and pepsin (Li et al. 2017). Enzymatic treatment with tannase is one-step solution to all these complications. Catechins have been hydrolyzed very efficiently by tannase from *A. niger* (Ni et al. 2015). An inferior Tieguanyin Oolong tea was undergone quality improvement with improved antioxidant property by the action of tannase enzymes (Li et al. 2017). Epigallocatechin gallate of green tea and yerba mate tea were hydrolyzed by the action of tannase enzyme from *Paecilomyces variotti* during which the antioxidant property of both the tea infusions got significantly amplified (Macedo et al. 2011). In another attempt, epigallocatechin gallate and epicatechin gallate were hydrolyzed by tannase to improve the sweet aftertaste, thereby improvising the general acceptability of green tea infusions (Zhang et al. 2016).

19.6.2 Fruit Juice

Tannins are abundant in skins and seeds of grape berries representing one of the most soluble classes of polyphenolics in them (Adams 2006). Blueberries, raspberry, and pomegranate during their storage form sediments, dark color, and bitter taste (Aguilar et al. 2007). These undesired characteristics can be removed by tannases during preparation and preservation of different fruit juices (Rout and Banerjee 2006). Pomegranate is known for its antioxidant potential; however, haze formation, sedimentation, color formation, bitterness, and astringency upon storage of pomegranate juice are contributed by the abundance of tannins. Conventional methods are inefficient for fruit juice debittering while enzymatic debittering using tannase was advantageous. However, treatment with tannase and gelatin in combination achieved more tannin degradation (49%) than treatment with tannase alone (25%) (Rout and Banerjee 2006). Clarification of cashew apple juice was achieved by de-tannification using tannase enzyme, thereby eliminating the astringency of the juice (Anwar 2015). Enzyme-treated cashew apple juice was suitable for consumption after 2 months of storage at 4°C (Campos et al. 2002). Aonla/myrobalan (*Phyllanthus emblica*) juice rich in vitamin C and tannins was treated with tannase from *A. niger* ITCC 6514.07, and 68. 8% of tannin was removed without dropping the vitamin C content. Nonenzymatic treatment of the juice on the other hand significantly reduced the vitamin C, suggesting the advantage of enzymatic treatment (Srivastava and Kar 2009). Biotransformation of polyphenols in orange juice by tannase from *P. variotti* is achieved, thereby enhancing its nutraceutical potential (Ferreira et al. 2013). Grapes are a rich source of tannins, and the tannase enzyme from *Penicillium montanense* was useful in clarification of grape juice by reducing 46% of the tannin content (Lima et al. 2014). 56% of total tannin was removed during the enzymatic clarification of pomegranate juice using tannase from *A. niger* MTCC 2425 (Nandi and Chatterjee 2016).

19.6.3 Bear and Wine Industry

Haze formation usually occurs during the storage of beer and wine due to the abundance of phenolic compounds in wine and beer. Tannase from *Aspergillus flavus* was employed to hydrolyze the polyphenols in chilled beer, thereby reducing the haze formation in beer (Masschelein and Batum 1981). As polyphenols are abundant in grape berries, the wine produced from them also contains these compounds leading to haze formation. Previously, chemical treatment was practiced for removal of haze formation, which is now replaced by enzymatic treatment with tannase to remove tannins like catechins and epicatechins. Acorn wine with superior quality was obtained by treating with tannase producing *Aspergillus* sp. AN-11 (Chae and Yu 1983). Flavor of beer was dramatically improved by treating with tannases, and Hennink et al. patented the process for this improved flavor stability (Hennink et al. 2004). Tannase producing *L. plantarum* was reported from grape must and wine, which might turn to be effective in haze removal (Vaquero et al. 2004).

19.6.4 Animal Feed

The presence of tannins in agro-industrial waste and plant materials which are used as animal feed make them unusable. Tannins are considered anti-nutritional factor since their interaction with macromolecules such as proteins and making them indigestible (Frutos et al. 2004). Their presence affects protein digestibility and inhibition of digestive enzymes causing the elimination of essential amino acids and proteins (Kumar and Singh 1984; Lekha and Lonsane 1997). All these lead to lower nutritional quality and toxicity of animal feed making lower feed intake and health issues in animals. Cellulosic material can be treated with tannase for reducing the tannin content as described by Xu (2009). Tannase enzymes can improve the nutritive value of animal feed by removing tannins.

19.6.5 Environmental Pollution

Agro-industrial waste residues as mentioned above are rich in polyphenols like tannins and represent one of the leading causes of environmental pollution. Direct release of these phenolics to the environment is hazardous (Kumar et al. 1999). Treating these agro-industrial and plant waste materials with tannase could be a solution for its degradation. Tannin polluting industrial waste from tannery wastewater is another cause of environmental pollution. Tannase production was successfully achieved using different agro-industrial waste materials (Sabu et al. 2006; Yadav et al. 2008). Production of tannase producing organisms on these

industrial wastes and its bioconversion could be an efficient and economical way of removing the pollutants.

19.6.6 Gallic Acid

As mentioned above, one of the major applications of tannases is gallic acid production. Gallic acid is commercially important for its applications in several industries. Gallic acid has got significant antioxidant and antibacterial properties. They also possess cytotoxicity against cancer cells (Beniwal et al. 2013). Apart from this, gallic acid acts as an intermediate for the production of various molecules of commercial significance. Trimethoprim (2,4, diamino 3,4,5 trimethoxy benzyl pyrimidine) is an antibacterial drug synthesized from gallic acid (Sittig 1988). Pyrogallol and gallate esters formed from gallic acid find their applications in food industries, cosmetic industries, etc. Pyrogallol has also been used as a photographic film developer (Beniwal et al. 2013).

19.7 Enzyme Immobilization

Enzyme immobilization has been practiced for a longer time for industrial applications. Major advantages are enhanced thermal and pH stability, simple separation of products, constant use of enzyme in packed-bed reactors, etc. Immobilizations of tannase from *A. oryzae* caused enhancement of thermal stability and also stability at lower pH. Even after repeated usage of 17 times, 85% residual activity was shown by the immobilized enzyme (Abdel-Naby et al. 1999). Immobilized tannase enzyme of *Rhizopus oryzae* was stable till 7 times of repeated use (Hota et al. 2007). Immobilized tannase from *P. variable* IARI 2031 retained 85% of its activity even after continuous usage of 9 times (Sharma et al. 2008). Immobilized tannase enzyme from *Staphylococcus lugdunensis* MTCC 3614 showed 80% residual activity after 7 cycles of usage and more than 50% residual activity after 15 cycles (Chaitanyakumar and Anbalagan 2016).

19.8 Conclusions and Future Perspectives

The discovery of tannase back in the nineteenth century and subsequent developments lead to quality improvement of many foods and beverage products, though commercially gallic acid synthesis is the main intention. When it comes to food applications, instantaneous tea preparation has been a great bottleneck. Tannase applications in tea preparation have acquired a maximum number of patents for the past 50 years. A large number of patents have been granted for food applications

such as wine, beer, and fruit juice production (Table 3). All of these projects are the immensity of tannase enzyme-based treatment, especially in the food sector. Usability of tannase-treated plant material as animal feed with improved quality is yet another application of these biomolecules. Tannase treatment can be useful in the reduction of environmental pollution caused by tannin-rich plant materials and tannery effluents. Tannase also has been used as a marker for testing colon cancer. With more and more patents granted each year, industrial application of tannase acquired a steady pace. This demands large-scale production of tannases; however, only limited studies regarding cloning and hyperproduction of tannases have been available. Recombinant tannase production for food applications has to achieve more pinnacles. Although tannases have been identified and studied for over a century and a half, only a few crystal structures were determined to date. Structural characterization has to be performed for a deeper understanding of the molecule. Protein engineering is yet another area to be explored for tannase enzymes. More efforts have to be put for development of enzymes with improved properties. The versatility of tannases underlines its economic importance.

References

Abdel-Naby MA, Sherif AA, El-Tanash AB, Mankarios AT (1999) Immobilization of *Aspergillus oryzae* tannase and properties of the immobilized enzyme. J Appl Microbiol 87(1):108–114

Abe T, Ueoka H, Shibata K (2009) Process for producing purified green tea extract. US20090081350A1

Aguilar CN, Gutierrez-Sanchez G (2001) Sources, properties, applications and potential uses of tannin acyl hydrolase. Food Sci Technol Int 7(5):373–382

Aguilar CN, Augur C, Favela-Torres E, Viniegra-González G (2001) Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochem 36(6):565–570

Aguilar CN, Rodríguez R, Gutiérrez-Sánchez G, Augur C, Favela-Torres E, Prado-Barragan LA, Ramírez-Coronel A, Contreras-Esquivel JC (2007) Microbial tannases: advances and perspectives. Appl Microbiol Biotechnol 76(1):47–59

Anwar YAS (2015) Effect of tannase on chemical parameters of cashew apple juice (*Anacardium occidentale* Linn). Alchemy Jurnal Penelitian Kimia 11(1):29–37

Arbenz A, Averous L (2015) Chemical modification of tannins to elaborate aromatic biobased macromolecular architectures. Green Chem 17(5):2626–2646

Bajpai B, Patil S (1996) Tannin acyl hydrolase (EC 3.1. 1.20) activity of Aspergillus, Penicillium, Fusarium and Trichoderma. World J Microbiol Biotechnol 12(3):217–220

Bajpai B, Patil S (1997) Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti. Enzyme Microb Technol 20(8):612–614

Banerjee D, Pati BR (2007) Optimization of tannase production by Aureobasidium pullulans DBS66. J Microbiol Biotechnol 17(6):1049–1053

Banerjee D, Mondal KC, Pati BR (2001) Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. J Basic Microbiol Int J Biochem Physiol Genet Morphol Ecol Microorganisms 41(6):313–318

Barmentlo B, Bel WJ, Hoogstad B, Pendlington S, Slater NKH (1993) Process of preparing a tea product. EP0391468B1

Barthomeuf C, Regerat F, Pourrat H (1994) Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. J Ferment Bioeng 77(3):320–323
Batra A, Saxena RK (2005) Potential tannase producers from the genera *Aspergillus* and *Penicillium*. Process Biochem 40(5):1553–1557
Begovic S, Duzic E (1976) Experimental proof of tanin decomposition owing to ferments present in mucosa of the rumen in bovines. Veterinaria (Yugoslavia)
Begovic S, Duzic E (1977) Purifying of tannase from the mucous membrane of the rumen and small intestines of bovines and a comparison of activity. Veterinaria (Yugoslavia)
Belmares-Cerda R, Reyes-Vega ML, Contreras-Esquivel JC, Rodriguez-Herrera R, Aguilar CN (2003) Effect of carbon source on tannase production by two strains of *Aspergillus niger*. Rev Mex Ing Quim 2:95–100
Belur PD, Gopal M, Nirmala KR, Basavaraj N (2010) Production of novel cell-associated tannase from newly isolated *Serratia ficaria* DTC. J Microbiol Biotechnol 20(4):732–736
Beniwal V, Kumar A, Goel G, Chhokar V (2013) A novel low molecular weight acido-thermophilic tannase from *Enterobacter cloaca*e MTCC 9125. Biocatal Agric Biotechnol 2(2):132–137
Bhat TK, Singh B, Sharma OP (1998) Microbial degradation of tannins—a current perspective. Biodegradation 9(5):343–357
Boadi DK, Neufeld RJ (2001) Encapsulation of tannase for the hydrolysis of tea tannins. Enzyme Microb Technol 28(7–8):590–595
Böer E, Bode R, Mock HP, Piontek M, Kunze G (2009) Atan1p—an extracellular tannase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the ATAN1 gene and characterization of the recombinant enzyme. Yeast 26(6):323–337
Bouwens EC, Trivedi K, van Vliet C, Winkel C (1999) Method of enhancing color in a tea-based foodstuff. US5879730A
Buzzini P, Arapitsas P, Goretti M, Branda E, Turchetti B, Pinelli P, Ieri F, Romani A (2008) Antimicrobial and antiviral activity of hydrolysable tannins. Mini Rev Med Chem 8(12):1179–1187
Campos DCP, Santos AS, Wolkoff DB, Matta VM, Cabral LMC, Couri S (2002) Cashew apple juice stabilization by microfiltration. Desalination 148(1–3):61–65
Chae SK, Yu TJ (1983) Experimental manufacture of acorn wine by fungal tannase. Korean J Food Sci Technol 15(4):326–332
Chaitanyakumar A, Anbalagan M (2016) Expression, purification and immobilization of tannase from *Staphylococcus lugdunensis* MTCC 3614. AMB Express 6(1):89
Chen GH, Yang CY, Lee SJ, Wu CC, Tzen JT (2014) Catechin content and the degree of its galloylation in oolong tea are inversely correlated with cultivation altitude. J Food Drug Anal 22(3):303–309
Chokotho L, Van Hasselt E (2005) The use of tannins in the local treatment of burn wounds-a pilot study. Malawi Med J J Med Assoc Malawi 17(1):19
Chung KT, Wei CI, Johnson MG (1998) Are tannins a double-edged sword in biology and health? Trends Food Sci Technol 9(4):168–175
Costa AM, Ribeiro WX, Kato E, Monteiro ARG, Peralta RM (2008) Production of tannase by *Aspergillus tamarii* in submerged cultures. Brazil Arch Biol Technol 51(2):399–404
Curiel JA, Rodríguez H, Acebrón I, Mancheño JM, De Las Rivas B, MUNoz ROSARIO (2009) Production and physicochemical properties of recombinant *Lactobacillus plantarum* tannase. J Agric Food Chem 57(14):6224–6230
Deschamps AM, Otuk G, Lebeault JM (1983) Production of tannase and degradation of chestnut tannin by bacteria. J Ferment Technol 61(1):55–59
Enemuor SC, Odibo FJC (2009) Culture conditions for the production of a tannase of *Aspergillus tamarii* IMI388810 (B). Afr J Biotech 8(11):2554–2557
Fan FY, Shi M, Nie Y, Zhao Y, Ye JH, Liang YR (2016) Differential behaviors of tea catechins under thermal processing: formation of non-enzymatic oligomers. Food Chem 196:347–354

Farias GM, Gorbea C, Elkins JR, Griffin GJ (1994) Purification, characterization, and substrate relationships of the tannase from *Cryphonectria parasitica*. Physiol Mol Plant Pathol 44(1):51–63

Fernbach MA (1900) Sur la tannase. Comptes rendus 131:1214–1215

Ferreira LR, Macedo JA, Ribeiro ML, Macedo GA (2013) Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res Int 51(2):526–535

Fitzpatrick DF (2004) Vasodilating compound and method of use. US6706756B1

Frutos P, Hervas G, Giráldez FJ, Mantecón AR (2004) Tannins and ruminant nutrition. Spanish J Agric Res 2(2):191–202

Fukuda M, Takahashi H, Kusaka R, Itaya E (2013) Green tea drink packed in container. EP2098121B1

Goel G, Kumar A, Beniwal V, Raghav M, Puniya AK, Singh K (2011) Degradation of tannic acid and purification and characterization of tannase from *Enterococcus faecalis*. Int Biodeterior Biodegradation 65(7):1061–1065

Goel G, Puniya AK, Singh K (2007) Phenotypic characterization of tannin–protein complex degrading bacteria from faeces of goat. Small Ruminant Res 69(1–3):217–220

Goel G, Puniya AK, Aguilar CN, Singh K (2005) Interaction of gut microflora with tannins in feeds. Naturwissenschaften 92(11):497–503

Gonçalves HB, Riul AJ, Quiapim AC, Jorge JA, Guimarães LHS (2012) Characterization of a thermostable extracellular tannase produced under submerged fermentation by *Aspergillus ochraceus*. Electron J Biotechnol 15(5):4-4

Gonçalves HB, Riul AJ, Terenzi HF, Jorge JA, Guimarães LHS (2011) Extracellular tannase from *Emericella nidulans* showing hypertolerance to temperature and organic solvents. J Mol Catal B Enzym 71(1–2):29–35

Goodsall CW, Jones TG, Mitei JK, Parry AD, Safford R, Thiru A (2003) Cold water infusing leaf tea. EP1150575B1

Goodsall CW, Jones TG, Mitei JK, Parry AD, Safford R, Thiru A (2001) Cold water infusing leaf tea. EP1150575A1

Griffiths DW (1991) Condensed tannins. In D'Mello FJP, Duffus CM, Duffus JH (eds) Toxic substances in crop plants. The Royal Society of Chemistry, Thomas Graham House, Science Park, Cambridge CB4 4WF, Cambridge, pp 180–201

Hadi TA, Banerjee R, Bhattacharyya BC (1994) Optimization of tannase biosynthesis by a newly isolated *Rhizopus oryzae*. Bioprocess Eng 11(6):239–243

Hagerman AE (2002) Tannin handbook, department of chemistry and biochemistry. Miami University, USA

Hara T, Nakayama M, Narishima N (2001) Fruit or vegetable juice-containing protein beverage. JP2001340069A

Haslam E, Stangroom JE (1966) The esterase and depsidase activities of tannase. Biochem J 99 (1):28

Hatamoto O, Watarai T, Kikuchi M, Mizusawa K, Sekine H (1996) Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. Gene 175(1):215–221

Hennink H, Schmedding DJM, Smith CJ, Van GMJMC (2004) Process for the production of beer having improved flavour stability. EP1252285B1

Hennink H, Schmedding DJM, Smith CJ, Van GMJMC (2001) Process for the production of beer having improved flavour stability. EP1122303A1

Hota SK, Dutta JR, Banerjee R (2007) Immobilization of tannase from *Rhizopus oryzae* and its efficiency to produce gallic acid from tannin rich agro-residues. Indian J Biotechnol 6(2):200–204

Huybrechts L (2001) Tooth whitening products and procedures. WO2001064175A1

Iibuchi S, Minoda Y, Yamada K (1972) Hydrolyzing pathway, substrate specificity and inhibition of tannin acyl hydrolase of Asp. oryzae No. 7. Agric Biol Chem 36(9):1553–1562

Iwamoto K, Tsuruta H, Nishitaini Y, Osawa R (2008) Identification and cloning of a gene encoding tannase (tannin acylhydrolase) from *Lactobacillus plantarum* ATCC 14917T. Syst Appl Microbiol 31(4):269–277

Jana A, Halder SK, Banerjee A, Paul T, Pati BR, Mondal KC, Mohapatra PKD (2014) Biosynthesis, structural architecture and biotechnological potential of bacterial tannase: a molecular advancement. Biores Technol 157:327–340

Jana A, Maity C, Halder SK, Das A, Pati BR, Mondal KC, Mohapatra PKD (2013) Structural characterization of thermostable, solvent tolerant, cytosafe tannase from *Bacillus subtilis* PAB2. Biochem Eng J 77:161–170

Jiménez N, Barcenilla JM, de Felipe FL, de las Rivas B, Muñoz R (2014) Characterization of a bacterial tannase from *Streptococcus gallolyticus* UCN34 suitable for tannin biodegradation. Appl Microbiol Biotechnol 98(14):6329–6337

Kasieczka-Burnecka M, Kuc K, Kalinowska H, Knap M, Turkiewicz M (2007) Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain *Verticillium* sp. P9. Appl Microbiol Biotechnol 77(1):77–89

Khanbabaee K, van Ree T (2001) Tannins: classification and definition. Natural Prod Rep 18 (6):641–649

Koseki T, Ichikawa K, Sasaki K, Shiono Y (2018) Characterization of a novel *Aspergillus oryzae* tannase expressed in *Pichia pastoris*. J Biosci Bioeng (in press)

Kumar R, Singh M (1984) Tannins: their adverse role in ruminant nutrition. J Agric Food Chem 32 (3):447–453

Kumar RA, Gunasekaran P, Lakshmanan M (1999) Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. J Basic Microbiol Int J Biochem Physiol Genet Morphol Ecol Microorganisms 39(3):161–168

Lane RW, Yamakoshi J, Kikuchi M, Mizusawa K, Henderson L, Smith M (1997) Safety evaluation of tannase enzyme preparation derived from *Aspergillus oryzae*. Food Chem Toxicol 35(2):207–212

Lehmberg GL, Balentine DA, Hang RS, Gobbo SA (1999) Enzyme extraction process for tea. US5919500A

Lehmberg GL, Ma SX (2000) Tea concentrate prepared by enzymatic extraction and containing xanthan gum which is stable at ambient temperature. US6024991A

Lekha PK, Lonsane BK (1997) Production and application of tannin acyl hydrolase: state of the art. Adv Appl Microbiol 44:216–260

Lewis JA, Starkey RL (1969) Decomposition of plant tannins by some soil microorganisms. Soil Sci 107(4):235–241

Li J, Xiao Q, Huang Y, Ni H, Wu C, Xiao A (2017) Tannase application in secondary enzymatic processing of inferior Tieguanyin oolong tea. Electron J Biotechnol 28:87–94

Lima JSD, Cruz R, Fonseca JC, Medeiros EVD, Maciel MDHC, Moreira KA, Motta CMDS (2014) Production, characterization of tannase from *Penicillium montanense* URM 6286 under SSF using agroindustrial wastes, and application in the clarification of grape juice (*Vitis vinifera* L.). Sci World J 2014

Liu F, Wang B, Ye Y, Pan L (2018) High level expression and characterization of tannase tan7 using *Aspergillus niger* SH-2 with low-background endogenous secretory proteins as the host. Protein Expr Purif 144:71–75

López MJA, Streitenberger SA, Peñalver MM, Martínez OP (2008) Process and apparatus for preparing pomegranate extracts. EP1967079A2

Lu MJ, Chen C (2007) Enzymatic tannase treatment of green tea increases in vitro inhibitory activity against N-nitrosation of dimethylamine. Process Biochem 42(9):1285–1290

Macedo JA, Battestin V, Ribeiro ML, Macedo GA (2011) Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. Food Chem 126(2):491–497

Madhavakrishna W, Bose S, Nayudamma Y (1960) Estimation of tannase and certain oxidizing enzymes in Indian vegetable tanstuffs. Bull Cent Leath Res Inst 7(1):11

Mahapatra K, Nanda RK, Bag SS, Banerjee R, Pandey A, Szakacs G (2005) Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori nakazawa*. Process Biochem 40(10):3251–3254
Mahapatra S, Banerjee D (2009) Extracellular tannase production by endophytic *Hyalopus* sp. J Gen Appl Microbiol 55(3):255–259
Mahendran B, Raman N, Kim DJ (2006) Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Appl Microbiol Biotechnol 70(4):444–450
Mahmoud AE, Fathy SA, Rashad MM, Ezz MK, Mohammed AT (2018) Purification and characterization of a novel tannase produced by *Kluyveromyces marxianus* using olive pomace as solid support, and its promising role in gallic acid production. Int J Biol Macromol 107:2342–2350
Marco MG, Rodríguez LV, Ramos EL, Renovato J, Cruz-Hernández MA, Rodríguez R, Contreras J, Aguilar CN (2009) A novel tannase from the xerophilic fungus *Aspergillus niger* GH1. J Microbiol Biotechnol 1:1–10
Maruyama E, Ueoka H, Shibata K (2010) Method for producing purified tea extract. US20100184167A1
Masschelein CA, Batum MS (1981) Enzymic degradation and participation of ester linked beer polyphenols in chill haze formation. In: Proceedings of the 18th congress of the European brewing convention, Copenhagen, 1981
Matoba Y, Tanaka N, Noda M, Higashikawa F, Kumagai T, Sugiyama M (2013) Crystallographic and mutational analyses of tannase from *Lactobacillus plantarum*. Proteins Struct Funct Bioinf 81(11):2052–2058
Matsuyama A., Nakamori K., Watabe N., 2000. Black tea drink containing fruit juice JP2000037164A
Molin G, Ahrné S, Jeppsson B (2009) Compositions comprising lactobacillus plantarum strains in combination with tannin and new *lactobacillus plantarum* strains. US7507572B2
Mondal KC, Pati BR (2000) Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. J Basic Microb Int J Biochem Physiol Genet Morphol Ecol Microorganisms 40(4):223–232
Mondal KC, Banerjee D, Banerjee R, Pati BR (2001) Production and characterization of tannase from *Bacillus cereus* KBR9. J Gen Appl Microbiol 47(5):263–267
Mondal KC, Banerjee R, Pati BR (2000) Tannase production by *Bacillus licheniformis*. Biotech Lett 22(9):767–769
Mueller-Harvey I, Hartley RD, Reed JD (1987) Characterisation of phenolic compounds, including flavonoids and tannins, of ten Ethiopian browse species by high performance liquid chromatography. J Sci Food Agric 39(1):1–14
Mullick A, Putrevu SM (2011) Process for the preparation of theaflavin-enhanced tea products. EP2096937B1
Nadaf NH, Ghosh JS (2011) Production, purification and characterization of tannase from *Rhodococcus* NCIM, 2891. Curr Res J Biol Sci 3(3):246–253
Nandi S, Chatterjee A (2016) Extraction, partial purification and application of tannase from *Aspergillus niger* MTCC 2425. Int J Food Sci Nutr 1(3):20–23
Nelson KE, Pell AN, Schofield P, Zinder S (1995) Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. Appl Environ Microbiol 61 (9):3293–3298
Ni H, Chen F, Jiang ZD, Cai MY, Yang YF, Xiao AF, Cai HN (2015) Biotransformation of tea catechins using *Aspergillus niger* tannase prepared by solid state fermentation on tea byproduct. LWT-Food Sci Technol 60(2):1206–1213
Nicolas P, Raetz E, Reymond S, Sauvageat JL (1997) Producing a tea extract. EP0777972A1
Nishitani Y, Osawa R (2003) A novel colorimetric method to quantify tannase activity of viable bacteria. J Microbiol Methods 54(2):281–284

Nishitani Y, Sasaki E, Fujisawa T, Osawa R (2004) Genotypic analyses of lactobacilli with a range of tannase activities isolated from human feces and fermented foods. Syst Appl Microbiol 27 (1):109–117

Noguchi N, Ohashi T, Shiratori T, Narui K, Hagiwara T, Ko M, Watanabe K, Miyahara T, Taira S, Moriyasu F, Sasatsu M (2007) Association of tannase-producing *Staphylococcus lugdunensis* with colon cancer and characterization of a novel tannase gene. J Gastroenterol 42(5):346–351

Okuda T, Ito H (2011) Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphenols related to tannins. Molecules 16(3):2191–2217

Ordoñez RM, Colombo I, Alberto MR, Isla MI (2011) Production of tannase from wood-degrading fungus using as substrate plant residues: purification and characterization. World J Microbiol Biotechnol 27(10):2325–2333

Pepi M, Lampariello LR, Altieri R, Esposito A, Perra G, Renzi M, Lobianco A, Feola A, Gasperini S, Focardi SE (2010) Tannic acid degradation by bacterial strains *Serratia* spp. and *Pantoea* sp. isolated from olive mill waste mixtures. Int Biodeterior Biodegradation 64(1):73–80

Pourrat H, Regerat F, Morvan P, Pourrat A (1987) Microbiological production of gallic acid from *Rhus coriaria* L. Biotech Lett 9(10):731–734

Pourrat H, Regerat F, Pourrat A, Jean D (1985) Production of galilc acid from tara by a strain of *Aspergillus niger*. J Ferment Technol 63(4):401–403

Purohit JS, Dutta JR, Nanda RK, Banerjee R (2006) Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae*. Biores Technol 97(6):795–801

Qiu Y, Niu H, Huang W, He Y, Wu XH (2011) Properties and secondary structure of tannase from *Penicillium herquei*. Biotechnol Bioprocess Eng 16(5):858

Quan D, Xiong WW (2007) Green tea formulations and methods of preparation. US7232585B2

Raghuwanshi S, Dutt K, Gupta P, Misra S, Saxena RK (2011) *Bacillus sphaericus*: the highest bacterial tannase producer with potential for gallic acid synthesis. J Biosci Bioeng 111(6):635–640

Ren B, Wu M, Wang Q, Peng X, Wen H, McKinstry WJ, Chen Q (2013) Crystal structure of tannase from *Lactobacillus plantarum*. J Mol Biol 425(15):2737–2751

Riul AJ, Gonçalves HB, Jorge JA, Guimarães LHS (2013) Characterization of a glucose-and solvent-tolerant extracellular tannase from *Aspergillus phoenicis*. J Mol Catal B Enzym 85:126–133

Rodríguez H, de las Rivas B, Gómez-Cordovés C, Muñoz R (2008) Characterization of tannase activity in cell-free extracts of *Lactobacillus plantarum* CECT 748T. Int J Food Microbiol 121 (1):92–98

Rodríguez-Durán LV, Valdivia-Urdiales B, Contreras-Esquivel JC, Rodríguez-Herrera R, Aguilar CN (2011) Novel strategies for upstream and downstream processing of tannin acyl hydrolase. Enzyme Res

Rout S, Banerjee R (2006) Production of tannase under MSSF and its application in fruit juice debittering. Indian J Biotechnol 5(3):351–356

Sabu A, Augur C, Swati C, Pandey A (2006) Tannase production by *Lactobacillus* sp. ASR-S1 under solid-state fermentation. Process Biochem 41(3):575–580

Sabu A, Pandey A, Daud MJ, Szakacs G (2005) Tamarind seed powder and palm kernel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. Bioresour Technol 96(11):1223–1228

Sanderson G, Coggon P (1974) Green tea conversion using tannase and natural tea enzymes. US3812266A

Sanderson GW, Hoefler AC, Graham HN, Coggon P (1977) Cold water extractable tea leaf and process. US4051264A

Sasatsu M, Noguchi M, Shiratori T (2006) Diagnostic agent and test method for colon cancer using tannase as index. US7090997B2

Saxena S, Saxena RK (2004) Statistical optimization of tannase production from *Penicillium variable* using fruits (chebulic myrobalan) of *Terminalia chebula*. Biotechnol Appl Biochem 39(1):99–106

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30(12):3875–3883
Scharbert S, Hofmann T (2005) Molecular definition of black tea taste by means of quantitative studies, taste reconstitution, and omission experiments. J Agric Food Chem 53(13):5377–5384
Schmedding D, Hennink H, Gestel MV, Smith C (2003) Process for the production of beer having improved flavour stability. US20030157217A1
Selwal MK, Yadav A, Selwal KK, Aggarwal NK, Gupta R, Gautam SK (2010) Optimization of cultural conditions for tannase production by *Pseudomonas aeruginosa* IIIB 8914 under submerged fermentation. World J Microbiol Biotechnol 26(4):599–605
Sharma KP, John PJ (2011) Purification and characterization of tannase and tannase gene from *Enterobacter* sp. Process Biochem 46(1):240–244
Sharma S, Agarwal L, Saxena RK (2008) Purification, immobilization and characterization of tannase from *Penicillium variable*. Bioresour Technol 99(7):2544–2551
Sharma S, Bhat TK, Dawra RK (1999) Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. World J Microbiol Biotechnol 15(6):673–677
Sharma S, Bhat TK, Dawra RK (2000) A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem 279(1):85–89
Shikata K, Sato H, Shibata K (2010) Process for producing purified tea extract. EP2225952A1
Shikata K, Sato H, Shibata K (2014) Process for producing purified tea extract. EP2225952B1
Shrikhande AJ, Wang H, Kupina SA (2010) Grape extract, dietary supplement thereof, and processes therefor. US7767235B2
Sittig M (1988) Trimethoprim. In: Sittig M (ed) Pharmaceutical manufacturing encylopedia. Noyes Publication, New Jersey, pp 282–284
Skene IK, Brooker JD (1995) Characterization of tannin acylhydrolase activity in the ruminal bacterium *Selenomonas ruminantium*. Anaerobe 1(6):321–327
Srivastava A, Kar R (2009) Characterization and application of tannase produced by *Aspergillus niger* ITCC 6514.07 on pomegranate rind. Brazil J Microbiol 40(4):782–789
Swain T (1977) Secondary compounds as protective agents. Ann Rev Plant Physiol 28(1):479–501
Takino Y (1976) Enzymatic solubilization of tea cream. US3959497A
Tieghem P (1867) Sur la fermentation gallique. CR Acad Sci (Paris) 65:1091–1094
Tsai CH (1988) Enzymatic treatment of black tea leaf. EP0135222B1
Vaquero I, Marcobal Á, Muñoz R (2004) Tannase activity by lactic acid bacteria isolated from grape must and wine. Int J Food Microbiol 96(2):199–204
Weetall HH (1989) Enzymatic synthesis of gallic acid esters. EP0137601B1
Wu M, Wang Q, McKinstry WJ, Ren B (2015) Characterization of a tannin acyl hydrolase from *Streptomyces sviceus* with substrate preference for digalloyl ester bonds. Appl Microbiol Biotechnol 99(6):2663–2672
Xu F (2009) Methods of reducing the inhibitory effect of a tannin of the enzymatic hydrolysis of cellulosic material. WO2009059175A2
Yadav A, Aggarwal NK, Kumar K, Kumar A (2008) Tannase production by *Aspergillus fumigatus* MA under solid-state fermentation. World J Microbiol Biotechnol 24(12):3023–3030
Yamada H, Adachi O, Watanabe M, Sato N (1968) Studies on fungal tannase: part I. Formation, purification and catalytic properties of tannase of *Aspergillus flavus*. Agric Biol Chem 32 (9):1070–1078
Yotsumoto Y (2009) Method of enzymatically treating green tea leaves. EP2036440A1
Zeida M, Wieser M, Yoshida T, Sugio T, Nagasawa T (1998) Purification and characterization of gallic acid decarboxylase from *Pantoea agglomerans* T71. Appl Environ Microbiol 64 (12):4743–4747
Zhang YN, Yin JF, Chen JX, Wang F, Du QZ, Jiang YW, Xu YQ (2016) Improving the sweet aftertaste of green tea infusion with tannase. Food Chem 192:470–476
Zhong X, Peng L, Zheng S, Sun Z, Ren Y, Dong M, Xu A (2004) Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. Protein Expr Purif 36(2):165–169

Chapter 20
Enzymes in the Design of Functional Foods or Their Constituents

Sarita G. Bhat and Venetia D'Rose

Abstract Food plays a major role in maintaining the health and wellbeing of human being. The food we have has a direct impact on the human gut microbiome. Stress also influences the gut health and is responsible for inflammatory reactions in the gut. Any dysbiosis in the gastrointestinal microbiota leads to several gut related diseases like diarrhoea, irritable bowel syndrome, and even colorectal cancer. Functional foods have the ability to rejuvenate and enrich the beneficial gut microorganisms, thereby alleviating the symptoms of various gut related diseases, diminishes the risk of cardiovascular diseases and improves health and wellbeing. Microbial enzyme processes use fermentation technology to engineer or enhance production of certain naturally occurring dietary substances to boost physiological benefits. These are functional foods and functional food ingredients. Functional foods enriched with probiotic, prebiotics, synbiotics and cobiotics as well as other plant and animal related food components have the ability to improve the consumer's health and well-being. Enzyme catalysed degradation of phytates to enhance bioavailability of iron in cereal based foods is an example. Certain lactic acid bacteria produce glucose, galactose and oligosaccharides (prebiotics) due to transgalactosidal and lactose hydrolysis activities of beta-galactosidase. Other examples include non-starch polysaccharides (NSP) from cereals such as β-glucan and arabinoxylan as dietary fibre constituents or for producing prebiotic compounds

Keywords Functional food · Probiotic · Prebiotic

S. G. Bhat (✉) · V. D'Rose
Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala 682022, India
e-mail: saritagbhat@gmail.com; sgbhat@cusat.ac.in

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_20

20.1 Introduction

Food, air and water are the three important requirements for survival of life forms. As humans, the value of the food that we consume depends on the balance of macro and micronutrients to ensure normal growth, metabolism and wellbeing. The quality of food, eating habits, production and consumption of food has health, societal and environmental influences. The gut is vital in digestion and absorption of food, as well as providing barricades to toxic and harmful molecules. Todays' fast paced life styles affect both physical and mental health, by impacting the gut heath due to increased inflammatory responses and causing irritable bowel disorders, colitis, colorectal cancers, cardiovascular diseases, etc.; while foods have a significant role in maintaining a healthy gut thereby alleviating the symptoms of stress and reducing inflammatory responses.

20.1.1 Human Gut Microbiomes

Large-scale sequencing based projects like the European Metagenomics of the human intestinal tract (MetaHIT) (Arumugam et al. 2011; Qin et al. 2010) and the US human Microbiome Project (HMP) (HMP Consortium 2012a, b) have given an understanding of the healthy gut microbiota and their altered state in disease conditions. The human gastro intestinal tract with its diversiform and heterogenous microbial community plays a pivotal role in human health. Bacteria exceeding more than thousand species and with multitude of genes than that in the human genome (Ley et al. 2006a; Qin et al. 2010), this community is related to a masked metabolic 'organ' due to their enormous influence on sustenance of humans, including physiology, dietetics and immune function. Our gut microbiome is suggested to coevolve with us (Ley et al. 2008) and changes therein have both beneficial and harmful consequences for human health.

Disruption of gut microbiota which results in dysbiosis is very significant to various pathological intestinal conditions which include obesity (Ley et al. 2006b; Zhang et al. 2009) malnutrition (Kau et al. 2011), Type 2 diabetes (Qin et al. 2012), chronic inflammatory diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), including ulcerative colitis (UC) Crohn's disease (CD) (Frank et al. 2007) and onset of colorectal cancer (Arthur et al. 2012; Castellarin et al. 2012; Kostic et al. 2012; McCoy et al. 2013).

Plant based foods like fruits, vegetables and whole grains are related to reduced risk of chronic diseases such as life style diseases like cardiovascular diseases, irritable bowel disease and cancer; but high consumption of cereal fiber did not necessarily lower the risk of colorectal cancer (Terry et al. 2001). Risk factors of CVD like obesity, hypercholesterolemia, hypertension and type 2 diabetes are the important, which are increased due to poor diets, smoking and alcohol consumption (Wang et al. 2007). To reduce hepatic cholesterol synthesis and to increase

excretion of cholesterol and bile acids, during defaecation diet modification along with life style changes are some options available for treatment of hypercholesterolemia.

Our gut microbiota play very crucial roles in human fitness and ailment. It is important to understand more precisely the connection between the gut microbiota and various intestinal maladies, especially the role of microbial populations in GI diseases and disorders. This will go a long way to develop appropriate therapeutic requirements. Therefore the rationale that modulating the gut biota could help alleviate or treat chronic diseases. Since microbial communities are established based on the microenvironments in their specific niches, the idea that gut health can be improved by modifying the gut community by the use of functional foods incorporated with probiotics, prebiotics, synbiotics and cobiotics and nutraceuticals, as well as faecal transplantation is currently a topic of considerable interest. Thus changed diets, functional foods with probiotics, prebiotics can be used modulate gut microbiomes, eliminate pathogens and therefore ensure host health (Guinane and Cotter 2013).

20.2 Functional Foods

Functional foods are those that have health promoting properties, beyond their normal nutritional value; foods that are modified, enriched, enhanced or fortified to provide more health benefits than normal diet (Hasler 2002). There are no one accepted definition for functional foods. Functional foods are recognized today as Foods for Specified Health Use (FOSHU). It was first initiated in Japan in the 1980s when confronted with heightened health care costs. Nearly 300 food products had been granted till July 2002. The National Academy of Sciences' Food and Nutrition Board defined functional foods as “any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. Committee on Opportunities in the Nutrition and Food Sciences, Food and Nutrition Board, Institute of Medicine (1994)”.

Functional foods may be classified into different types based on the presence of bioactive ingredients and source of origin. Naturally occurring foods, are conventional foods with certain frequently existing bioactive components. Processed foods are suitably modified or altered by adding, removing a bioactive ingredient in it or by changing their bioavailability (Henry 2010).

Functional foods can also be categorized into animal based foods and plant based foods based on their source of origin. Certain class of functional foods are much more preferred among consumers. The consumption of functional foods is influenced by various factors, their use in the past, rising cost of healthcare and public awareness about health and wellness. Functional food category comprises of various products in dairy, energy beverages, bakery, confectionery, and the baby food industry (Jaiswal and Sharma).

20.2.1 Animal Based Functional Components

Several animal derived foods contain specific nutrients and (or) non-nutrients which benefit human health, beyond what is traditionally known as nutritional effects. The current nutritional approach is to obtain PUFA(n-3)-enriched and CLA-enriched milk, meat and eggs to enhance their functionality. As in fatty fish salmon the two primary (n-3) fatty acids such as eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) are also found in fishes like tuna, mackerel, sardines and herring (Gibson et al. 2004). DHA is part of the phospholipids of all cellular membranes and is necessary for the brain and retinal development in infants (FAO 2007). Research have been done on the physiologic effects of (n-3) fatty acids in chronic conditions such as cancer, rheumatoid arthritis, psoriasis, Crohn's disease, cognitive dysfunction and cardiovascular disease (Cencic and Chingwaru 2010), and their role in reducing the risks of cardiovascular disease is well studied. PUFA protect against damaging effects of the metabolic syndrome and lower the risk of CVD (Clarke et al. 2001). This is by acting as energy partitioners, i.e. directing fatty acids away from triacylglycerol storage toward their oxidation; also enhancing glucose conversion to glycogen.

Another class of biologically active animal-derived components are probiotics. They are living microorganisms with health benefits (Hosono 1992).These include the lactic acid bacteria (LABs), various strains of *Lactobacillus acidophilus*, strains of *Lactobacillus johnsonii* La1, *Lactobacillus reuteri, L.GG*, and *Lactobacillus casei Shirota,* many of which are incorporated into functional foods. The prophylactic influence of these functional ingredients have been studied in cancer, intestinal tract function, immunity, stomach health, cholesterol reduction and high blood pressure (Lee 2009).

Other functional ingredients are prebiotics. Prebiotics are nondigestible food ingredients that benefit the host by selectively stimulating the growth and/or activity of certain beneficial bacteria in the colon, thereby improving health. Prebiotics include short-chain carbohydrates such as fructooligosaccharides (FOS), mannanoligosacharrides (MOS), galactooligosaccharides (GOS), and inulin. Inulin is a longer chain version of FOS which serve as substrate in the colon for the endogenous colonic bacteria. "Synbiotics," on the other hand are mixtures of probiotics and prebiotics that augment the host health.

Conjugated linoleic acid (CLA) is another functional component with potent antimutagenic agent (Sanders et al. 2014), which are abundant in dairy products and foods derived from ruminant animals (EFSA 2005). CLA have hypocholesterolemic and (or) antiatherogenic properties. Previous studies in rabbits fed with atherogenic diet, CLA decreased blood total cholesterol, LDL-cholesterol and cholesterol deposition in the aorta wall (Lee et al. 1994). Studies have shown that mammary carcinogenesis is inhibited in animals by CLA (Anadón et al. 2006) which also has the ability to increase bone density in animal models (EFSA 2013).

20.2.2 Plant Based Functional Components

The only few plant based food components which bear FDA-approved health claims include oat soluble (β-glucan) fiber (EFSA 2017), soluble fiber from psyllium seed husk (Schachtsiek et al. 2004), soy protein (Oelschlaeger 2010) and sterol- and stanol-ester–fortified margarine (Cremonini et al. 2002); but there are many others such as cranberries, garlic, nuts, grapes and chocolate with growing clinical evidence supporting their potential health benefits. Others include tea (catechins), lycopene from tomatoes, and the carotenoids lutein and zeaxanthin from green leafy vegetables. In vitro and in vivo (animal) studies support the preventive effect of flaxseed lignans against cancer (Upadrasta and Madempudi 2016), citrus fruit limonoids (Khalesi et al. 2014) and other cruciferous vegetable phytochemicals such as isothiocyanates and indoles (Ruan et al. 2015). Cruciferous vegetables like broccoli, brussel sprouts and cabbage contain high levels of sulforaphane. Sulforaphane is a compound within the isothiocyanate group of organosulfur compounds which has been shown to be a potent inducer of Phase II detoxifying enzymes in the liver, which in turn accelerate the inactivation of toxic substances and therefore their elimination from the body (Lima-Filho et al. 2000). Table 20.1 gives the plant based functional components and their action.

Table 20.1 Plant based functional components and their action

Plant source	Active component	Action	References
Cranberries	Tannins (proanthocyanidins)	Prevention of *E. coli* from adherence to the epithelial cells lining the urinary tract	Schoster et al. (2013)
		Antiadhesion properties have benefits in oral cavity	JimmySaint-Cyr et al. (2017)
Garlic (*Allium sativum*)	Active organosulfur components (e.g., allicin, allylic sulfides)	Modest blood pressure—lowering effect in clinical studies	Carter et al. (2017), Chingwaru and Vidmar (2017)
		Inhibit the activity of *Helicobacter pylori*	
		Reduce blood cholesterol	De Montijo-Prieto et al. (2015), Thomas and Greer (2010)
Almonds		Reduces total cholesterol and LDL cholesterol	Kumar et al. (2013), Nase et al. (2001)
Walnuts		Decreases in total and LDL cholesterol, lower the risk of CHD	Li and Gu (2016), Gu et al. (2015)
Red wine	Polyphenolics	Antioxidant, reduce the risk of heart disease	Pompei et al. (2007)

(continued)

Table 20.1 (continued)

Plant source	Active component	Action	References
Grape juice	Phenolic antioxidant compounds	Reduce platelet aggregation	Nova et al. (2007)
Chocolate	Polyphenolic, flavonoids (procyanidins)	Potential benefits to heart health, reduce oxidative stress on LDL cholesterol	Mishra and Lambert (1996), Ouwehand et al. (1999)
Tomatoes	Lycopenes	Antioxidant	Isolauri et al. (2001)
		Reduced risk of prostate, lung and stomach cancers	Collado et al. (2007), Begley et al. (2006), Weinberg (1997)
Spinach, cooked cabbage	Lutein	Reduced risk of age-related macular degeneration (AMD) and cataract	Marteau and Shanahan (2003), Schatzmayr et al. (2006)

20.2.3 Probiotics

According to FDA/WHO probiotics is defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host". To substantiate the health claims and the benefits they have jointly set the guidelines to effectively evaluate probiotics in foods. Mechanism of action and properties of probiotics is depicted in Table 20.2. *Lactobacillus rhamnosus*, *L. reuteri*, bifidobacteria, strains of *L. casei*, *L. acidophilus*-group, *Bacillus coagulans, Escherichia coli* strain Nissle 1917, *Enterococcus faecium* SF68, yeast *Saccharomyces boulardii* are some of the probiotic strains used (Pandey et al. 2015). Among probiotics the genus Bacillus which are bacterial spore formers are also included. These probiotics are added either individually or as combinations to foods to make functional food products, mostly fermented foods.

An ideal probiotic organism should be GRAS (generally regarded as safe), preferably be a LAB, be genetically stable, have antigenotoxic property, be acid and bile tolerant, should have the ability to adhere to gut lining, have a short generation time, should be robust and capable of surviving under processing conditions (Pandey et al. 2015). The FAO/WHO guidelines on probiotics insist on the following activities to evaluate probiotics in food to substantiate their health claims. These include "identification of the strain, functional characterization of the strain (s) for safety and probiotic attributes, validation of health benefits in human studies and labelling of efficacy claims and content for the entire shelf life." Table 20.3 lists the various applications of probiotic strains.

Table 20.2 Mechanism of action and properties of probiotics (Sánchez et al . 2017; He et al. 2002; Hess et al. 2004; Neish et al. 2000)

Mechanism of action	Biological effects
Antimicrobial activity	Decrease luminal pH Secretion of antimicrobial peptides Nutrient competition Block adhesion
Increase in barrier function	Increase in mucus production Increase in IgA and defensin production Increase in mucin production
Immunomodulation	Cytokine production T helper and Treg response Effect on epithelial cells • By acting on TLR • Production of cytokines • Activation of antiapoptotic Akt/protein kinase B Effect on dendritic cells • Production of IL-10 Effect on Macrophage • Increased production of IL-10 • Production of IFNγ
Production of antimicrobial substances	Bacteriocin (inhibition of pathogen replication) Deconjugated bile acids Anti-adhesive effects (increased mucin production) Anti-invasive effects
Neurotransmitter production	GABA Tryptophan Serotonin Catecholamine Acetylcholine

Table 20.3 Applications of probiotic strains

Probiotic	Area of application	Function	References
L. plantarum	Rat	Decreased total cholesterol and LDL-cholesterol	Kumar et al. (2011)
	Human	Prevention of endotoxin production	Lee et al. (2013)
	Human	Reduction of irritable bowel syndrome symptoms	Ortiz et al. (2014)
	In vitro	Cholesterol assimilation	Kumar et al. (2011), Tomaro-Duchesneau et al. (2014a, b)
	Rat	Decreased LDL, VLDL, and increased HDL with decrease in deposition of cholesterol and triglyceridein liver and aorta	Mohania et al. (2013)

(continued)

Table 20.3 (continued)

Probiotic	Area of application	Function	References
L. acidophilus	Human	Treating of travellers' diarrhoea	McFarland (2007)
		Reducing stay of children with acute diarrhea in hospital	Phavichitr et al. (2013)
		Treatment of bacterial vaginosis	Homayouni et al. (2014)
		Treatment of *C. difficile*-associated diarrhea	Cortés-Zavaleta et al. (2014)
		Decrease in incidence of febrile urinary tract infections in children	Mohseni et al. (2013)
		Decrease in irritable bowel syndrome symptoms	Ortiz et al. (2014)
L. casei		Treatment of functional constipation in adults	Chmielewska and Szajewska (2010)
		Treatment of *C. difficile*-associated diarrhea	Wu (2013)
		Reduction of irritable bowel syndrome symptoms	Ortiz et al. (2014)
L. fermentum		Prevention and treatment of bacterial vaginosis	Homayouni et al. (2014)
		Antistaphylococcal action (*L. fermentum* ATCC11739)	Chen et al. (2013)
		Potential for reduction of insulin resistance and hypercholesterolemia (*L. fermentum* NCIMB5221)	Tomaro-Duchesneau et al. (2014a, b)
L. rhamnosus		Reduction of viral-associated pulmonary damage	Zelaya et al. (2014)
		Reduction of risk for developing allergic disease	Licciardi et al. (2013)
		Weight loss of obese women	Sanchez et al. (2013, 2014, Sánchez et al. 2017)
		Protection of human colonic muscle from Lipopolysaccharide-induced damage	Ammoscato et al. (2013)
		Sepsis in very low birth weight infants	Leroy et al. (2008)
		Pediatric antibiotic associated diarrhea	
L. acidophilus *L. bulgaricus* *L. casei*	In vitro	Assimilation of cholesterol Attachment of cholesterol to cell surface Disrupt the formation of cholesterol micelle Deconjugation of bile salt Exhibited bile salthydrolase activity	Lye et al. (2010)

(continued)

Table 20.3 (continued)

Probiotic	Area of application	Function	References
Lactobacillus lactis	Human	Crohn's disease	Gotteland et al. (2006)
Lactobacillus reuteri	Human	Shortened acute diarrhoea	Marteau et al. (2001)
Enterococcus faecium	Human	Decreased duration of acute diarrhoea from gastroenteritis	
Bifidobacterium. infantis	Human	Decrease in irritable bowel syndrome symptoms	Wu (2013)
B. bifidum	Human	Reduction of total cholesterol	Bordoni et al. (2013)
B. longum	Human	Prevention and treatment of necrotizing enterocolitis in newborns	Di Gioia et al. (2014)
		Reduction of irritable bowel syndrome symptoms Treatment of gastrointestinal diseases	Wu et al. (2013), Yu et al. (2013)
B. coagulans	Human	Treatment of diarrhoea associated with antibiotic	Hempel et al. (2012)
		Bacterial vaginosis	RatnaSudha et al. (2012)
B. subtilis	Human	*H. pylori* eradication	Tompkins et al. (2010)
		Production of nitric oxide	Clark and Hodgkin (2014)
Saccharomyces cerevisiae Boulardii	Human	Reduces the risk and duration of antibiotic-associated diarrhea Affects *Clostridium difficile* or antibiotic-associated diarrhea by altering the gut Microbiota in a healthy mode	Fric (2007), Tuohy et al. (2003)

20.2.4 Prebiotics

According to FAO/WHO prebiotics are defined as a non-living food component which gives health to the host because of their ability to modulate the gut microbial diversity. Although, they are part of the dietary fibers, i.e. the non digestable part of the food, there is not much information regarding these diverse carbohydrate components with regard to their sources, their fermentation patterns, their modulatory effects as well as their dosage for augmenting health. However according to Pokusaeva and coworkers, not many fulfil the criteria for prebiotics except perhaps inulin, fructooligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Pokusaeva et al. 2011). The properties of an ideal prebiotic are depicted in Fig. 20.1

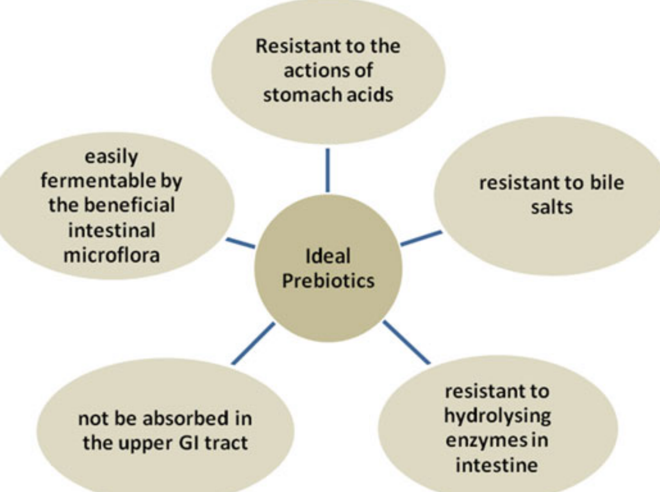


Fig. 20.1 Properties of an ideal prebiotic. Adapted from Kuo (2013)

Sources of prebiotics include human milk, soybeans, inulin sources, bananas oats, unrefined wheat and barley, non-digestible carbohydrates, animal colostrum and in particular non-digestible oligosaccharides. Prebiotics selectively enhances the growth and/or activity of some microorganisms in the gut, especially lactobacilli and bifidobacteria by the fiber components (DeVrese and Schrezenmeir 2008). The mechanism of action of prebiotics and their biological effects is given in Table 20.4.

The health benefits of prebitoics include reduction of inflammation associated with intestinal bowel disorder and protective effects to prevent colon cancer (Peña 2007); increased bioavailability and uptake of minerals, lowering of the risk of cardiovascular disease, preventing obesity (Pokusaeva et al. 2011). Table 20.5 outlines gives a list of some of the prebiotics studied and their role in improving health

Table 20.4 The mechanism of action and biological effects of prebiotics

Mechanism of action	Biological effects
Changes in intestinal microbiota	Positive effect on development of beneficial bacteria and thus on host health
Inhibition of carcinogenesis	Reduced risk of colorectal cancers and other tumors, anti-inflammatory properties against colorectal carcinoma cells
Immunomodulation	Support of the immune system, increased count of lymphocytes/ leukocytes in gut-associated lymphoid tissues (GALTs) and in peripheral blood, increased secretion of IgA, stimulate the phagocytic function of intra-inflammatory macrophages
Nutrient absorption effects	Reduced risk of obesity and metabolic syndrome
Pathogen inhibition	Protection against infections

Source Markowiak and Śliżewska (2017)

Table 20.5 Some prebiotics and their role in health

Prebiotic	Applications	Reference
MOS (Mannanoligosaccharides)	Lowers *Salmonella* infection	Fernandez et al. (2002)
	Elevated intestinal microbial community and intestinal morphology development	Baurhoo et al. (2009)
	Enhanced weight gain	Sims et al. (2004)
FOS (Fructooligosaccharides)	Increased weight gain and feed conversion increased LAB and Bifidobacteria	Xu et al. (2003)
	Improved villi height and crypt depth	Hanning (2012)
	Crohn's disease (reduced disease activity index)	Lindsay et al. (2006)
FOS, MOS	Elevated GALT immunity and increased IgG and IgM levels	Janardhana et al. (2009)
	Elevated LAB and Bifidobacteria	Kim et al. (2011)
GOS (Galactooligosaccharides)	Lactose fermenting Bifidobacterium, Faecalibacterium, and in crease in Lactobacillus	Azcarate-Peril et al. (2017)
GOS, FOS	Decrease in occurence of acute diarrhoea and upper respiratory tract infections	Bruzzese et al. (2006)
	Prebiotic formula well tolerated, normal growth trend toward a higher percentage of Bifidobacterium and a lower percentage of *E. coli* in stool, suppresses Clostridium in stool	Costalos et al. (2008)
	The concentration of secretory IgA was higher in the prebiotic group than the control; also, Bifidobacterium percentage was higher than the control and Clostridium was lower	Scholtens et al. (2008)
Inulin	Increased mucin mRNA expression of jejunum, increased cecum IgA level, increased intestinal immune function	Huang et al. (2015)
	Growth inhibition and induction of apoptosis in human colorectal carcinoma	Munjal et al. (2009a, b)
TOS (Transgalacto-oligosaccharide)	Ca absorption increased in Postmenopausal women	

The rare disaccharide kojibiose (α-1,2-glucobiose) was produced with the help of enzyme sucrose phosphorylase from the bulk sugars sucrose and glucose. The enzyme prevented the formation of other glucobioses and was therefore economical. L341I_Q345S is a double mutant which was the outcome of a semi-rational mutagenesis as well as a low- throughput screen. This mutant has a 95% selectivity for Kojibiose and gave a yield of 74% (Verhaeghe et al. 2016).

In animals probiotic organisms with cellulolytic enzymes might allow digestion of plant fibers and current diets more efficiently (Chassy 1987). The pelleting process is accelerated by heat resistant probiotic strains in the mass production of probiotic animal feeds.

Oligosaccharides from Inulin: Inulin a storage polysaccharide found in plants like chicory and dahlia is a fructose polymer (β-2,1 linked) terminated by a sucrose residue is a low digestable carbohydrate. Inulin produced from the chicory is used as a component of a chocolate in Europe (alternative for cocoa butter). Oligosaccharide DFA III (di-fructose di-anhydride III) is formed from inulin with the help of inulin fructotransferase (DFA III-producing) [EC 4.2.2.18] (Harguchi 2016). The special functionality of the oligosaccharide DFA III was studied using rats (Suzuki et al. 1998). A combination of oligosaccharide DFA III (di-fructose di-anhydride III) and calcium promotes the absorption of calcium from intestines from tight junctions and promotes the assimilation of calcium. Calcium absorption is increased in the intestine with help of organic acids formed by microorganisms in the intestine from DFA III. More than two decades ago, a beet sugar company in Hokkaido, Japan employed the inulin fructotransferase (DFA III producing) produced by *Arthrobacter* sp. H65-7 for oligosaccharide DFA III production (Yokota et al. 1991). A supplement containing DFA III and minerals (Fe, Ca, Mg, and so on) was developed in Japan by a functional food producer in 2004 and is being sold in pharmacy and convenience stores in Japan (Fig 20.2).

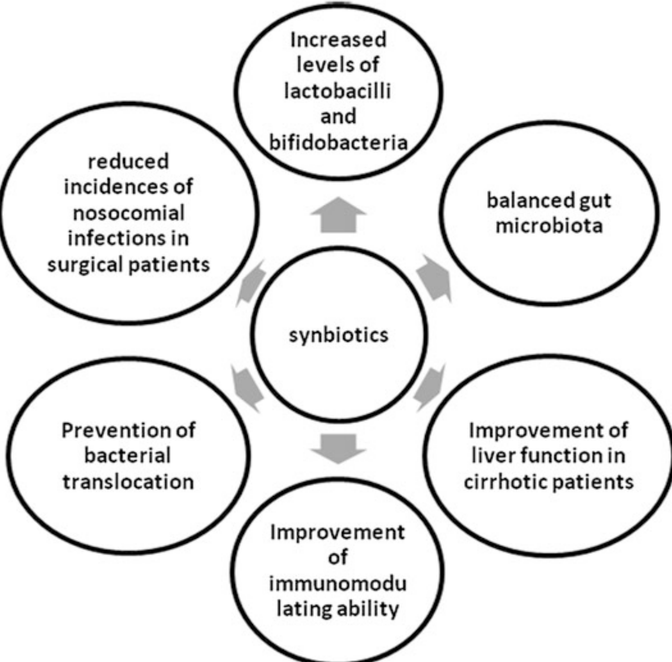


Fig. 20.2 The health benefits on synbiotics consumption by humans. Adapted from Zhang et al. (2010)

20.2.5 Synbiotics

A synbiotic is a combination of one or more prebiotic materials(s) and probiotic strain(s) to deliver health benetics to the host. In this type of product, there is synergy between the selective (probiotic) growth enhancing ability of the prebiotic, that benefits the host by enhancing the growth and survival of the microbe accelerating the growth and/or activating the metabolism of one or a few of implanted benefial microbes (Cencic and Chingwaru 2010). The survival of the probiotic bacteria are enhanced during the passage through the upper intestinal tract is greatly improved by the synbiotics. The viability of probiotics are influenced by factors like pH, H_2O_2, organic acids, oxygen, moisture and stress (Romeo et al. 2010). *Lacbobacilli, Bifidobacteria* spp., *S. boulardii*, *B. coagulans* etc. are some probiotic strains used in symbiotic formulations, while oligosaccharides like fructo oligosaccharide (FOS), GOS and xylose oligosaccharide (XOS), inulin, prebiotics from natural sources like chicory are the major prebiotics. Figure 20.2 shows the health benefits on synbiotics consumption by humans and the application of synbiotics is tabled in Table 20.6.

Table 20.6 Health benefits of some synbiotics

Synbiotic	Applications	Reference
L. rhamnosus CGMCC 1.3724, inulin	Weight loss and reduction in leptin	Sanchez et al. (2014)
L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum, L. bulgaricus, FOS	Decrease in BMI z-score and waist circumference	Safavi et al. (2013)
L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum, L. bulgaricus, FOS	The levels of fasting blood sugar and insulin resistance improved significantly	Eslamparast et al. (2014a, b)
L. sporogenes, inulin	Significant reduction in serum insulin levels, HOMA-IR (homeostasis model assessment of insulin resistance), and homeostatic model assessment cell function	Tajadadi-Ebrahimi et al. (2014)
L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum, L. bulgaricus, FOS	Inhibition of NF-κB and reduction of TNF-α in Non-alcoholic fatty liver disease	Eslamparast et al. (2014a, b)
B. lactis B94, inulin	Elimination of *Helicobacter pylori*	Ustundag et al. (2017)
L. rhamnosus GG, B. lactis Bb12, inulin	Increased *L. rhamnosus* and *B. lactis* in faeces, reduction in *C. perfringens*, prevents increased secretion of IL-2 in polypectomized patients, increased production of interferon-γ in cancer patients	Rafter et al. (2007)

20.2.6 Cobiotics

Cobiotics can be called next generation probiotics. These are combinations of one or more prebiotic, one or more probiotic microbes as well as an additional functional component(s), all of which work synergistically to provide health benefits by encouraging the growth of beneficial bacteria in the gut. Human gut dysbiosis is also associated with type 2 diabetes and insulin resistance. In the colon, fermentation of the water soluble dietary fibres like inulin and beta-glucan takes place with a increase in viscosity attributed to beta-glucan. Green way and coworkers conducted a study to understand the role of cobiotic in type 2 diabetes and insulin resistance. This cobiotic constituting purified inulin, sugar-free blueberry pomace extract, and an oat preparation of purified beta-glucan was consumed to correct the gastrointestinal dysbiosis, along with metformin for diabetes control. Blueberries with their inherent antioxidant property have the ability improve insulin sensitivity. This study showed that "safe food supplement can increase the efficacy of metformin and its tolerability" and therefore has tremendous public health implications (Greenway et al. 2014).

20.2.7 Functional and Restructured Meats

Functional meat: Meat is a great source of fatty acids, minerals, dietary fiber, antioxidants, and bioactive peptides (Decker and Park 2010). Hence functional meat are a viable option producers and consumers alike, such as. Iron, zinc, conjugated linoleic acids, and vitamin B are a major source of bioactive compounds in muscle foods (Jiménez-Colmenero et al. 2001). Meat and meat-based products are restructured by adding beneficial components and removing potentially harmful compounds. The functional value of meat is improved by the addition of compounds like selenium, conjugated linolenic acid, n3 fatty acids, and vitamin E in the animal diet or by adding ingredients like vegetable proteins, lactic acid bacteria and dietary fibres during meat processing. Also processes of fermentation, curing and aging, and enzymatic hydrolysis during meat and meat products processing generates peptides (Zhang et al. 2010). These peptides in addition to normal nutrition have antimicrobial properties, lowering hypertension, cholesterol-lowering property, antioxidant properties, immunomodulatory effects, etc., (Hartmann and Meisel 2007). ACE inhibitory peptides is one such example which helps maintain blood pressure (Ogbru and Marks 2008) by inhibiting the conversion of angiotensin I to angiotensin II. This type of peptides have also been isolated from whey proteins, fish, pork, chicken and eggs, and beef, etc. (Ghassem et al. 2011; Jang and Lee 2005; Saiga et al. 2003; Vercruysse et al. 2005). These small peptides reach the blood vessels by crossing the digestive epithelial barrier and (Yust et al. 2003). Enzymatic methods and the choice of enzymes such as trypsin, chymotrypsin,

pepsin, pancreatin, thermolysin, and proteinase A to synthesize bioactive peptides from meat depend on meat type.

Restructuring meat and fish: Enzymes for restructuring meat and fish include thrombin and transglutaminases. Thrombin is a serine protease that catalyzes the conversion of soluble fibrinogen into insoluble fibrin fibres and other coagulation reactions. Both thrombin and fibrinogen can be sourced from cattle and pig blood plasma obtained from slaughterhouses or from fish tissues (Manseth et al. 2003). For restructured fish or meat fibrinogen is converted into a fibrin gel by the action of thrombin which then interacts with collagen and binds the pieces of meat or fish. The binding strength of the reconstituted piece is strongest at pH near 7, with larger particle sizes and if the fibrin gel runs parallel to the available collagen in meat (Boles and Shand 1998; Chen and Lin 2002), and is successful if the meat or fish has been previously frozen (Boles and Shand 1999).

The property of the transglutaminase to incorporate amino acids and cross-link insoluble protein polymers is used with surimi or other protein-rich foods to bind proteins together or to give restructured fish or meat pieces desired shapes and forms resembling crab or steak. Transglutaminase is of great use inv binding protein-rich foods such as red meat, poultry and seafood pieces. It is also used in binding casein, soybean globulins and egg proteins (Motoki and Seguro 1998; Yokoyama et al. 2004). Their applications enhanced retention of capsaicin in capsaicin-enriched layered noodles (Li et al. 2013) and is important for the gelation process and cross-linking of actomyosin during surimi manufacture (Nielsen and Nielsen 2012).

20.3 Enzymes in Food Industry

Enzymes being biocatalyst have found multiple applications in the food industry. Proteases, amylases, lipases, pectinases, transglutaminases, xylanses, lactases, invertases, and many more. Proteins are another important and essential nutrient in a diet. The improvement of the physical and functional properties of foods can be through chemical or enzymatic modifications. However chemical modifications are not preferred for food applications due to the nonspecific chemicals or reagents, other side reactions, difficulty of removal of residual chemicals, to name a few. Enzymes are preferred due to their specificity, fast reactions and mild conditions as well as their biological nature in improving the functional as well as physical properties of proteins.

Proteases and transglutaminases are frequently used for modifying the polypeptide backbone. Solubility, gelation, emulsification and foaming are functional properties of proteins, which are closely related to their size, structural conformation, and level and distribution of ionic charges. Enzymatically hydrolyzed proteins are classified based on the molecular weight distribution of the resultant hydrolysate. Larger peptides (2–5 kDa) are mainly used as functional ingredients or in personal care products. Medium sized peptides (1–2 kDa) are used

in clinical nutrition. Smaller peptides have pharamceutical applications. For the preparation of protein hydrolysates high nutritional value alkaline microbial proteases have been used. Protein hydrolysates have multiple nutritional applications and have a role in blood pressure regulation, in infant food formulations, therapeutic dietary products and in fortification of fruit juices and soft drinks (Ward 1985; Neklyudov et al. 2000; Sujith and Hymavathi 2013). Angiotensin-converting enzyme (ACE) inhibitors, used in the treatment of hypertension are produced fromproteases obtained from whey protein hydrolysates of *B. licheniformis* and *Aspergillus orza* with (Erdmann et al. 2008). Fish protein hydrolysates and peptides obtained from fishery resources because of its wide availability and abundance are therefore commobly used as food supplements, functional ingredients and to enhance flavour in food and beverage industry. The protein hydrolysate of fish *Rastrelliger kanagurta* backbones exhibited potent antioxidant properties on proteolytic digestion with pepsin and papain (Sheriff et al. 2014).

In the food industry lipase is associated with its use in the preparation of food additives. Vitamin A esters were produced with a general yield of 78%, when reacting for 7 h at 30 °C using a recombinant lipase from immobilized *Candida antarctica* ZJB09193 produced in the host *Pichia pastoris*, (Liu et al. 2012). Lipases also catalyse the production of anti-oxidants such as isoascorbic acid esters which are used as food additives (Sun et al. 2013). In the dairy industry lipases helps in modifying the fatty acid chain lengths, to augment the flavor of cheeses, and to stimulate cheese ripening (Ghosh et al. 1996; Sharma et al. 2001).

α-L-arabinofuranosidases are arabinose-releasing enzymes which are involved in the hydrolysis of L-arabinose linkages. (Chavez et al. 2006; Raweesri et al. 2008) of importance in food industry as L-arabinose can be used as a food additive because its sweet taste and its low uptake due to poor absorption by the human body (Matuso et al. 2000). others and have important health implications-arabinose can be used as antiglycemic agent as it can competitively and selectively inhibit intestinal sucrase, thus reducing the glycaemic response by dose dependently suppressing the increase of blood glucose level after sucrose ingestion (Seri et al. 1996; Shin et al. 2003). Pentosans added to the dough may be moderately hydrolysed by wheat flour enzymes and also by exogenous enzymes like α-L-arabinofuranosidases (Fessas and Schiraldi 1998; Jiménez and Martinez-Anaya 1999), producing free pentoses (mainly arabinose and xylose), making available soluble carbohydrates in the dough (Gobbetti et al. 1999; Jiménez and Martinez-Anaya 1999; Martinez-Anaya and Devesa 1999; Gobbetti et al. 2000) needed for the metabolism of sourdough lactic acid bacteria of *Lactobacillus* sp. (Gobbetti et al. 1999), increasing the acidification rates and the production of acetic acid. Resveratrol is a stilbenoid, a type of phenolic compound in grapes with properties that contribute to the reduction of the risks of coronary heart disease by reducing systolic blood pressure and other human health-related processes. During the wine production process, arabinofuranosidase increase the resveratrol content of white wine in addition to increasing the aroma.

Lignocellulosic substrates such as cellulose, hemicellulose, pectin, and lignin which are made up of polysaccharides and phenolic polymers are abundantly

present in fruits and vegetables and are hydrolyzed by various lignocellulolytic enzymes during processing. Lignocellulolytic enzymes such as cellulase, hemicellulase, and pectinase together represent more than 20% of worldwide sales of commercially available enzymes and have a wide range of important potential applications in the food processing industries ranging from the fruit and vegetable juice industry to the baking industry These enzymes and their cocktail are used in juice processing to accelerate juice extraction, to increase processing efficiency and in solid settling or removal, for generating a clear and visually attractive final product. (Polizeli et al. 2005; Jaramillo et al. 2015; Toushik et al. 2017). Functional use of cellulases and hemicellulases was to produce animal feeds and later to produce food, textiles, and paper in the 1980s (Bhat 2000; Polizeli et al. 2005). The cellulase complex comprises 3 different enzymes such as endo-(1,4)- β-D glucanase (EG) and exo (1,4)-β-D-glucanase (CBG) enzymes, which initially act synergistically to convert cellulose chains into cello-oligosaccharides, and then *β*-glucosidase (BG) hydrolyzes the cello-oligosaccharides into simple sugars or glucoses. β-Glucosidases can improve the aroma of wines by modifying glycosylated precursors. (Sathya and Khan 2014; Sindhu et al. 2016). Texture, flavor, and aroma properties of fruits and vegetables can be enhanced by reducing excessive bitterness of citrus fruits by infusion of enzymes such as pectinases and β-glucosidases (Baker and wicker 1996). Cocktail of enzymes pectinases, cellulases, and hemicellulases are also used for increasing extraction of olive oil. Use of macerating enzymes not only improves the cloud stability but also decreases their viscosity rapidly (Godfrey 1996) Pectinase and cellulase enzymes disrupts the cell wall of fruits and vegetables and releases the carotenoids in the chloroplasts and in cell fluids. Important polysaccharides are synthesized from breakdown of xylan, the most abundant hemicellulose in the cell wall of fruits and vegetables, by the action of two hemicellulase enzymes, endoxylanases and exoxylanases. Endo-1,4-β-xylanase from *Thermomyces lanuginosus* supplementation improved the nutritive value of wheat based broiler diets (Francesch et al. 2012). Pectin is another complex, hydrocolloid acidic polysaccharide present in plants. Most pectin-degrading enzymes are classified into 3 major groups of glycoside hydrolases. Ligninolytic enzymes which degrade lignin in cell walls comprises of three oxidative enzymes; lignin peroxidase (LiP) commonly known as ligninase, manganese peroxidase (MnP) and laccase (Niladevi 2009). Laccases are involved in enhancing or modifying the colour appearance of food or beverage for clarifying fruit juices, beer and wine by elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity (Rodríguez and Toca 2006).Lignin peroxidase (LiP) and manganese peroxidase (MnP) is used to produce natural aromatic flavours (Lesage-Meessen et al. 1996).

20.3.1 *Enzymes in the Production of Bioactive Peptides from Milk and Whey Proteins*

Casein and whey proteins have ignited a lot of interest in the scientific community due their various physiological and biological effects, both in vitro and in vivo in animals. Whey contains β-lactoglobulin, α-lactalbumin and minor proteins with various biological activities. These are mainly released due to enzymatic digestion or on fermentation of milk. Some of the bioactive peptides include casomorphins, casokinins casoxins, lactokinins, lactoferricins, isracidin casocidicins, casoplatelins, to name a few (FitzGerald and Meisel 2003). Four different strategies can be used to produce these peptides - use of proteinase and exopeptidase *for* in vitro digestion of milk proteins, digestion of milk proteins by GI proteinases or peptidases in vivo; by bacterial proteinase and peptidase during production of fermented milk products and by chemical synthesis of known bioactive peptides (FitzGerald and Meisel 2003; Meisel 2001). Casein and whey protein-derived peptides have opioid, antimicrobial, antiappetizing hypocholesterolemic immunoregulatory, antihypertensive, antioxidant and antithrombotic effects (Clare and Swaisgood 2000; Hernández-Ledesma et al. 2005; FitzGerald and Meisel 2003). Figure 20.3 gives the bioactivity of the milk peptides.

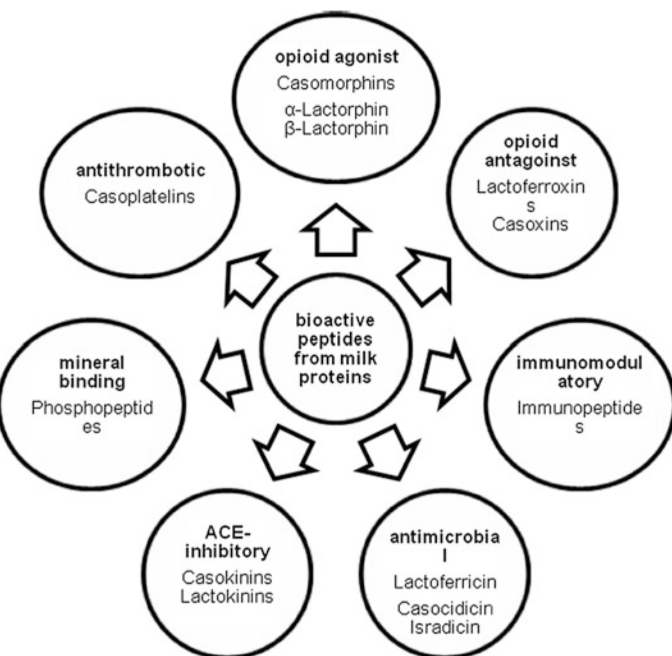


Fig. 20.3 Some bioactive peptides from milk and their activity. Adapted from FitzGerald and Meisel (2003)

20.3.2 Enzyme-Mediated Phytate Degradation in Cereal Based Foods

Myo-inositol hexaphosphate the main storage form of phosphorus in plant-based foods is also known as phytate and contributes to about 80% of the total phosphorous in cereal seeds. The overall negative charge attributed to the phosphate groups is involved in chelating calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts thereby limiting their availability. Numerous studies have shown that phytate have various therapeutic effects which include anticancer activity, anti-oxidant activity, mineral binding ability, reducing pH, cell cycle arrest, production of natural killer cells, hypolipidaemic effect, antiviral activity, protection against tooth decay and prevention of kidney stones (Kumar et al. 2010). Despite this, phytates exhibits beneficial effects in treating colon cancer, AIDS, Alzheimer's disease, Arthritis and Parkinson's diseases (Meyers and Rasmussen 2008). Phytases of both plants and microbial origin, not only degrade phytate into inositol and free orthophosphates but also makes available calcium, magnesium, protein and lipid. These enzymes are structurally very different, with alpha/beta form (Plant phytases) and beta form (bacterial phytases) (Lei et al. 2007). Phytate degradation was observed in probiotic bacteria isolated functional foods such as sourdough, fermented bread and dairy products. This could help in improved mineral uptake in humans. Many authors report that the addition of sourdough and lactic acid bacteria with high phytate degrading activity during the breadmaking process can reduce the phytic acid content in bread (Katina et al. 2005; Corsetti and Settanni 2007; Palacios et al. 2008a, b).

Most LABS (Anastasio et al. 2010; Reale et al. 2004) *and Lactobacillus brevis* and *Bacillus subtilis* in fermented food products are capable of producing higher amount of phytase (Shimizu 1992; Hong et al. 2011; Lee et al. 2006). Phytases produced by most probiotic organisms are very specific towards phytate and are stimulated by Ca^{2+} ions for effective activity Recombinant phytases can also be used for intestinal phytate utilization. An exogenous phytase used for phytate degradation improved iron absorption from cereal porridges prepared with water, but not with milk, except from high-tannin sorghum (Hurrell et al. 2003).

In cereal based foods traditional processing methods suck as soaking cereal flour prior to heating activate native phytases, which enhances Zinc bioavailability, as degradation products of phytates have reduced affinity for zinc. The effect of increasing the soaking time for wheat bater at 10 °C for 0–48 h and its effect on roti making with wheat, millet and sorghum flour was investigated. Degradation of IP6 in rotimaking without soaking for all three cereals was only 14–19% with marginal decrease in zinc solubility (Agte and Joshi 1997).

20.3.3 Degradation of Non-starch Polysaccharides (NSP)

Nonstarch polysaccharides (NSPs)are present in many foods and they are the major part of dietary fiber. Nonstarch polysaccharidesare complex polysaccharides other than the starch which include cellulose, pectins, glucans, gums, mucilages, inulin, and chitin, among others and have important health implications for humans. The NSP content of bran and white rice is about 24 and 0.1% respectively. NSPs, the primary constituent of dietary fiber, acts as a prebiotic substrate for different groups of colonic bacteria which also helps in improving fecal biomass excretion are raised by increasing the intake of indigestible polysaccharide in humans (Rao et al. 1994).

Dietary NSPs are water soluble and shows high level of viscosity and are fermentable into short chain fatty acids (SCFAs) with the help of anaerobic bacteria present in human intestine. The common SCFAs produced are acetate, propionate, and butyrate by inhibiting growth of the pathogenic organism and also the formation of toxic breakdown products. SCFAs also increases mineral absorption, maintains normal bowel structure and function, prevents or alleviates diarrhea by promoting sodium and water absorption, by stimulating pancreatic secretions and other gastrointestinal hormones (Butzner et al. 1996) These characteristics may help to reduce the risk of several fatal and serious diet related diseases like coronary heart disease, colo-rectal cancer, inflammatory bowel disease, breast cancer, tumor formation, mineral related abnormalities, and disordered laxation. Cellulose and hemicellulose being effective as laxatives soluble NSPs, especially mixed-link β-glucans lower plasma cholesterol levels and help to normalize blood glucose and insulin levels. Most of the NSP in wheat, barley, rye and maize are primarily comprised of insoluble arabinoxylan whilst in oats a soluble β-glucan predominated. Barley and rye contained both arabinoxylan and β-glucan. The NSP content of breakfast cereal products depend on the quality of the flours and grains from which they were made.

Arabinoxylan is the most prevalent type of NSP in broiler feeds and hence reduces the digestibility of nutrients in the diets (Salim et al. 2010). Enzymes such as Xylanase, amylase and protease are used to target the NSP fraction of the diet. Enzymes act by breaking down components of the cell wall which releases encapsulated nutrients, and reduce digesta viscosity which increases digesta passage rate which allows improved access the bird's endogenous enzymes to nutrients and reduces bacterial proliferation in the small intestine. Xylanase-supplemented bird diets improved not only digestion, but also growth performance (Kiarie et al. 2014). Hydrolysis of starch in feeds by Amylase also improved its digestibility (Gracia et al. 2003). Mode of action of proteases in the gastrointestinal tract is unclear (Adeola and Cowieson 2011). A combination of xylanase, amylase and protease improved nutrient utilization and solubilization of NSP in the gut (Olukosi et al. 2015).

The application of probiotics (also known as direct-fed microbials (DFMs) also influences poultry health by altering the gut environment, modulating the immune

system, and removing dysbiosis (Lee et al. 2010), and the use of *Bacillus* strains reduced inflammatory markers (Lee et al. 2010), as well as lowered mortality and increased body weight in poultry (Dersjant-Li et al. 2015).Although enzymes are known to have a prebiotic effect in the broiler gut (Romero et al. 2013, 2014), there is only little work on the combination of probiotics and enzymes in poultry diets. *Bacillus* spp. are gaining interest in human health related functional food research due to their enhanced tolerance and survivability under hostile environment of gastrointestinal tract. Another study which compared the effect of a commercially available *Bacillus*-based probiotic product, given alone or in combination with either of 2 multi-enzyme supplements, on growth performance, digestibility of nutrients and energy, disappearance of NSP, and gut microbial composition in broilers which showed that the combination of *Bacillus* probiotics and exogenous enzymes led to increased and significant effects on the apparent ileal digestibility of nutrients and energy than their delivery alone. (Wealleans et al. 2017).

20.4 Conclusion

With the world trying to get more out of the food that they consumed, functional foods that provide more than just nutritive value are here to stay. 'We are what we eat' and and our food choices combined with the stresses associated with the fast lifestyles today can maar health and reduce well being. The ability of food consumed to selectively regulate and control the gut microbiome by either enhancing the beneficial bacteria or the promoting the bad ones, and thereby make or harm human health is being seriously studied today. Functional foods, probiotics, prebiotics, synbiotics and cobiotics add and enrich the aleady complex food components and help to reduce the risks of several disorders. However, every functional component needs to be researched for all their modes of action, and roles in augmenting health and well being before being considered as a food ingredient.

References

Adeola O, Cowieson AJ (2011) Board-invited review: opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. J Anim Sci 89(10):3189–3218

Agte VV, Joshi SR (1997) Effect of traditional food processing on phytate degradation in wheat and millets. J Agric Food Chem 45(5):1659–1661. https://doi.org/10.1021/jf9605308

Ammoscato F, Scirocco A, Altomare A, Matarrese P, Petitta C, Ascione B, Caronna R, Guarino M, Marignani M, Cicala M et al (2013) *Lactobacillus rhamnosus* protects human colonic muscle from pathogen lipopolysaccharide-induced damage. Neurogastroenterol Motil 2013. https://doi.org/10.1111/nmo.12232

Anadón A, Martínez-Larrańaga MR, Martínez MA (2006) Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul Toxicol Pharmacol 45:91–95. https://doi.org/10.1016/j.yrtph.2006.02.004

Anastasio M, Pepe O, Cirillo T, Palomba S, Blaiotta G, Villani F (2010) Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. J Food Sci 75:M28–M35. https://doi.org/10.1111/j.1750-3841.2009.01402.x

Arthur J, Perez-Chanona E, Muhlbauer M, Tomkovich S, Uronis J, Fan T et al (2012) Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338:120–123

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende D et al (2011) Enterotypes of the human gut microbiome. Nature 473:174–180

Azcarate-Peril MA, Ritter AJ, Savaiano D, Monteagudo-Mera A, Anderson C, Agness ST, Klaenhammer TR (2017) Impact of short-chain galactooligosaccharides on the gut microbiome of lactose-intolerant individuals. Proc Natl Acad Sci USA 2017 114:E367–E375

Baker RA, Wicker L (1996) Current and potential applications of enzyme infusion in the food industry. Trends Food Sci Technol 7(9):279–284

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

Begley M, Hill C, Gahan CGM (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72:1729–1738. https://doi.org/10.1128/AEM.72.3.1729-1738.2006

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

Boles JA, Shand PJ (1998) Effects of comminution method and raw binder system in restructured beef. Meat Sci 49:297–307

Boles JA, Shand PJ (1999) Effects of raw binder system, meat cut and prior freezing on restructured beef. Meat Sci 53:233–239

Bordoni A, Amaretti A, Leonardi A, Boschetti E, Danesi F, Matteuzzi D, Roncaglia L, Raimondi S, Rossi M (2013) Cholesterol-lowering probiotics: in vitro selection and in vivo testing of bifidobacteria. Appl Microbiol Biotechnol 9:8273–8281

Bruzzese E, Volpicelli M, Salvini F, Bisceglia M, Lionetti P, Cinquetti M, Iacono G, Guarino A (2006) Early administration of GOS/FOS prevents intestinal and respiratory infections in infants. J Pediatr Gastroenterol Nutr 42:E95

Butzner JD, Parmar R, Bell CJ, Dalal V (1996) Butyrate enema therapy stimulates mucosal repair in experimental colitis in the rat. Gut 38:568–573

Carter A, Adams M, La Ragione RM, WoodWard MJ (2017) Colonisation of poultry by *Salmonella*Enteritidis S1400 is reduced by combined administration of *Lactobacillus salivarius* 59 and *Enterococcus faecium* PXN-33. Vet Microbiol 199:100–107. https://doi.org/10.1016/j.vetmic.2016.12.029

Castellarin M, Warren R, Freeman J, Dreolini L, Krzywinski M, Strauss J et al (2012) Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res 22:299–306

Cencic A, Chingwaru W (2010) The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients 2:611–625. https://doi.org/10.3390/nu2060611

Chassy BM (1987) Prospects for the genetic manipulation of lactobacilli. FEMS Microbiol Rev 46:197–312

Chen MJ, Lin CW (2002) Factors affecting the water-holding capacity of fibrinogen/plasma protein gels optimized by response surface methodology. J Food Sci 67:2579–2582

Chen PW, Jheng TT, Shyu CL, Mao FC (2013) Synergistic antibacterial efficacies of the combination of bovine lactoferrin or its hydrolysate with probiotic secretion in curbing the growth of meticillin-resistant Staphylococcus aureus. J Med Microbiol 62:1845–1851

Chingwaru W, Vidmar J (2017) Potential of Zimbabwean commercial probiotic products and strains of *Lactobacillus plantarum* as prophylaxis and therapy against diarrhoea caused by *Escherichia coli* in children. Asian Pac J Trop Med 10:57–63. https://doi.org/10.1016/j.apjtm.2016.12.009

Chmielewska A, Szajewska H (2010) Systematic review of randomised controlled trials: probiotics for functional constipation. World J Gastroenterol 16:69–75

Clare DA, Swaisgood HE (2000) Bioactive milk peptides: a prospectus1. J Dairy Sci 83(6):1187–1195
Clark LC, Hodgkin J (2014) Commensals, probiotics and pathogens in the *Caenorhabditis elegans* model. Cell Microbiol 16:27–38
Clarke SD (2001) Polyunsaturated fatty acid regulatuion of gene transcription: a molecular mechanism to improve the metabolic syndrome. J Nutr 131:1129–1132
Collado MC, Meriluoto J, Salminen S (2007) Interactions between pathogens and lactic acid bacteria: aggregation and coaggregation abilities. Eur J Food Res Technol 226:1065–1073. https://doi.org/10.1007/s00217-007-0632-x
Committee on Opportunities in the Nutrition and Food Sciences, Food and Nutrition Board, Institute of Medicine (1994) Enhancing the food supply. In: Thomas PR, Earl R (eds) Opportunities in the nutrition and food sciences: research challenges and the next generation of Investigators. National Academy Press, Washington, DC, pp 98–142
Corsetti A, Settanni L (2007) Lactobacilli in sourdough fermentation. Food Res Int 40(5):539–558
Cortés-Zavaleta O, López-Malo A, Hernández-Mendoza A, García HS (2014) Antifungal activity of Lactobacilli and its relationship with 3-phenyllactic acid production. Int J Food Microbiol 173:30–35
Costalos C, Kapiki A, Apostolou M, Papathoma E (2008) The effect of a prebiotic supplemented formula on growth and stool microbiology of term infants. Early Hum Dev 84:45–49
Cremonini F, di Caro S, Nista EC, Bartolozzi F, Capelli G, Gasbarrini G, Gasbarrini A (2002) Meta-analysis: the effect of probiotic administration on antibiotic associated diarrhoea. Aliment Pharmacol Ther 16:1461–1467. https://doi.org/10.1046/j.1365-2036.2002.01318.x
De Montijo-Prieto S, Moreno E, Bergillos-Meca T, Lasserrot A, Ruiz-López M, Ruiz-Bravo A, Jimenez-Valera M (2015) A *Lactobacillus plantarum* strain isolated from kefir protects against intestinal infection with *Yersinia enterocolitica* O9 and modulates immunity in mice. Res Microbiol 166:626–632. https://doi.org/10.1016/j.resmic.2015.07.010
Decker EA, Park Y (2010) Healthier meat products as functional foods. Meat Sci 86(1):49–55
Dersjant-Li Y, Awati A, Schulze H, Partridge G (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. J Sci Food Agric 95(5):878–896
DeVrese M, Schrezenmeir J (2008) Probiotics, prebiotics, and synbiotics. in food biotechnology (pp. 1–66). Springer Berlin Heidelberg
Di Gioia D, Aloisio I, Mazzola G, Biavati B (2014) Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. Appl Microbiol Biotechnol 98: 563–577
Erdmann K, Cheung BW, Schröder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. J Nutrit Biochem 19(10):643–654
Eslamparast T, Zamani F, Hekmatdoost A, Sharafkhah M, Eghtesad S, Malekzadeh R, Poustchi H (2014a) Effects of synbiotic supplementation on insulin resistance in subjects with the metabolic syndrome: a randomised, double-blind, placebo-controlled pilot study. Br J Nutr 112:438–445
Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hetmatdoost A (2014b) Synbioticsupplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilotstudy. Am J Clin Nutr 99:535–542
European Food Safety Authority (EFSA) (2005) Opinion of the scientific committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives. EFSA J 226:1–12
European Food Safety Authority (EFSA) (2013) Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA J 15:1–108
European Food Safety Authority (EFSA) (2017) Scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017 update). EFSA J 15:1–177

Fernandez F, Hinton M, Gils BV (2002) Dietary mannan-oligosaccharides and their effect on chicken caecalmicroflora in relation to Salmonella Enteritidis colonization. Avian Pathology 31 (1):49–58

Fessas D, Schiraldi A (1998) Texture and staling of wheat bread crumb: effects of water extractable proteins and pentosans'. ThermochimicaActa 323(1–2):17–26

FitzGerald RJ, Meisel H (2003) Milk protein hydrolysates and bioactive peptides. In: Fox PF, McSweeney PLH (eds), Advanced dairy chemistry. 3rd edn Chap 14. Kluwer Academic Plenum, London, UK, 675–691

Francesch M, Perez-Vendrell AM, Broz J (2012) Effects of a mono-component endo-xylanase supplementation on the nutritive value of wheat-based broiler diets. British Poultry Sci 53 (6):809–816

Food and Agriculture Organization (2007) FAO technical meeting on prebiotics: Food Quality and Standards Service (AGNS), Food and Agriculture Organization of the United Nations (FAO) FAO, Rome, Italy, 15–16 Sept 2007. FAO Technical Meeting Report

Frank D, St Amand A, Feldman R, Boedeker E, Harpaz N, Pace N (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Nat Acad Sci USA 104:13780–13785

Fric P (2007) Probiotics and prebiotics—renaissance of a therapeutic principle. Cent Eur J Med 2:237–270

Gibson GR, Probert HM, van Loo J, Rastall RA, Roberfroid M (2004) Dietary modulation of the human colonic microbiota: updating the concept of the prebiotics. Nutr Res Rev 17:259–275. https://doi.org/10.1079/NRR200479

Ghassem M, Arihara K, Babji AS, Said M, Ibrahim S (2011) Purification and identification of ACE inhibitory peptides from Haruan (Channastriatus) myofibrillar protein hydrolysate using HPLC–ESI-TOF MS/MS. Food Chem 129(4):1770–1777

Ghosh, B.C. and S. Singh, 1996. Effect of different levels of titrable acidity in curd from buffalo milk at stretching on Mozzarella cheese. J. Food Sci. Technol., 33: 70-72

Gobbetti M, Folkertsma B, Fox PF, Corsetti A, Smacchi E, De Angelis M, Cortini M (1999) Microbiology and biochemistry of Fossa (pit) cheese. Int Dairy J 9(11):763–773

Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F (2000) Production of Angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by Lactobacillus delbrueckiisubsp. bulgaricus SS1 and Lactococcuslactissubsp. cremoris FT4. Appl Environ Microbiol 66 (9):3898–3904

Godfrey T (1996) Textiles; baking; and key characteristics of enzymes. In: Godfrey T, West S (eds) Industrial enzymology

Gotteland M, Brunser O, Cruchet S (2006) Systematic review: are probiotics useful in controlling gastric colonization by *Helicobacter pylori*? Aliment Pharmacol Ther 23:1077–1086

Gracia MI, Aranibar M, Lazaro R, Medel P, Mateos GG (2003) Alpha-amylase supplementation of broiler diets based on corn. Poultry Sci 82(3):436-442

Greenway F, Wang S, Heiman M (2014) A novel cobiotic containing a prebiotic and an antioxidant augments the glucose control and gastrointestinal tolerability of metformin: a case report. Benef Microbes (1):29–32. https://doi.org/10.3920/bm2012.0063

Gu Q, Zhang C, Song D, Li P, Zhu X (2015) Enhancing vitamin B12 content in soy-yogurt by *Lactobacillus reuteri*. Int J Food Microbiol 206:56–59. https://doi.org/10.1016/j.ijfoodmicro.2015.04.033

Guinane CM, Cotter PD (2013) Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therap Adv Gastroenterol 6(4):295–308. https://doi.org/10.1177/1756283X13482996

Hanning I, Clement A, Owens C, Park SH, Pendleton S, Scott EE, Almeida G, Gonzalez Gil F, Ricke SC (2012) Assessment of production performance in 2 breeds of broilers fed prebiotics as feed additives. Poultry Sci 91(12):3295–3299

Haraguchi K (2016) Functional oligosaccharide DFA III forming enzyme produced by microorganisms. J Exp Food Chem 2:115. https://doi.org/10.4172/2472-0542.1000115

Hartmann R, Meisel H (2007) Food-derived peptides with biological activity: from research to food applications. Curr Opin Biotechnol 18(2):163–169
Hasler CM (2002) Functional foods: benefits, concerns and challenges—a position paper from the American Council on Science and Health. J Nutr 132(12):3772–3781 (1 Dec 2002)
He F, Morita H, Ouwehand AC, Hosoda M, Hiramatsu M, Kurisaki JI, Isolauri E, Benno Y, Salminen S (2002) Stimulation of the secretion of pro-inflammatory cytokines by Bifidobacterium strains. Microbiol Immunol 46(11):781–785
Hempel S, Newberry SJ, Maher AR, Wang Z, Miles JN, Shanman R, Johnsen B, Shekelle PG (2012) Probiotics for the prevention and treatment of antibiotic- associated diarrhea: a systematic review and meta-analysis. JAMA 307:1959–1969
Henry CJ (2010) Functional foods. Eur J Clin Nutr 64(7):657–9. https://doi.org/10.1038/ejcn.2010.101
Hernández-Ledesma B, Dávalos A, Bartolomé B, Amigo L (2005) Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. J Agric Food Chem 53(3):588–593
Hess P, Altenhöfer A, Khan AS, Daryab N, Kim KS, Hacker J, Oelschlaeger TA (2004) A Salmonella fim homologue in *Citrobacter freundii* mediates invasion in vitro and crossing of the blood-brain barrier in the rat pup model. Infect Immun 72(9):5298–5307
Homayouni A, Bastani P, Ziyadi S, Mohammad-Alizadeh-Charandabi S, Ghalibaf M, Mortazavian AM, Mehrabany EV (2014) Effects of probiotics on the recurrence of bacterial vaginosis: a review. J Low Genit Tract Dis 18:79–86
Hong SW, Chu IH, Chung KS (2011) Purification and biochemical characterization of thermostable phytase from newly isolated *Bacillus subtilis* CF92. J Korean Soc Appl Biol Chem 54:89–94. https://doi.org/10.3839/jksabc.2011.012
Hosono A (1992) Fermented milk in the orient. In: Nakazawa Y, Hosono A (eds) Functions of fermented milk: challengers for the health sciences. Elsevier Science Publishers Ltd., Barking, UK, pp 61–78
Huang Q, Wei Y, Lv Y, Wang Y, Hu T (2015) Effect of dietary inulin supplements on growth performance and intestinal immunological parameters of broiler chickens. Livestock Science 180:172–176
Human Microbiome Project (HMP) Consortium (2012a) Structure, function and diversity of the healthy human microbiome. Nature 486:207–214
Human Microbiome Project (HMP) Consortium (2012b) A framework for the human microbiome research. Nature 486:215–221
Hurrell RF, Reddy MB, Juillerat MA, Cook JD (2003) Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. Am J Clin Nutr 77(5):1213-1219
Isolauri E, Sutas Y, Kankaanpaa P, Arvilommi H, Salminen S (2001) Probiotics: effects on immunity. Am J Clin Nutr 73:444–450. https://doi.org/10.1016/j.bpg.2003.10.006
Jaramillo-Bustos DP, del Socorro Cruz-Cansino N, Ramírez-Moreno E, León-Rivera JE, Delgado-Olivares L, Alanís-García E, Ariza-Ortega JA (2015) Shelf life, physicochemical, microbiological and antioxidant properties of purple cactus pear (Opuntiaficusindica) juice after thermoultrasound treatment. Ultrasonicssonochemistry 27:277–286
Janardhana V, Broadway MM, Bruce MP, Lowenthal JW, Geier MS, Hughes RJ, Bean AG (2009) Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J Nutr 139(7):1404–1409
Jang A, Lee M (2005) Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. Meat Sci 69(4):653–661
JimmySaint-Cyr M, Haddad N, Taminiau B, Poezevara T, Quesne S, Amelot M, Daube G, Chemaly M, Dousset X, Guyard-Nicodème M (2017) Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control *Campylobacter jejuni* in broilers. Int J Food Microbiol 247:9–17. https://doi.org/10.1016/j.ijfoodmicro.2016.07.003
Jiménez T, Martinez-Anaya MA (1999, June) Enzymes, a key to improve bread and dough quality: degradation by products and relationship with quality. In 17th ICC Conference of the Cereal Across the Continents (pp. 6-9)

Jiménez-Colmenero F, Carballo J, Cofrades S (2001) Healthier meat and meat products: their role as functional foods. Meat Sci 59(1):5–13
Katina et al. (2005) Sourdough: a tool for the improved flavour, texture and shelf-life of wheat bread
Kau A, Ahern P, Griffin N, Goodman A, Gordon J (2011) Human nutrition, the gut microbiome and the immune system. Nature 474:327–336
Kiarie E, Romero LF, Ravindran V (2014) Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. Poultry Sci 93(5):1186–1196
Khalesi S, Sun J, Buys N, Jayasinghe R (2014) Effect of probiotics on blood pressure. A systematic review and meta-analysis of randomized, controlled trials. Hypertension 70:1–13. https://doi.org/10.1161/hypertensionaha.114.03469
Kim GB, Seo YM, Kim CH, Paik IK (2011) Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult Sci 90(1):75–82
Kostic A, Gevers D, Pedamallu C, Michaud M, Duke F, Earl A et al (2012) Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res 22:292–298
Kumar V, Sinha AK, Makkar HP, Becker K (2010) Dietary roles of phytate and phytase in human nutrition: a review. Food Chem 120(4):945–959
Kumar R, Grover S, Batish VK (2011) Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing Lactobacillus plantarum strains in Sprague-Dawley rats. Br J Nutr 105(4):561–573. https://doi.org/10.1017/S0007114510003740
Kumar S, Bansal A, Chakrabarti A, Singhi S (2013) Evaluation of efficacy of probiotics in prevention of *Candida* colonization in a PICU—a randomized controlled trial. Crit Care Med 41:565–572. https://doi.org/10.1097/CCM.0b013e31826a409c
Kuo SM (2013) The interplay between fiber and the intestinal microbiome in the inflammatory response. Adv Nutr: Intern Rev J. 4(1):16–28. https://doi.org/10.3945/an.112.003046
Lee YK (2009) Selection and maintenance of probiotic microorganisms. In: Lee YK, Salminen S (eds) Handbook of probiotics and prebiotics. Wiley-VCH; Weinheim, Germany, pp 177–187
Lee SH, Kwon HS, Koo KT, Kang BH, Kim TY (2006) Characterization of phytase from *Bacillus coagulans* IDCC 1201. Korean J Microbiol Biotechnol 34:28–34
Lee SJ, Bose S, Seo JG, Chung WS, Lim CY, Kim H (2013) The effects of co-administration of probiotics with herbal medicine on obesity, metabolic endotoxemia and dysbiosis: a randomized double-blind controlled clinical trial. Clin Nutr. https://doi.org/10.1016/j.clnu.2013.12.006
Lee K, Lillehoj HS, Siragusa GR (2010) J Poultry Sci (Korea) 47:106–114
Lei XG, Porres JM, Mullaney EJ, Brinch-Pedersen H (2007) Phytase: source, structure and application. In: Polaina J, MacCabe AP (eds) Industrial enzymes-structure, function and applications. Springer, Netherlands, pp 505–529
Leroy F, Falony G, de Vuyst L (2008) Latest developments in probiotics. In: Toldrà F (ed) Meat biotechnology, Springer Science + Business Media, LLC. 217–229 pp
Lesage-Meessen L, Delattre M, Haon M, Thibault J-F, Ceccaldi BC, Brunerie P, Asther M (1996) A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. J Biotechnol 50(2–3):107–113
Ley R, Hamady M, Lozupone C, Turnbaugh P, Ramey R, Bircher J et al. (2008) Evolution of mammals and their gut microbes. Science 320:1647–1651
Ley R, Peterson D, Gordon J (2006a) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848
Ley R, Turnbaugh P, Klein S, Gordon J (2006b) Microbial ecology: human gut microbes associated with obesity. Nature 444:1022–1023
Li P, Gu Q (2016) Complete genome sequence of *Lactobacillus plantarum* LZ95, a potential probiotic strain producing bacteriocins and B-group vitamin riboflavin. J Biotechnol 229:1–2. https://doi.org/10.1016/j.jbiotec.2016.04.048

Li L-Y, Easa AM, Liong M-T, Tan T-C, Foo W-T (2013) The use of microbial transglutaminase and soy protein isolate to enhance retention of capsaicin in capsaicin-enriched layered noodles. Food Hydrocoll 30:495–503

Licciardi PV, Ismail IH, Balloch A, Mui M, Hoe E, Lamb K, Tang ML (2013) Maternal supplementation with LGG reduces vaccine-specific immune responses in infants at high-risk of developing allergic disease. Front Immunol 4. https://doi.org/10.3389/fimmu.2013.00381

Lima-Filho JVM, Vieira EC, Nicoli JR (2000) *Saccharomyces boulardii* and *Escherichia coli*combinations against experimential infections with *Shigella flexneri* and *Salmonella enteritidis* subsp. Typhimurium J Appl Microbiol 88:365–370. https://doi.org/10.1046/j.1365-2672.2000.00973.x

Lindsay JO, Whelan K, Stagg AJ, Gobin P, Al-Hassi HO, Rayment N, Forbes A (2006) Clinical, microbiological, and immunological effects of fructo-oligosaccharide in patients with Crohn's disease. Gut 55:348–355

Lye HS, Rahmat-Ali GR, Leong MT (2010) Mechanisms of cholesterol removal by lactobacilli under conditions that mimic the human gastro intestinal tract. Int Dairy J 20(3):169–175. https://doi.org/10.1016/j.idairyj.2009.10.003

Manseth E, Skjervold PO, Fjear SO, Brosstad FR, Odegaard OR, Flensgrud R (2003) Developing a fish meat-binding agent: purification of salmon thrombin. J Food Sci 68:1648–1652

Markowiak P, Śliżewska K (2017) Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients 9(9):1021

Marteau P, Shanahan F (2003) Basic aspects and pharmacology of probiotics: an overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract Res Clin Gastroenterol 17:725–740. https://doi.org/10.1016/S1521-6918(03)00055-6.%5d

Marteau PR, Vrese MD, Cellier CJ, Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73(2):430s–436s

McCoy A, Araujo-Perez F, Azcarate-Peril A, Yeh J, Sandler R, Keku T (2013) Fusobacterium is associated with colorectal adenomas. PLoS ONE 8:e53653

McFarland LV (2007) Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Med Infect Dis 5:97–105

Meisel H (2001) Bioactive peptides from milk proteins: a producers. Aus J Dairy Technol 56 (2):83–92

Meisel H, FitzGerald RJ (2003) Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. Curr Pharm Des 9(16):1289–1296

Mishra C, Lambert J (1996) Production of anti-microbial substances by probiotics. Asia Pac J Clin Nutr 5:20–24

Mohania D, Kansal VK, Shah D, Nagpal R, Kumar M, Gautam AK, Singh B, Behare PV (2013) Therapeutic effect of probiotic dahi on plasma, aortic, and hepatic lipid profile of hypercholesterolemic rats. J Cardiovasc Pharmacol Ther 18(5):490–497. https://doi.org/10.1177/1074248413487431

Mohseni MJ, Aryan Z, Emamzadeh-Fard S, Paydary K, Mofid V, Joudaki H, Kajbafzadeh AM (2013) Combination of probiotics and antibiotics in the prevention of recurrent urinary tract infection in children. Iran J Pediatr 23:430–438

Motoki M, Seguro K (1998) Transglutaminase and its use for food processing. Trends Food Sci Technol 9:204–210

Munjal U, Glei M, Pool-Zobel BL, Scharlau D (2009a) Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon tumour cells of different stages of carcinogenesis. Br J Nutr 27:1–9

Munjal U, Glei M, Pool-Zobel BL, Scharlau D (2009b) Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon tumour cells of different stages of carcinogenesis. Br J Nutr 102(5):663–671

Nase L, Hatakka K, Savilahti E (2001) Effect of long-term consumption of *Lactobacillus* GG in milk on dental caries and caries risk in children. Caries Res 35:412–420. https://doi.org/10.1159/000047484

Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, Madara JL (2000) Prokaryotic regulation of epithelial responses by inhibition of IκB-α ubiquitination. Science 289(5484):1560–1563

Nielsen MK, Nielsen HH (2012) Seafood enzymes. In: Simpson BK, Nollet LML, Toldrá F, Benjakul S, Paliyath G, Hui YH (eds) Food biochemistry & food processing, 2nd ed. Blackwell Publishing, Ames, IO, pp 247–262

Niladevi KN (2009) Ligninolytic enzymes. In: Biotechnology for agro-industrial residues utilisation. Springer, Dordrecht, pp 397–414

Nova E, Warnberg J, Gomez-Martinez S, Diaz LE, Romeo J, Marcos A (2007) Immunodulatory effects of probiotics in different stages of life. Br J Nutr 98:S90–S95. https://doi.org/10.1017/S0007114507832983

Oelschlaeger TA (2010) Mechanisms of probiotic actions—a review. Int J Med Microbiol 300:57–62. https://doi.org/10.1016/j.ijmm.2009.08.005

Ortiz L, Ruiz F, Pascual L, Barberis L (2014) Effect of two probiotic strains of Lactobacillus on in vitro adherence of Listeria monocytogenes, *Streptococcus agalactiae*, and *Staphylococcus aureus* to vaginal epithelial cells. Curr Microbiol. https://doi.org/10.1007/s00284-014-0524-9

Ouwehand AC, Kirjavainen PV, Shortt C, Salminen S (1999) Probiotics: mechanisms and established effects. Int Dairy J 9:43–52. https://doi.org/10.1016/S0958-6946(99)00043-6

Palacios MC, Haros M, Sanz Y, Rosell CM (2008a) Selection of lactic acid bacteria with high phytate degrading activity for application in whole wheat breadmaking. LWT-Food Sci Technol 41(1):82–92

Palacios, MC, Haros M, Rosell CM, Sanz Y (2008b) Selection of phytate-degrading human bifidobacteria and application in whole wheat dough fermentation. Food Microbiol 25(1): 169–176

Pandey KR, Naik SR, Vakil BV (2015) Probiotics, prebiotics and synbiotics—a review. J Food Sci Technol 52(12):7577–7587. https://doi.org/10.1007/s13197-015-1921-1

Peña AS (2007) Intestinal flora, probiotics, prebiotics, synbiotics and novel foods. Rev Esp Enferm Dig 99(11):653

Phavichitr N, Puwdee P, Tantibhaedhyangkul R (2013) Cost-benefit analysis of the probiotic treatment of children hospitalized for acute diarrhea in Bangkok, Thailand. Southeast Asian J Trop Med Public Health 44:1065–1071

Pokusaeva K, Fitzgerald GF, van Sinderen D (2011) Carbohydrate metabolism in Bifidobacteria. Gen Nutr. 6(3):285–306. https://doi.org/10.1007/s12263-010-0206-6

Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M (2007) Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol 73:179–185. https://doi.org/10.1128/AEM.01763-06

Qin J, Li R, Raes J, Arumugam M, Burgdorf K, Manichanh C et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65

Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F et al (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490:55–60

Rafter J, Bennett M, Caderni G, Clune I, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan G, Pool-Zobel B et al (2007) Dietary synbiotics reduce cancer risk factors in polypectomized andcolon cancer patients. Am J Clin Nutr 85:488–496

Rao AV, Shiwnarain N, Koo M, Jenkins DJA (1994) Effect of fiber rich foods on the composition of intestinal microflora. Nutr Res 14:523–535

RatnaSudha M, Yelikar KA, Deshpande S (2012) Clinical study of Bacillus coagulans unique IS-2 (ATCC PTA-11748) in the treatment of patients with bacterial vaginosis. Indian J Microbiol 52:396–399

Reale A, Mannina L, Tremonte P, Sobolev AP, Succi M, Sorrentino E, Coppola R (2004) Phytate degradation by lactic acid bacteria and yeasts during the wholemeal dough fermentation: a 31 P NMR study. J Agric Food Chem 52:6300–6305. https://doi.org/10.1021/jf049551p

Rodríguez S, Toca JL (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24(5):500–513

Romeo J, Nova E, Wärnberg J, Gómez-Martínez S, DíazLigia LE, Marcos A (2010) Immunomodulatory effect of fibres, probiotics and synbiotics in different life-stages. Nutr Hosp 25(3):341–9
Ruan Y, Sun J, He J, Chen F, Chen R, Chen H (2015) Effect of probiotics on glycemic control: a systematic review and meta-analysis of randomized, controlled trials. PLoS ONE 10:e0132121. https://doi.org/10.1371/journal.pone.0132121
Safavi M, Farajian S, Kelishadi R, Mirlohi M, Hashemipour M (2013) The effects of synbiotic supplementation on some cardio metabolic risk factors in overweight and obese children: a randomized triple-masked controlled trial. Int J Food Sci Nutr 64:687–693
Sanchez M, Darimont C, Drapeau V, Emady-Azar S, Lepage M, Rezzonico E, Ngom-Bru C, Berger B, Philippe L, Ammon-Zuffrey C et al (2013) Effect of *Lactobacillus rhamnosus* CGMCC1.3724 supplementation on weight loss and maintenance in obese men and women. Br J Nutr 111:1507–1519
Sanchez M, Darimont C, Drapeau V, Emady-Azar S, Lepage M, Rezzonico E, Ngom-Bru C, Berger B, Philippe L, Ammon-Zuffrey C et al (2014) Effect of *Lactobacillus rhamnosus* CGMCC1.3724 supplementation on weight loss and maintenance in obese men and women. Br J Nutr 111:1507–1519
Sánchez B, Delgado S, Blanco-Míguez A, Lourenço A, Gueimonde M, Margolles A (2017) Probiotics, gut microbiota, and their influence on host health and disease. Mol Nutr Food Res 61(1):1600240
Sanders ME, Lenoir-Wijnkoop I, Salminen S, Merenstein DJ, Gibson GR, Petschow BW, Nieuwdorp M, Tancredi DJ, Cifelli CJ, Jacques P, et al (2014) Probiotics and prebiotics: prospects for public health and nutritional recommendations. Ann NY Acad Sci:1309:19–29. https://doi.org/10.1111/nyas.12377
Schachtsiek M, Hammes WP, Hertel C (2004) Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein CPF mediating coaggregation with and aggregation among pathogens. Appl Environ Microbiol 70:7078–7085. https://doi.org/10.1128/AEM.70.12.7078-7085.2004
Schatzmayr G, Zehner F, Taubel M, Schatzmayr D, Klimitsch A, Loibner AP, Binder EM (2006) Microbiologicals for deactivating mycotoxins. Mol Nutr Food Res 50:543–551. https://doi.org/10.1002/mnfr.200500181
Scholtens PA, Alliet P, Raes M, Alles MS, Kroes H, Boehm G, Knippels LM, Knol J, Vandenplas Y (2008) Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. J Nutr 138:1141–1147
Schoster A, Kokotovic B, Permin A, Pedersen PD, Dal Bello F, Guardabassi L (2013) In vitro inhibition of *Clostridium difficile* and *Clostridium perfringens* by commercial probiotic strains. Anaerobe 20:36–41. https://doi.org/10.1016/j.anaerobe.2013.02.006
Sheriff SA, Sundaram B, Ramamoorthy B, Ponnusamy P (2014) Synthesis and in vitro antioxidant functions of protein hydrolysate from backbones of Rastrelligerkanagurta by proteolytic enzymes. Saudi J Biol Sci 21(1):19–26
Shimizu M (1992) Purification and characterization of phytase from *Bacillus suhtilis* (natto) N–77. Biosci Biotechnol Biochem 56:1266–1269. https://doi.org/10.1271/bbb.56.1266
Sims MD, Dawson KA, Newman KE, Spring P, Hoogell DM (2004) Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. Poult Sci 83(7):1148–1154
Suzuki T, Hara T, Kasai T, Tomita F (1998) Effects of difructose anhydride III on calcium absorption on small and large intestine of rats. Biosci Biotech Biochem 62:837–842
Tajadadi-Ebrahimi M, Bahmani F, Shakeri H, Hadaegh H, Hijijafari M, Abedi F, Asemi Z (2014) Effects ofdaily consumption of synbiotic bread on insulin metabolism and serum high-sensitivity C-reactive protein among diabetic patients: a double-blind, randomized, controlled clinical trial. Ann Nutr Metab 65:34–41

Terry P, Giovannucci E, Michels KB, Bergkvist L, Hansen H, Holmberg L, Wolk A (2001) Fruit, vegetables, dietary fiber, and risk of colorectal cancer. J Natl Cancer Inst 93:525–533. https://doi.org/10.1093/jnci/93.7.525
Thomas DW, Greer F (2010) Probiotics and prebiotics in pediatrics. Pediatrics 126:1217–1231. https://doi.org/10.1542/peds.2010-2548
Tomaro-Duchesneau C, Jones ML, Shah D, Jain P, Saha S, Prakash S (2014a) Cholesterol assimilation by Lactobacillus probiotic bacteria: an in vitro investigation. Bio Med Res Int 2014:1–9. https://doi.org/10.1155/2014/380316
Tomaro-Duchesneau C, Saha S, Malhotra M, Jones ML, Labbé A, Rodes L, Kahouli I, Prakash S (2014) Effect of orally administered *L. fermentum* NCIMB 5221 on markers of metabolic syndrome: an in vivo analysis using ZDF rats. Appl Microbiol Biotechnol 98:115–126
Tompkins TA, Xu X, Ahmarani JA (2010) Comprehensive review of post-market clinical studies performed in adults with an Asian probiotic formulation. Benef Microbes 1:93–106
Toushik SH, Lee KT, Lee JS, Kim KS (2017) Functional applications of lignocellulolytic enzymes in the fruit and vegetable processing industries. J Food Sci 82(3):585–593
Tuohy KM, Probert HM, Smejkal CW, Gibson GR (2003) Using probiotics and prebiotics to improve gut health. Drug Discov Today 8:692–699
Upadrasta A, Madempudi RS (2016) Probiotics and blood pressure: current insights. Integr Blood Press Control 9:33–42. https://doi.org/10.2147/IBPC.S73246
Ustundag GH, Altuntas H, Soysal YD, Kokturk F (2017) The effects of synbiotic "Bifidobacteriumlactis B94plus Inulin" addition on standard triple therapy of Helicobacter pylori eradication in children. Can J Gastroenterol Hepatol 2017:8130596
van den Heuvel EGHM, Schoterman MHC, Muijs T (2000) Trans galactooligosaccharides stimulate calcium absorption in postmenopausal women. J Nutr 2000 130:2938–2942
Verhaeghe T, De Winter K, Berland M, De Vreese R, D'hooghe M, Offmann B, Desmet T (2016) Converting bulk sugars into prebiotics: semi-rational design of a transglucosylase with controlled selectivity. Chem Commun (Camb) 52(18):3687–3689. https://doi.org/10.1039/c5cc09940d
Wang CZ, Mehendale SR, Yuan CS (2007) Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness. Am J Chin Med 35(4):543–58
Wealleans AL, Walsh MC, Romero LF, Ravindran V (2017) Comparative effects of two multi-enzyme combinations and a *Bacillus* probiotic on growth performance, digestibility of energy and nutrients, disappearance of non-starch polysaccharides, and gut microflora in broiler chickens. Poult Sci 96(12):4287–4297. https://doi.org/10.3382/ps/pex226
Weinberg ED (1997) The *Lactobacillus* anomaly: total iron abstinence. Perspect Biol Med 40:578–583. https://doi.org/10.1353/pbm.1997.0072
Wu ZJ, DU X, Zheng J (2013) Role of Lactobacillus in the prevention of Clostridium difficile-associated diarrhea: a meta-analysis of randomized controlled trials. Chin Med J (Engl) 126:4154–4161
Xu ZR, Hu CH, Xia MS, Zhan XA, Wang MQ (2003) Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult Sci 82 (6):1030–1036
Yokota A, Enomoto K, Tomita F (1991) Purification and properties of an inulin fructotransferase (depolymerizing) from Arthrobacter sp. H65-7. J Ferment Bioeng 72:262–265
Yokoyama K, Nio N, Kikuchi Y (2004) Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol 67:447–454
Yu H, Liu L, Chang Z, Wang S, Wen B, Yin P, Liu D, Chen B, Zhang J (2013) Genome sequence of the bacterium *Bifidobacterium longum* strain CMCC P0001, a probiotic strain used for treating gastrointestinal disease. Genome Announc. https://doi.org/10.1128/genomea.00716-13
Zelaya H, Tsukida K, Chiba E, Marranzino G, Alvarez S, Kitazawa H, Agüero G, Villena J (2014) Immunobiotic Lactobacilli reduce viral-associated pulmonary damage through the modulation of inflammation-coagulation interactions. Int Immunopharmacol 19:161–173
Zhang H, DiBaise J, Zuccolo A, Kudrna D, Braidotti M, Yu Y et al (2009) Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci USA 106:2365–2370

Chapter 21
Biorefinery Approach for Red Seaweeds Biomass as Source for Enzymes Production: Food and Biofuels Industry

Arturo Siller-Sánchez, Héctor A. Ruiz, Cristóbal N. Aguilar and Rosa M. Rodríguez-Jasso

Abstract The biorefineries of seaweeds biomass in recent years have attracted attention, in this case, red seaweeds are a source of sulphated galactans such as agar and carrageenan that are relevant polysaccharides commonly applied as powerful gelling agents and stabilizers, particularly, for food and nutraceutical purposes, moreover, the rich quantity of cellulose are also studied as a source for biofuels production. Moreover, recent reports have focused on the wide biological activities of these polysaccharides as antithrombotics, antimetastasics, antivirals, anti-inflammatories, and anticoagulants agents, based on the molecular weight and sulphation degree. Galactan hydrolases specific for polyanionic and insoluble polysaccharides are mainly classified as agarases and carrageenases. The extraction and production by different technologies of red seaweed hydrolytic enzymes open the way to a new field of applications in terms of the biorefinery concept for food and biofuels applications. This chapter provides a comprehensive overview of various aspects related to the red seaweed biomass including sugar composition and relevance and its hydrolases enzymes extraction and production, as well as the biotransformation of the biomolecules obtained and its application, properties, and uses under a biorefinery approach.

Keywords Red seaweed · Polysaccharides · Hydrolases · Food industry Biorefinery

A. Siller-Sánchez · H. A. Ruiz · C. N. Aguilar · R. M. Rodríguez-Jasso (✉)
Biorefinery Group, Food Research Department, Faculty of Chemical Sciences, Autonomous University of Coahuila, Blvd. V. Carranza e Ing. José Cárdenas Valdés, 25280 Saltillo, Coahuila, Mexico
e-mail: rrodriguezjasso@uadec.edu.mx

H. A. Ruiz · R. M. Rodríguez-Jasso
Cluster of Bioalcoholes, Mexican Centre for Innovation in Bioenergy (Cemie-Bio), Mexico, Mexico

© Springer Nature Singapore Pte Ltd. 2019

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_21

21.1 Introduction

The importance of red seaweed biomass rests mainly in obtaining hydrocolloids, which are known as sulphated galactans such as agar and carrageenan, and they are found within the cell wall of seaweed. These polysaccharides can vary in the chemical structure, the molecular weight of the compounds obtained according to the extraction method used, the degree of esterification of sulfate, the position of the glycosidic bond, the sulfate groups, and other substituents (Mollah et al. 2009).

The most important use that is given to this type of compounds is reflected in the food industry as well as in biotechnology area, and in the last years in the concept of biorefinery (Ruiz et al. 2015; Cervantes-Cisneros et al. 2017). The use in the food industry is mainly due to the physical properties that these compounds provide for being a powerful gelling agent and thickener, as well as the use in the biotechnological industry with a potential for microorganism cultures (Rengasamy et al. 2014). The biorefinery concept offers a wide range of products which helps in waste reduction as well as decrease pollution. It also helps from structural modifications to the area of uses of the main products and increases the commercial value.

On the other hand, enzymes can provide an opportunity to modify these compounds. These enzymes could be used in the untreated seaweeds biomass in the extraction of galactans, providing reductions in the time of obtaining products, decrease in the energy cost process compared to other methods of compounds structural modification, decreasing in the use of solvents, increasing the yield in the production, and preserving or increasing the biological activity (de Borba Gurpilhares et al. 2016).

One of the most promising industries is biofuels, in which the red seaweed biomass (rich in cellulose) can be used as substrate and hydrolyzed by enzymes to release fermentable sugars for the production of biofuels as bioethanol (Ruiz et al. 2015), also the sulfactated galactans by depolymerization to monosaccharides can be used for biofuels production of biofuels. In terms of biorefinery concept, the oligosaccharides from this biomass can present very interesting biological activities, taking into account that these would vary by the molecular structure, and then it is important to analyze the structures of both poly- and oligosaccharides and develop strategies for producing pure oligosaccharides with biological activities (Courtois 2009). This review aims to offer an updated view of the chemical structural properties presented by the products containing red seaweed biomass and present a discussion of the methods used for structural modification focused mainly on enzymatic modification to be used in different areas as food and biofuels according to the concept of biorefinery.

21.2 Seaweed

Also known as “macroalgae”, the seaweeds are multicellular organisms, aquatic, eukaryotic, photosynthetic, found mainly in the oceanic coasts, attached to rocks or solid surfaces, at different depths, arriving to find algae up to 25 m deep, where the limited light availability (Ledermann et al. 2017), also the seaweeds have also been found floating at sea level near the coasts.

Approximately there are between 10,000 species of seaweed in nature (Sudhakar et al. 2018). Seaweed are divided into three large groups: Green seaweed (*Chlorophyta*), Brown seaweed (*Phaeophyta*), and Red seaweed (*Rodophyta*). It is possible to distinguish the polysaccharides of these biomasses, which are divided into two large groups, according to their function, as they can be those that give structure and those that serve for energy storage. Within the structural polysaccharides, we can mainly find cellulose, agar, and carrageenan, among others, which are inside the cell wall, as well as for energy storage highlight the starch and laminarin, among others. The classification and polysaccharides quantity and quality are what gives the commercial importance to the seaweed species and seasonal collected region.

Compared with terrestrial biomass, it has some very noticeable differences, such as the capacity to retain water, since seaweeds have the capacity to store water between 90 and 70%, compared to terrestrial biomass, which varies enormously depending on the type of terrestrial plant (Milledge et al. 2014). The largest amount of metals within the chemical composition of the seaweed can be approximately 50–20% dry weight (Cesário et al. 2018). Also, one of the most important characteristics present in the seaweed, unlike the terrestrial biomass, is the lignin content. This polymer provides that characteristic stiffness in terrestrial biomass, which in seaweed is not necessary. This reflects as one of the greatest advantages that has been raised within some industries, as it is in biofuels, by simplifying the process of extraction of polysaccharides and to facilitate later stages such as enzymatic saccharification. Another advantage that is found in seaweed is the fact that it does not need arable land for its production, does not compete with food crops, as well as does not require fresh water, as it grows in seawater. It also presents a higher photosynthetic velocity with which the ability to absorb CO_2 benefits. As it is possible to see, it has some advantages that allow it to be used in different areas and industries to be used in the concept of biorefinery.

21.2.1 Global Distribution of Seaweed

The distribution of seaweed in the world is divided by four main zones, which are the western and eastern zones of the Atlantic and the western and eastern zones of the Pacific (Hayashi et al. 2014). The diversity of seaweed species varies according to the region in which it is found; at present, 42 countries are seaweed producers

being the most relevant considering the five continents China, Indonesia, Philippines, Japan, South Korea, North Korea, Malaysia, France, Ireland, Norway, Spain, Portugal, Argentina, Chile, Brazil, Mexico, and Australia (Boonstra 2015; Nedumaran and Arulbalachandran 2015).

It has been found 221 species of usable seaweed, where 145 species are applied within the food industry and 110 species are focused in phycocolloids industry (Nedumaran and Arulbalachandran 2015). Statistics published by FAO in 2014 reported a production of 26 million tons of fresh seaweed, worth USD 7.3 million annually (Baghel et al. 2016). However, recent studies have shown that seaweed is also rich in other metabolites such as lipids, proteins, pigments, oligosaccharides, and minerals, which give them greater economic benefit in terms of a biorefinery (Griffiths et al. 2016).

21.2.2 The Three Types of Seaweed

21.2.2.1 Green Seaweed

Green seaweed has the characteristic form as, a simple stem, thin sheets like paper, these seaweeds are the most similar to terrestrial plants, for example, in the coloration, which is given by chlorophyll "A", as well as it contains carotenoids type "B", these compounds have been found among 7,000 species, which are mainly found in bays, tide pools and mainly in shallow waters.

The main compositions find as energy reserve is based in polysaccharides as starch with concentrations of 1–4%. The structural polysaccharides such as ulvan and cellulose have percentages of 8–29 and 38–52% dry weight, respectively (Cesário et al. 2018). Ulvan is a sulphated heteropolysaccharide considered as a hydrocolloid, being soluble in water, with possible applications in chemistry, biomedical, agricultural, and pharmaceutical area (Cardoso et al. 2014). However, compared with other hydrocolloids, there are fewer commercial applications reported. Ulvan is mainly composed of L-rhamnose, xylose, glucose, uronic acid, and iduronic acid. Some examples of green seaweed are *Ulva lactuca*, *Ulva rigida*, *Ulva pertusa*, *Cladophora rupestris*, among others.

21.2.2.2 Brown Seaweed

Brown seaweed has complex stems since there are organisms that grow up to 100 m high, allowing it to survive the air exposure. The coloration presented in this seaweed group is found from olive green to dark brown, the chemical components responsible for this range of coloration are: Chlorophyll type "A" and type "C", which provide the green tones and fucoxanthins content provide more yellowish-brown tones, that cover the coloration that gives the chlorophyll. There are known about 1,500 species around the world that are able to resist different

temperatures found in both temperate zones and in polar areas. This type of seaweed has been found in places such as North America, Europe, the Gulf of Mexico, and in the middle Atlantic (Sudhakar et al. 2018). They are found mainly on rocky shores, forming what is known as "seaweed forest" (due to the longitude that reaches the seaweed), which becomes the habitat of countless species of fish, marine mammals, and invertebrates.

The main composition of these seaweeds is based on energy reserves polysaccharides such as laminarin and mannitol. Laminarin represents 35% in dry weight in brown seaweed, it is a polysaccharide constituted by 20–25 glucose units, it is soluble in water and at the end of this molecule it is possible to find bound at the reducing end of the glucose unit to mannitol, which is a polyalcohol (also called as alcoholic sugar). Laminarin looking for the biological activities it presents, such as antioxidant, anti-tumor, antimicrobial, immune modulation, and anticoagulant properties (Cardoso et al. 2014). Recently, the interest is focused on its prebiotic capacity, not being digested by the human digestive system, thus stimulating the growth of the favorable intestinal microbiota. Mannitol is used mainly in the food industry as a common sweetener, mainly in chewing gum, to replace glucose, for diabetics.

Brown seaweed is also composed of structural polysaccharides with commercial importance such as alginate and fucoidan. Alginate represents 40% in dry weight (Davis et al. 2003; Draget 2009), it is composed mainly of two acids, mannuronic acid, and glucuronic acid, these can be found alternately or in groups of a single acid, depending on the species of seaweed from which they are obtained, which differs from the properties presented (Draget 2009), as well as some species are known to produce high quality alginates, which are used to increase the viscosity of aqueous solutions, so it is used in both textile and food industries to form gels and gelatins.

Fucoidan is a highly branched heterogeneous sulfated polysaccharide linked by 1-2 bonds of α-fucose-4-sulfated linked to different compounds, such as D-xylose, D-galactose, D-mannose, and glucuronic acid depending on the species from which it is obtained, the importance of fucoidan is due to its therapeutic properties such as anti-inflammatory and anticoagulant (Cumashi et al. 2007). Some examples of brown seaweed relevant species are *Laminaria*, *Saccharina*, *Alaria*, *Fucus*, *Macrocystis*, *Sargassum*, among others.

21.2.2.3 Red Seaweed

Red seaweed growth as a filament, sheets of cells, and it is considered to be a parasite for other seaweed, reaching a growth height up to 1–5 m. These seaweeds have a red, pink color, and even purple in some case, the compounds responsible for the color are chlorophyll "A" and phycobilins (Schubert et al. 2006), which allows the photosynthesis in very low depths and with low temperatures, where this type of seaweed normally grows, although it is also found in warm waters with few depths, it is the most abundant seaweed with approximately 4,000 species

(Sudhakar et al. 2018). The main energy reserve polysaccharides are florid starch, similar to the branched fraction of amylopectin from plant starches (Kravchenko et al. 2013). The structural polysaccharides are a very particular group with different physical, chemical, and biological qualities, known as galactan (Jiao et al. 2011), which it is discussed in the following sections.

The concept of biorefinery integrates processes of bioconversion of algal biomass, for the production of fuels, energy, and chemical products are of great added value. The red seaweed biomass could be exploited under the biorefinery approach due to the different stages of valorization and products for different purposes, such as for food and feed uses, additives, supplements, as well as fertilizers, cosmetics, and pharmaceutical products, in conjunction with the production of biofuels. Seeking to achieve a proper platform for the separation recovery of the maximum products that allow to be a profitable biomass by obtaining different high value products, which can be obtained through different types of sustainable strategies to recover lipids, proteins, pigments, minerals, and the fraction of carbohydrates that for certain species is the most searched for food and fuels purpose.

21.2.3 Chemical Composition of Red Seaweed

Red seaweed present natural macromolecules that have complex biochemical structures. Among the most important polysaccharides of red seaweed are agar, carrageenan, and cellulose, molecules that include glucose and galactose among their monosaccharides, and less quantity of xylans and mannan (Usov 2011). These polysaccharides are sought for the properties that this biomass confers, as well as the applications that could be given to them, just as it is known that one crucial factor that defines the possible use is jeopardized by the extraction and recovery procedure and applied technology to obtain its structural compounds. In Table 21.1 is described the used treatment and the focal purpose of red seaweed polysaccharides from different species and regions under a biorefinery approach. It means that depending on the treatment and the combination of process stages applied in the biomass, it can be used to obtain different high-value products in order to be a sustainable biomass.

21.2.3.1 Agar

Discovered in the year 1658 in Japan, technically called Agar-Agar, better known as Agar, used mainly for its colloidal properties being the first phycocolloids discovered and used food additive approved by the Food and Drug Administration (Koch 1882) and the relevance of this polysaccharide was detonated when the application for microbiology area was discovered (Armisen and Galatas 2009). Defined as a hydrocolloid, it is a strong gelling agent extracted from red seaweed of the genera *Gelidium*, *Gelidiella*, *Pterocladia*, *Gracilaria*, *Ahnfeltia* (Armisen 1995).

Table 21.1 Polysaccharides in red seaweed under a biorefinery approach

Polymer	Seaweed species	Country	Treatment	Application	Reference
Agar	*Gracilaria salicornia*	Thailand	Autoclave at 121 °C for 15 min	Photosynthetic efficiency and agar properties	Chirapart and Praiboon (2018)
Agar	*Gracialaria greville*	China	50 °C for 2 h with water, after neutral protease (90 U/mL, neutral protease/ supernatant)	Rheological properties	Huang et al. (2017)
Agar	*Gracilariopsis lemaneiformis*	China	6% NaOH at 80 °C for 2 h	Antioxidant and immunomodulatory activities	Shi et al. (2017)
Agar	*Gelidium amansii*	South Korea	121 °C for 20, 40, 60, and 80 min	Bioethanol production	Kim et al. (2015)
Agar	*Gracilaria verrucosa*	Korea	Thermal acid hydrolysis with 270 mM sulfuric acid at 121 °C for 60 min	Bioethanol production	Ra et al. (2015)
Sulfated Agarose	*Polysiphonia senti culosa*	China	Distilled water at 80 °C for 3 h (3 times)	Structure and immunomodulatory activity	Zhao et al. (2017)
Agar	*Gracilaria vermiculophylla*	Portugal	t (20–10 min), T (90–70 °C), solvent (40–20 mL)	Optimization of extraction	Sousa et al. (2010)
Agar	*Gelidium latifolium*	Turkey	Water bath plus magnetic stirrer extraction with 95 °C, 350 rpm for 6 h. Autoclave extraction with 110 °C for 30 min	Gelling and melting temperature, pH, viscosity, and sulfate contents	Öğretmen and Duyar (2018)
Agarose	*Gelidium amansii*	Malaysia	0.1 M HCl solution and sonicated at 30 kHz, 40% amplitude for 30 min	Physicochemical properties analysis	Chew et al. (2018)
Agar	*Hydropuntia cornea*	México	T (80, 90 and 100 °C) and for t (2, 3 and 4 h)	Physicochemical properties analysis	Pereira-Pacheco et al. (2007)

(continued)

Table 21.1 (continued)

Polymer	Seaweed species	Country	Treatment	Application	Reference
Carrageenan	*Kappaphycus alvarezii*	Brazil	First 6% KOH solution (w/v) for 24 h at 25 °C (“cold” alkali transformation) after 65 °C for 2 h at 120 rpm with water	Chemical analysis and biorefinery	Masarin et al. (2016)
Carrageenan	*Kappaphycus alvarezii*	Brazil	First 6% KOH solution (w/v) for 24 h at 25 °C (“cold” alkali transformation) after 65 °C for 2 h at 120 rpm with water	Chemical, structural for future bioenergy generation	Roldán et al. (2017)
Carrageenan	*Chondrus armatus Ahnfeltiopsis flabelliformis Tichocarpus crinitus*	Japan	90 °C for 2 h in a boiling water bath	Matrices for the Inclusion of Echinochrome	Yermak et al. (2017)
Carrageenan	*Kappaphycus alvarezii*	India	Autoclave at 0.9 N H2SO4 at 100 °C	Bioethanol production	Khambhaty et al. (2012)
Kappa-Carrageenan	*Hypnea musciformis*	Brazil	Papain digestion (6 h, 60 °C)	Antioxidant, antimicrobial, cytotoxic, anticancer and neuroprotective activities	Souza et al. (2018)
Carrageenan	*Mastocarpus stellatus*	Spain	Enzymatic hydrolysis for 3 h, using protease 2.4 L (EC 3.4.21.14) pH 8, 50 °C	Obtaining bioactive ingredients	Blanco-Pascual et al. (2014)
Carrageenan	*Hypnea musciformis*	Bangladesh	Ultrasonic assisted extraction t of 10 and 20 min and ultrasonic powers of 400 and 500 W	Characterization of its chemical, rheological properties and antioxidant activities	Rafiquzzaman et al. (2016)
Carrageenan	*Solieria chordalis*	France	Microwave-assisted extraction with T (90 and 105 °C) and t (10, 20, and 25 min)	Anti-herpetic activity (HSV-1)	Boulho et al. (2017)

(continued)

Table 21.1 (continued)

Polymer	Seaweed species	Country	Treatment	Application	Reference
Carrageenan	*Eucheuma spinosum*	Indonesia	95 °C for 3 h with adequate stirring	Chemical and physical characteristics	Diharmi et al. (2017)
Carrageenan	*Hypnea musciformis*	México	Microwave-assisted extraction T (85, 95, and 105 °C) and T (10 and 20 min)	Optimization of extraction conditions	Vázquez-Delfín et al. (2014)
Cellulose	*Gelidium elegans*	Malaysia	3 stages alkalization, bleaching treatment and acid hydrolysis treatment	Morphological analysis of new cellulose nanomaterial	Chen et al. (2016)
Cellulose	*Gracilaria verrucosa*	India	Pulp obtained from agar extraction with alkali treatment	Bioethanol production	Kumar et al. (2013)
Cellulose	*Gracilaria dura* *Gelidium pusillum*	India	Integrated biorefinery process	Biorefining of marine macroalgal biomass	Baghel et al. (2015)
Cellulose	*Gelidium corneum* *Gelidium amansii*	Republic of Korea Morocco	The extraction process is similar to the pulping process used in the manufacture of wood pulp	Pulp can be an alternative source of raw material for papermaking	Seo et al. (2010)
Sulfated xylomannan	*Scinaia hatei*	India	Depigmentation, washing, separation, dialysate, precipitate and fractional.	Anti-herpetic activity	Mandal et al. (2008)

This polysaccharide is a molecule consisting of two fractions: Agarose and Agaropectin. Agarose is a long molecule composed of D-galactose linked by α(1-3) bonds with 3,6-anhydro-L-galactose molecules which in turn is bound by β(1-4) bonds to the following molecule of D-galactose. The agarose is the one that contains the high gelling power, unlike agaropectin that has low gelling power in water, this is due to its constitution of D- and L-galactose in regularly substituted methyl, sulfate, and pyruvate groups, which are responsible for affecting the gelling property. The amount of sulfate varies from 5 to 8% (Armisen and Galatas 2009; Hamer et al. 1977). Figure 21.1a shows the chemical structure of agarose. The agarose industrially is extracted by the methods freezing-thawing and the syneresis method. The industrial methods are more used than the separation of water agar, since the way of extraction is similar for both methods, in which the plant material is placed inside tanks with water, which is brought to boiling temperature for several hours, so that the agar dissolves in the water (Armisen and Galatas 2009). The freeze-thaw method is based on the decrease in temperature allowing the agar to gel, then the temperature is mostly lowered for freezing, followed by thawing to carry out the separation of the water. The method of syneresis, also known as pressure-filtering, it is based on the absorption of water in the gel, which can be removed by force applied properly by separating the water from the agar. Therefore, the method of extraction, the season in which it is collected, the genus of seaweed, the concentration of agarose, the concentration of agaropectin and the concentration of sulphated compounds, the molecular weight of the molecule, the temperatures of gelation, and fusion are important parameters for the use and application of this polysaccharide (Pereira-Pacheco et al. 2007).

21.2.3.2 Carrageenans

Discovered in the year 1810 in Ireland, named at that time with the common name of Carrigan or Carrageen, which means "Little Rock" known today as carrageenan, this polysaccharide is a hydrocolloid used mainly in the food industry, for its physical properties such as stabilizer and gelling agent. These properties are used in the meat industry, in sausages and pates to give the consistency and stability that presents this type of products, this polysaccharide can also be used to give more viscosity to dairy desserts, it is also used to improve the texture as in cottage cheese (Necas and Motosikova 2013). The food industry uses most of the world production of carrageenans, this is around 70–80% (Campo et al. 2009). The rest of the production has uses in another type of industry, such as pharmaceuticals, cosmetics, and textiles. Carrageenans are extracted from *Kappaphycus*, *Gigartina*, *Euchema*, *Chondrus,* and *Hypnea*. Reports indicate that a proportion of up to 50% of dry weight can be found as carrageenans (Rhein-Knudsen et al. 2015). Carrageenans are molecules that belong to the family of sulfated linear hydrophilic galactans. It consists mainly of very complex repetitive disaccharide units, formed by molecules of 3,6-anhydro-D-galactose linked by (1,4) glucosidic bonds to D-galactose, which

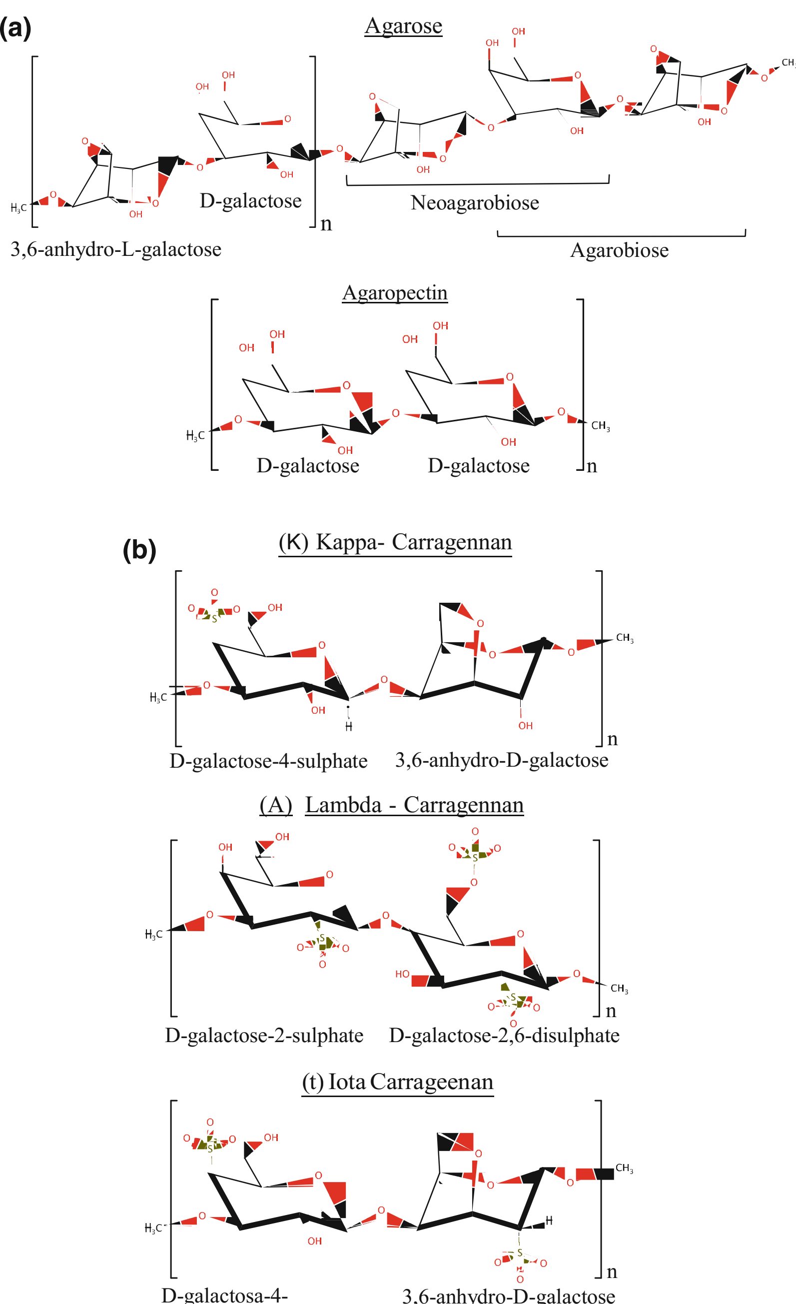


Fig. 21.1 Structure of red seaweed polysaccharides: **a** agar, sulfated polysaccharide composed of agarose molecules (molecule in greater proportion) and agaropectin (where the L- and D-configuration can vary in the molecule, as well as changes in substituents affecting gelation); **b** carrageenans, sulfated polysaccharides found in three different types (kappa, iota, and lambda) defined by the sulfate content; **c** cellulose, structural polysaccharide composed of glucose; **d** xylan and mannan, structural polysaccharides composed of xylose and mannose, respectively

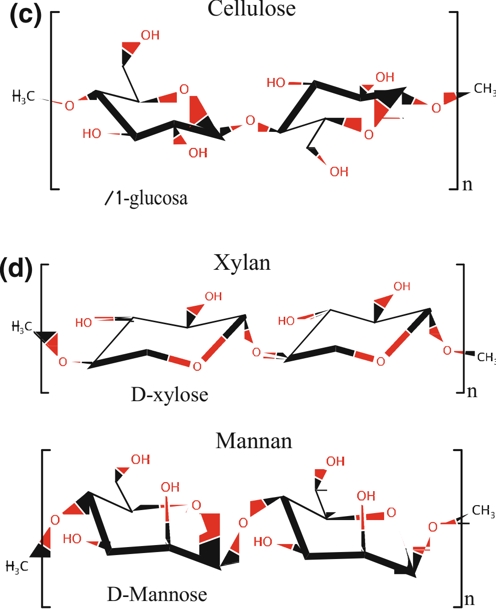


Fig. 21.1 (continued)

in turn is linked by (1,3) glucosidic bonds. The classification of these compounds is given by the presence of sulfate groups, based on the position and number thereof.

The base structures are shown in Fig. 21.1b, which consists of three main carrageenans classified as κ-, ι- and λ-. The factors that influence the properties of these molecules are the sulfate esters substitution, which gives different properties, with higher levels of esters translates into lower solubility temperature, as well as a lower resistance to jellified (Chauhan and Saxena 2016; Necas and Motosikova 2013). There are reports of commercial carrageenans that indicate that the amounts of sulfate in κ-carrageenan is already 38% (w/w), ι-carrageenan 32% (w/w), and λ-carrageenan 22% (w/w) (McHugh 2003), as well as for dimers, it is known that each carrageenan (κ-, ι- and λ-.) has one, two, and three sulfate ester groups, respectively, therefore, the sulfated content corresponds to 20, 33 and 44% (w/w), respectively (Campo et al. 2009; Chauhan and Saxena 2016). Also salt concentration and type of salt (NaCl, MgCl2, CaCl2, SrCl2, and KCl) can modify the physical properties of this type of compound, causing different effects on gelation (Bixler and Porse 2011). The best results to promote gelation are given in *k*-carrageenan with the addition of KCl and for i-carrageenan with Ca^{2+} ions, unlike l-carrageenan gelation is not affected by the salts addition (Chauhan and Saxena 2016).

The commercial production of the different types of carrageenans is obtained from different sources of seaweed, such as *k*-carrageenan is obtained mainly from *Kappaphycus alvarezii*, i-carrageenan from the species of *Euchema denticulatum*

and l-carrageenan has been reported in *Gigartina* and *Chondrus* species. However, the major effect in chemical structure and the physical properties are given by the extraction method and the parameters used (temperature, pH and residence time) (Kara et al. 2006). One of the methods used to obtain commercial carrageenan is the freeze-thaw method, where the solution obtained from an alkali treatment is completely frozen, thawed, the water is eliminated, and the compound is obtained. Another method for the separation of carrageenan extracted by the alkali treatment is known as the method of alcohol precipitation, this method consists of placing the solution with carrageenans in 2-propanol and other alcohols, which leads to its precipitation (Chauhan and Saxena 2016). Extraction with hot water has also been applied as pretreatment (water and temperature), where it is evaporated to reduce the volume and introduce impregnated rows with KCl (1.0–1.5%), where the gel separation is carried out (Hilliou et al. 2006).

21.2.3.3 Cellulose

Linear polysaccharide formed by β-D-glucans linked by β-(1,4) glycosidic bonds (Fig. 21.1c) with different degrees of polymerization, joined by hydrogen bridges, which gives a crystalline structure, based on this and the numerous hydroxyl groups contained, the formation of microfibrils is favored, which provide a resistance, giving structure to the cell wall. Cellulose is the most predominant organic polymer in terrestrial plant composition, reaching values of up to 30–50% (Rowe et al. 2009). The cellulose contained in red seaweed are presented in values of 13–19% in dry weight and the importance of this polymer lies mainly in the hydrolysis process to obtain monosaccharides for fermentation and the particularity low content of lignin, which is used in the development of biofuels to reduce the dependence on fossil biofuels (Michelin et al. 2015).

21.2.3.4 Xylans and Mannan

Hemicelluloses are a group of structural heteropolysaccharides with β-1,4 binding in their monomers (Fig. 21.1d). Inside red seaweed, it is possible to find groups of polysaccharides such as xyloglucans, glucuronoxylans, xylans, mannans, glucomannans, and galactoglucomannans (John et al. 2011). These chemical structures are insoluble in water and form strong non-covalent associations with cellulose microfibers, their main role being to give greater resistance to the wall. Xyloglucans are the most abundant hemicelluloses in the cell wall and are composed mostly of xylose.

21.2.4 Economic Aspects in Seaweed Polysaccharides

Seaweed industry has an annual global value of USD 5.5–6 billion, mainly used for food, phycocolloids, fertilizer, animal feed additives, cosmetics, and medicines. Products from seaweeds have become very attractive for the food industry; especially for its high capacities to act as thickening and gelling agents that help to improve rheological characteristics of certain products; moreover, it has been reported as sources to provide health benefits acting as anticoagulants, antioxidants, anti-tumor, antimicrobials, etc. Moreover, it is important to mention that an area under development with an increased interest is the use of seaweeds for bioalcohols production with significant and positive results, the process is based principally on the biorefinery concept in order to produce biofuels and high added value compounds (Usman et al. 2017; Ruíz et al. 2015; Cervantes-Cisneros et al. 2017; Pereira et al. 2013; Ruiz et al. 2013). Polysaccharides extracted from seaweeds have become very attractive to the industrial sector especially for its rheological characteristics, the most commercial compounds are carrageenan, alginate, and agar because they have important properties such as their ability to form gels, such as stabilizers, thickeners, and emulsifiers, allowing them to be widely used in food industry (Cervantes-Cisneros et al. 2017; Pereira et al. 2013; Vega-Villasante et al. 2010) (Table 21.2).

The production of agar is mainly food industry occupying 90% of the total production (Quitral et al. 2012), where the cost of the product can be relatively cheap, reaching a price of US $18 per kg, however, the application in fine chemistry has been increasing, such as DNA research and electrophoresis causing a significantly change in the price of this product, due to the high quality of agarose produced for this purpose, which can reach prices of up to US $5,000 per kg (McHugh 2003). The agar world production for 2009 was around 10,000 tons with an approximate value of $175 million (Web of science 2018; The Sea Weed Site 2015).

The production of carrageenan exceeded 60,000 tons in the world with a value of more than US $626 million per year (The Sea Weed Site 2015), with a price of US $10.4 per kg, due to its physicochemical properties the different forms of carrageenan are used in the food industry (jam, bread dough, cheese, and ice cream), also in pet food in canned foods and its binder and no comedogenic characteristic is also used in cosmetic industry. Currently, the attention of these compounds has been based for pharmaceutical used, either in the incorporation to matrices to control the release of drugs (53), or for the inhibition of viruses such as the dengue, herpes, and human papillomavirus.

Table 21.2 Most relevant applications of seaweed polysaccharides in industry

Polysaccharide	Property	Application in the food industry	Health benefits for other industries	Reference
Red seaweed				
Agar	Gel	Japanese cuisine, in meat and fish products to mimic gelatin and other jelly products	– Dietary fiber – Decrease cholesterol and blood lipids – Prevent heart disease, atherosclerosis, and hyperlipidemia – Antiviral activity against dengue virus and papillomavirus	Cervantes-Cisneros et al. (2017), Rhein-Knudsen et al. (2015), Barbeyron et al. (2001), Naumoff (2004)
Carrageenan	Gelling agents, thickeners, and stabilizer	Jellies		
Brown seaweed				
Laminaran	Dietary fiber	As an additive	– Help to reduce cholesterol absorption in the intestine and blood – Anti-apototic Immuno-stimulatory – Good source of dietary fiber – Anticoagulant – Antioxidant – Anti-inflammatory – Anti-tumor activity	Barbeyron et al. (2001), Peters et al. (2001), Gómez-Ordóñez (2013)
	Stabilizers	Manufacture of ice cream	– Aid in wound healing	Barbeyron et al. (2001), Naumoff (2004), Peters et al. (2001), Mayakrishnan et al. (2013)
	Thickeners	Sauces, syrups, toppings for ice cream and pie fillings	– Tissue regeneration with the addition of collagen – Anti-tumor	
Alginate	Mixtures of calcium salts and sodium alginate	Emulsifiers in water/oil solutions for mayonnaise and salad dressings preparation		
		Supports the formation of gel retarder		
		Jellies, instant desserts		

(continued)

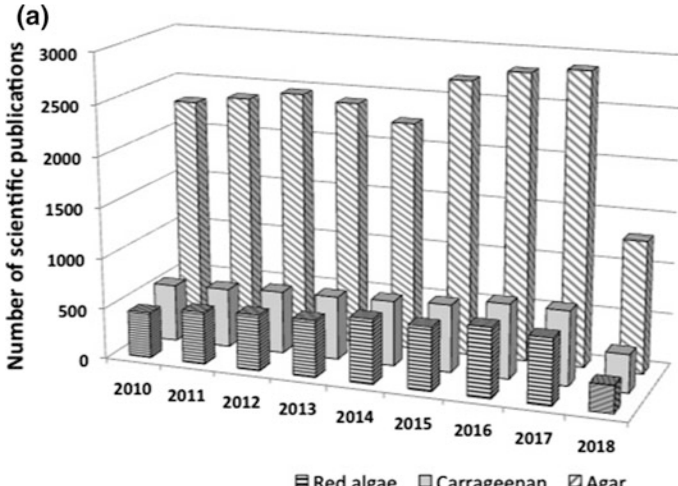
Table 21.2 (continued)

Polysaccharide	Property	Application in the food industry	Health benefits for other industries	Reference
	Gel	Food production restructured as meats, chicken nuggets, meat pies, fish fillet restructured		
	Films and coatings	Conservation of different food matrix		

21.2.5 *Enzymatic Modification of Sulfated Polysaccharide for Bioactivity Enhance, Application for Food and Biofuel*

Something distinctive and of greater importance within the approach of the biorefinery, refers to the obtaining of products of high value-added, by means of these products economically profitable, however, to obtain these products it is possible by structural improvements in order change its chemical, biological, and physical properties, these modifications can be achieved by different methodologies, described below, with greater importance to the modification by enzymes. The literature with respect to the modification of red seaweed compounds since 2010, showed an increasing interest in carrageenan and agar as biomolecules, with a notable higher prevalence in agar-based studies. Figure 21.2a, b showed the published research reported from 2010 to 2018 in the ISI Web of Knowledge with the keywords "red seaweed/seaweed", "carrageenan", "agar", "Carrageenases", "agarases", "red seaweed and enzyme", "carrageenan and enzyme", "agar and enzyme" (Mohamed et al. 2012). Additionally, it confirms that there are only few studies, less than 100 publications, based on the specific topics of hydrolytic enzymes of sulfated polysaccharides from red seaweed. These research reports have been based on carrageenases and agarases either for the polysaccharides extraction or modification or the enzymes production from marine biomass, among others.

The studied enzymes in Fig. 21.2 were based on the classifications of galactan hydrolases with the ability to hydrolyze the C–O, C–N, and C–C bonds (Table 21.3). These group of enzymes have the capacity to degrade both galactans and sulphated galactans present in red seaweed, knowing the nature of the applied substrate (Gupta and Abu-Ghannam 2011), as well as the chemical structures it is reduced to a more specific group of enzymes that are described below.



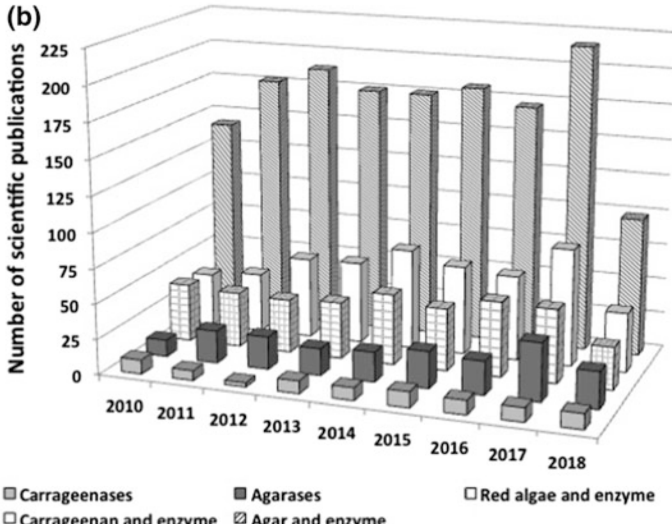


Fig. 21.2 Number of scientific publications published on the topics related with red polysaccharides and enzymes as a function of publication years, taken from ISI Web of Knowledge. **a** Search topic from first to third row: red fifth row: carrageenases, agarases, red seaweed seaweed/seaweed, carrageenan, agar. **b** Search topic from first to rases, seaweed, and enzyme, carrageenan and enzyme, agar and enzyme

Table 21.3 Enzymes used in polysaccharides modification

Microorganism	Enzyme	Catalytic reaction	Fermentation	Reference
Alteromonas sp. SY37-12	β-agarase	Hydrolysis of (1,4)-β-D-linkages in agarose	In plate, 35 °C, pH 7.0, 1.5% agar	Wang et al. (2006)
Thalassomonas sp. JAMB-A33	α-agarase	Endohydrolysis of (1,3)-a-D-linkages in agarose	Liquid culture 75 rpm, pH 8.5, 45 °C and 0.1% agar	Ohta et al. (2005)
Pseudomonas aeruginosa AGLSL-11	β-agarase	Hydrolysis of (1,4)-β-D-linkages in agarose	Liquid culture 170 rpm, pH 8.30 °C and 0.3% w/v agar	Lakshmikanth et al. (2006)
Agarivorans albus YKW-34	β-agarase	Hydrolysis of (1,4)-β-D-linkages in agarose	Liquid culture 120 rpm, pH 8.40 °C and 0.23% agar	Fu et al. (2008)
Agarivorans sp. HZ105	β-agarase	Hydrolysis of (1,4)-β-D-linkages in agarose	Liquid culture 175 rpm, 25 °C and 1% agar	Hu et al. (2009)
Zolbellia galacta	ι-carrageenase	Endohydrolysis of (1,4)-β-D-linkages in ι-carrageenase	Liquid culture pH 7.2, 37 °C and 2% carragenan	Barbeyron et al. (2000)
Pseudomonas atlantica T6c	Sulfatase	Eliminates sulfate from D-galactose 4-sulfate, producing D-galactose	Liquid culture 20 °C, 0.1% w/v κ-, ι-, or λ-carrageenan	Préchoux et al. (2013)
Pseudoalteromonas carrageenovora	λ-carrageenase	Hydrolysis of (1,4)-β-D-linkages in λ-carrageenase	Liquid culture 20 °C, 0.2% w/v λ-carrageenan	Guibet et al. (2007)

21.2.5.1 Alfa Galactosidase

Also known as melibioses, it is an exoglycosidase with the ability to break the α-(1,6) galactosidic bonds, including the oligosaccharides of galactose, melibiose, raffinose, stachyose, galactomannans, and galactoglucomannans (Kadam et al. 2015). These enzymes have several applications, which can be divided between medical applications and industrial applications. In medical applications, it is used for the treatment of Fabry's Disease (Ermakova et al. 2013), as it is also applied as digestive enzyme to degrade the sugars with galactose residues that reach the large intestine (Levine and Weisman 2004), as well as has the ability to convert the groups blood, as they are from group "B" in group "O", for blood transfusions (Gong et al. 2005), another important application in medicine is to prevent xenoreception in xenotransplantation (Parker et al. 1995).

In the industrial applications, these enzymes are used to increase the nutritional value of vegetables, as it is a rich source of oligosaccharides of raffinose, stachyose, melibiose (LeBlanc et al. 2004), it is used in sugar beet industry, by eliminating raffinose to improve the crystallization process for sugar, also is reported for the partial enzymatic hydrolysis of guar gum to eliminate the side chains of galactose addition, without altering the mannose chain (Pai and Khan 2002), there are also records in industry of the paper as catalyzer for galactose release in the soft mellow and to help whiteness, in synergy with other enzymes (Clarke et al. 2000), another efficient application that is reported is as cleaning, for both clothing and surfaces (McDonald and Poulose 2010).

Based on the sequence of amino acids, these enzymes are inculcated within the four different families, which are GH4, GH27, GH36, and GH57. Even so most of the enzymes belong to the families GH27 and GH36, the majority is of eukaryotic origin, there are reports of different sources of production, some varieties of plants, animals, both invertebrates and vertebrates are mostly studied with microorganisms, bacteria, fungi, and yeast, as well as the largest producers for industrial uses (Anisha 2017). For the production of α-agarases, there are studies focused to look for microorganisms that have the ability to produce these enzyme of interest, for example, the use of X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside), which is reported as a qualitative test as a preliminary assay in nutritious agar plates that help to identify if the microorganism has the ability to hydrolyze the correct bond and release a chromophore that indicates the production of galactosidase (Anisha et al. 2010). Moreover, in quantitative test, the most commonly used substrate is a synthetic chromogenic called *p*-nitrophenyl-α-D-galactopyranoside (pNPG), where the hydrolyzate of this substrate releases *p*-nitrophenol that can be quantified spectrophotometrically.

21.2.5.2 Beta Galactosidase Enzyme

Also known as lactase, enzymes with the ability to break the β-(1,4) galactosidic bonds, mostly used for the degradation of lactose in dairy products and their derivatives. The main industrial applications are in the dairy industry, although it also has some medical applications. One of the most important applications is the use of immobilized enzymes to place in different reactors configurations for dairy industry and the process of obtaining lactose-free milk, since the economic factor of the process is very affected in the price of the enzyme, thus seeking to recycle the enzyme, enlarge its useful life and make more feasible the manufacture of lactose-hydrolyzed products, both in milk and whey products (Husain 2010; Şener et al. 2006). Other, cases are based on the implementation of bacterial strains that express this type of enzymes to add to dairy products in order to create defatted products such as cheese and yogurt. Most applications for this type of enzyme is closely related to Morquio syndrome type “B”, which is a recessive autosomal

disease where there is a deficiency or lack of this enzyme in the body to break down the long sugar chains, which triggers intestinal secretions, gas, abdominal pain, and diarrhea (Cappello and Marzio 2005).

21.2.5.3 Agarases Enzymes

Classified as α-agarase (EC 3.2.1.158) and β-agarase (EC 3.2.1.81), according to the hydrolyzing bond within the agar molecule, which is constituted by repetitive units of β-D-galactose and 3,6-anhydro-α-L-galactose, hence α-agarase hydrolyses the bond α(1-3), obtaining agarooligosaccharides and β-agarase hydrolyses the β(1-4) bond, obtaining neoagarooligosaccharides. There are reports where the agarases are produced from a high range of marine microorganisms, mostly gram-negative bacteria, isolated from environments such as marine sediments, marine seaweed, marine mollusk, and marine soil. Agarases belong to 3 different families, GH16, GH50, and GH86, where the great majority of agarase belongs to the GH16 family, there are more types belonging to this family, such as glucanase, galactosidase, laminarase, and carrageenase), whereas in families GH50 and GH86, it is possible to find agarases as the only type of enzyme.

The agarases have application in different industries such as the medical, cosmetic, and food. Among the applications that can give it are the agarases for the recovery of DNA bands of the agarose gel for biological investigations (Sugano et al. 1993; Gold 1992). These enzymes are used for the cell wall degradation of seaweed, and thus recover substances of great interest with important biological activities, such as unsaturated fatty acids, vitamins and some pigments such as carotenoids. Moreover, agarases have been used to obtain protoplasts, which are isolated from marine seaweed, for physiological and cytological studies, as excellent tools for plant breeding, cell fusion and gene manipulation (Araki et al. 1998). Another application that can be given is to recover oligosaccharides from agar, both neoagarooligosaccharides and agarooligosaccharides, these compounds have been reported for their physiological and biological activities, as well as their high economic value. There are reports that mentioned the effect of neoagarooligosaccharides between 6 and 4 units as a good antioxidant (Wu and Pan 2004; Wu et al. 2005) that can be used in healthier foods for inhibiting the growth of bacteria, slowing down the degradation of starch, as well as for the low caloric intake. So far, the use of raw materials for bioethanol is focused on the use of cellulose. However, enzymes can hydrolyze and degrade the agar in agarooligosaccharides and galactose, which can later be used as bioethanol material (Mollah et al. 2009). The production of agarases, as already known, it is based on the use of agar as a substrate where it is possible to find certain microorganisms that have the ability to produce these enzymes, there are both qualitative and quantitative techniques to determine the production capacity of microorganisms.

Different agarases enzymes have been obtained from different microorganisms (Pai and Khan 2002; Clarke et al. 2000; McDonald and Poulose 2010) and some mechanisms of action have been well characterized and reported. As already

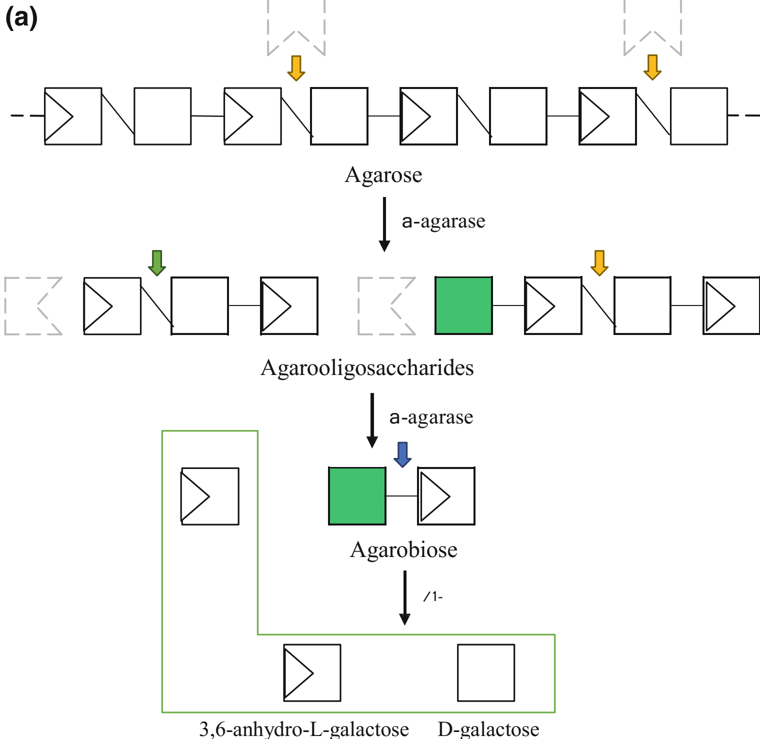


Fig. 21.3 Mechanisms of enzymatic action, **a** mechanism for β-agarase, **b** mechanism for α-agarase (also enzyme agarolytic galactosidase), **c** mechanism for κ-carrageenase, **d** mechanism for ι-carrageenan, **e** mechanism for λ-carrageenan and **f** explanation of the figures used for each diagram

known, they are classified into α- and β-agarases, by their action of inclusion in the links α-1,3 and β-1,4, respectively (Fig. 21.3a, b). Ekborg et al. (2006) demonstrated the behavior of β-agarase where it describes a β-agarase enzyme with endo-type depolymerize action, hydrolyzing the agarose in neoagarooligosaccharides, as a first step in the elucidation of the action mechanism for monomers recovery, followed by the action of β-agarase enzyme identified by, exo type, with the ability to hydrolyze neoagarooligosaccharides, as the next step for hydrolysis (Ekborg et al. 2006; Kim et al. 2010; Pluvinage et al. 2013; Michel et al. 2006), there are report of enzymes with the ability to hydrolyze neoagarobiose in different monosaccharides from agarose, as well as α-agarases also can be used for the hydrolysis, the enzymatic action of neoagarobiose is shown in Fig. 21.3b.

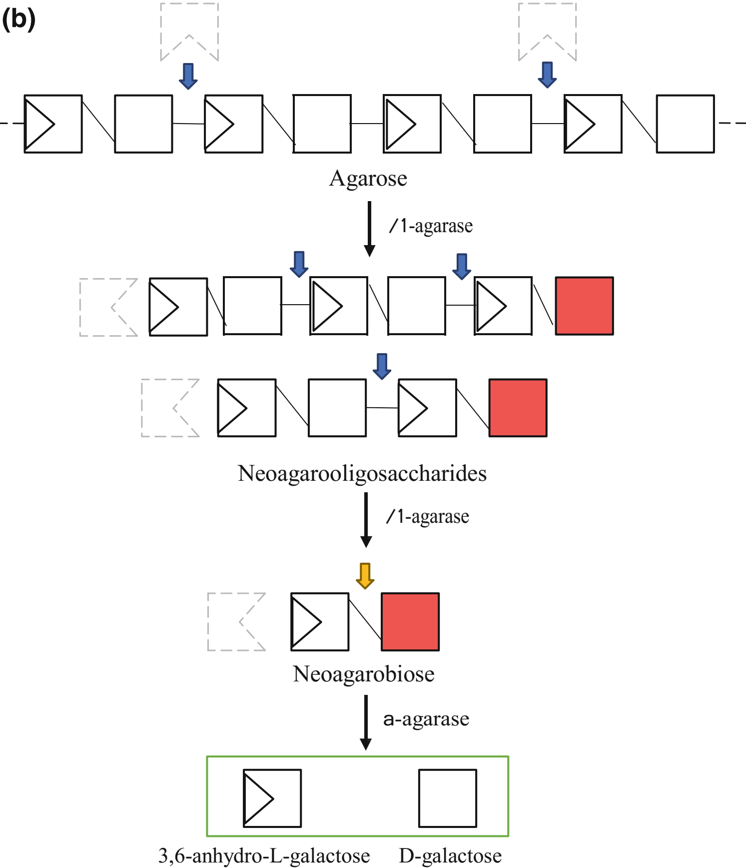


Fig. 21.3 (continued)

21.2.5.4 Carrageenase Enzyme

Enzymes with the ability to break the bonds of carrageenan, which is divided into three types, as carrageenases are added in three categories, corresponding to each type, κ-carrageenase (EC 3.2.1.83), ι-carrageenase (EC. 3.2.1.157) and λ-carrageenase (EC: 3.2.1.162), depending on each substrate (Michel et al. 2006). All are endolithic enzymes with the ability to break the β(1,4) bond by which their molecules are bound. The applications that are given to this type of enzymes, mainly lies in oligosaccharides obtaining of which undergo a change in their biological activities, within which there are several investigations that report anti-tumor, immuno-stimulant, antiviral, and antioxidant activities (Yuan and Song 2005; Yuan et al. 2005, 2011; Sun et al. 2015; Wang et al. 2011; Pérez-Riverol et al. 2014). Another application is the isolation and preparation of seaweed protoplasts by the combination effect with the use of other enzymes, the cell

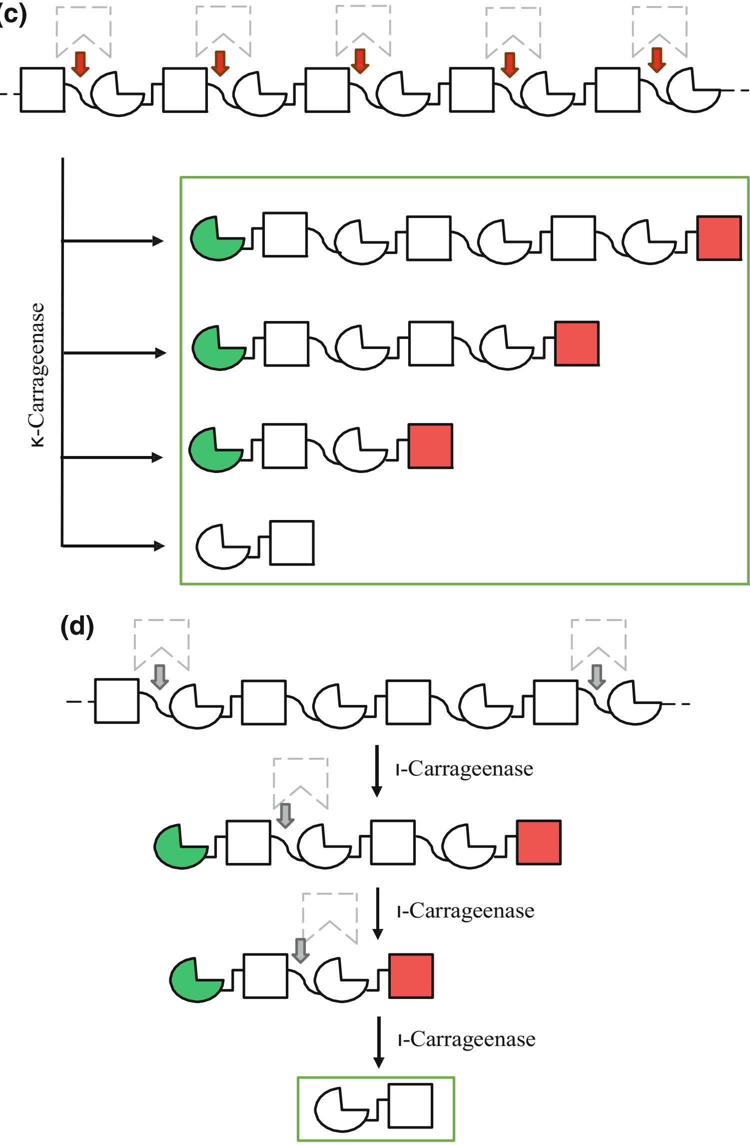


Fig. 21.3 (continued)

degradation, genetic engineering experiments, as well as extraction of target interesting compounds as vitamins and pigments such as carotenoids. Another application is a reference to the investigations that are carried out to elucidate the fine structure of carrageenan (Anastyuk et al. 2011). Among industrial applications include the use of these enzymes in the textile industry where they are applied for

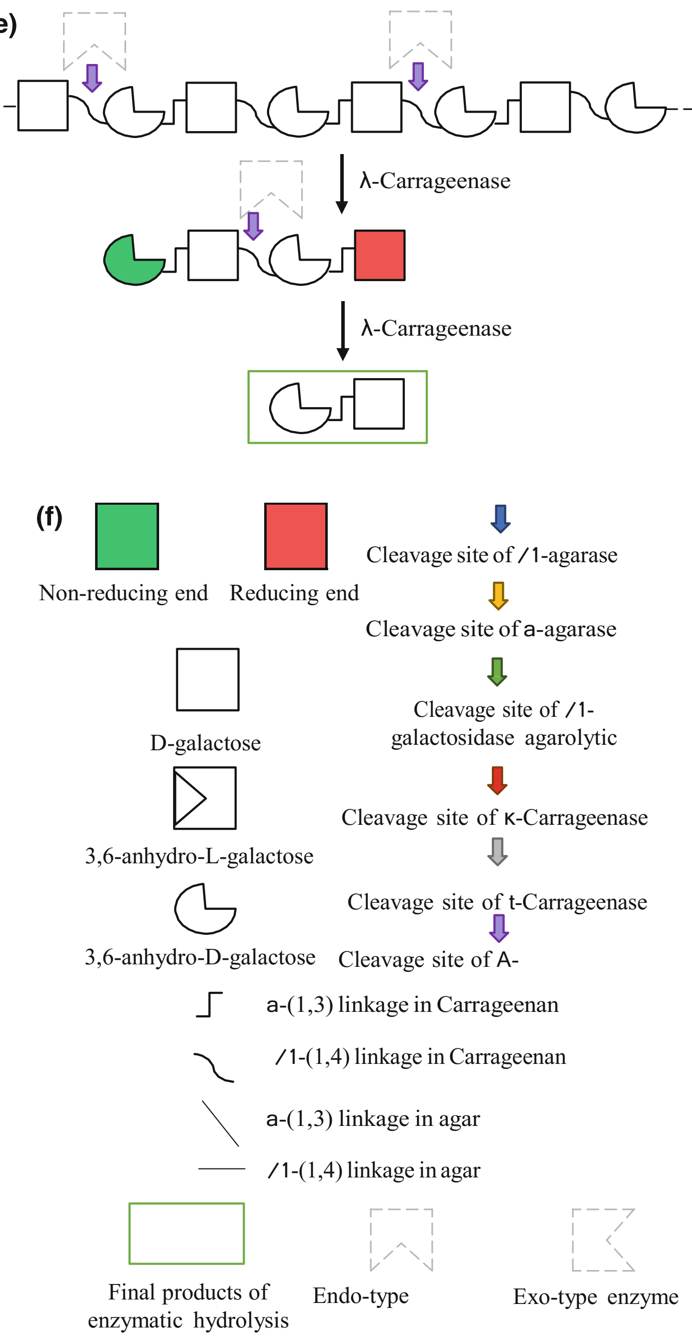


Fig. 21.3 (continued)

the treatment of the carrageenan used during the textile printing and to modify its physical characteristics as thickener (Pedersen et al. 1995).

The production of carrageenases is mainly performed by marine bacteria (Greer and Yaphe 1984; Gauthier et al. 1995; Jouanneau et al. 2010; Smith et al. 2005), although marine microorganisms have also been found with the capacity to produce them. The main substrate for these three types of enzymes is carrageenan. However, in order to find the microorganisms that are able to produce these enzymes, both quantitative and qualitative techniques are described to find this information. For the qualitative test, an alcian blue solution can be used for enzymes determination.

The enzymes responsible for hydrolyzing the main chains of the carrageenan polymers must be very specific, giving the complexity of this type of molecules, with respect to the substituents that may contain (Chauhan and Saxena 2016). Enzymes that degrade this type of compounds have already mentioned above, which are k-carrageenase, i-carrageenase, and l-carrageenase, these three enzymes are endo (Zhu et al. 2018; Barbeyron et al. 2000), so these enzymes only have the ability to break the internal glycosidic bonds, not counting on the ability to hydrolyze the units of the ends (either reducing or non-reducing), then the hydrolysis of these molecules can only be carried to find units of disaccharides, as its most simplified form. The major reports for these enzymes are corresponding to the enzyme *k*-carrageenase, where some authors have reported that this enzyme does not follow a pattern to hydrolyze, but hydrolyses random links (Fig. 21.3c), some reports have been found with this enzyme from 2 to 10 of polymerization degrees, the most common being to obtain oligosaccharides with a degree of polymerization of 6 (Chauhan and Saxena 2016; Guibet et al. 2007). While for i- and l-carrageenase enzymes, lower degrees of polymerization are reported (2 and 4 degrees of polymerization), this is because the hydrolysis carried out by these two enzymes occurs successively (Fig. 21.3d, e), which means that the enzyme slides along the polysaccharide chain, carrying out more organized hydrolysis (Barbeyron et al. 2000; Michel et al. 2001).

21.2.6 Physical, Chemical, and Thermal Methods for Sulfated Polysaccharide Modification

Other modification methods to alter the structure, bioactivity, yields, position of substituents, and the degree of polymerization, it cannot only be carried out by hydrolysis of specific bonds, although it is part of the disadvantages since it is not so specific, it will describe some used methods, which seeks higher yields, preserve structures, modification of bioactivities, and especially reduction of production costs.

21.2.6.1 Physical Methods

This method is based on the application of force, whether mechanical, sonic or otherwise, to break the cell wall of marine seaweed and to obtain galactans. The most common techniques are ground, ultrasound and supercritical fluids (de Borba Gurpilhares et al. 2016). Exposure to gamma rays also weakens the cell wall due to the chain reaction due to the high activity generated by the radicals during irradiation and allows easier release of the soluble compounds. These methods are mainly used in order to improve the care of the environment, although they end up being used as a pretreatment for the extraction of compounds. The main effect of these methods is related to the polymer size of the obtained galactan, finding changes in physical properties, such as viscosity (the most important property of this polysaccharide) with an ultrasound process (Lii et al. 1999).

21.2.6.2 Chemical and Thermal Methods

Mostly used simultaneously, this method to improve the process extraction, the principle of the chemical method is based on the destruction of the cell wall of the seaweed, when the acid interacts with the intramolecular hydrogen bonds to break them, all with the same objective to obtain the highest amount of polysaccharides and with changes in the structures, to obtain better results of extraction, different studies taking into account four parameters for this treatment, which are: (1) the chemical that is used, (2) the concentration of the acid or base, (3) the resident time, (4) the operational temperature (Harun et al. 2014).

In general, for this type of treatment, strong inorganic acids such as sulfuric acid, hydrochloric acid, nitric acid, and phosphoric acid are used (Harun et al. 2014; Wei et al. 2013). Although this type of treatment gives good results, it has certain disadvantages, for example, the use of a neutralizer to use it after the process, causing environmental problems. On the other hand, the use of weak acids such as citric acid, which have certain advantages because they are cheap, simple, respectful with the environment and can be used more easily in combination with other methods (enzymes) (Sirajunnisa and Surendhiran 2016; Kwon et al. 2016). As well as there are studies that reported the use of alkali treatments with hydroxides or carbonates. Other type of process is the thermal treatments, which are carried out only with water at high temperatures, for the extraction of compounds, considering as a green technology. However, when high temperatures and high pressures are associated, the Millard reactions may occur, forming unwanted molecules as furans, or unwanted combinations could be found with the free radicals derived from the polysaccharides, generating molecules that are not sought or affect the properties, which seeks to improve (Jiang et al. 2016).

21.3 Conclusions and Future Trends

This chapter allowed us to describe the scientific relevance of red seaweed biomass, seaweed polysaccharides that are a promising raw material for biorefinery due to the wide applications of these macromolecules classified by their number of units (molecular weight) and substituents (sulfation) that define its degree of gelling and functionality principally for food, pharmaceutical, medicine, textiles, and biofuels as a biorefinery sustainable global concept. However, the variety of the biomass source and the extraction procedure caused a high degree of structural and chemical complexity in these polysaccharide gels and the degree of sophistication of the enzymes involved in the degradation of agar and carrageenans clearly points to the complexity of the substrate. The challenges in the hydrolytic enzymes of sulfated galactans are based on defining the complete mode of action conjugated with the understanding of its molecular mechanism, including the study of other enzymes that may intervene in the degradation of this matrix that contains, such as sulphatases or carbohydrate esterases, which are barely reported. In addition, the use of alternative bioprocesses and technologies to produce these enzymes using red seaweed biomass is still an area of opportunity to develop.

Acknowledgements Financial support is gratefully acknowledged from the Energy Sustainability Fund 2014-05 (CONACYT-SENER), Mexican Centre for Innovation in Bioenergy (Cemie-Bio), Cluster of Bioalcohols (Ref. 249564). We gratefully acknowledge support for this research by the Mexican Science and Technology Council (CONACYT, Mexico) for the Infrastructure Project—INFR201601 (Ref. 269461) and Basic Science Project—2015-01 (Ref. 254808). Author Arturo Siller would like to thank the Mexican Science and Technology Council (CONACY, Mexico) for their master fellowship support.

References

Anastyuk SD, Barabanova AO, Correc G, Nazarenko EL, Davydova VN, Helbert W, Yermak IM (2011) Analysis of structural heterogeneity of κ/β-carrageenan oligosaccharides from *Tichocarpus crinitus* by negative-ion ESI and tandem MALDI mass spectrometry. Carbohyd Polym 86(2):546–554. https://doi.org/10.1016/j.carbpol.2011.04.081

Anisha GS (2017) α-Galactosidases. In: Current developments in biotechnology and bioengineering, pp 369–394. https://doi.org/10.1016/b978-0-444-63662-1.00016-6

Anisha GS, John RP, Pandey A (2010) Alpha-galactosidase: a food and feed enzyme. Handbook of nutritional biochemistry: genomics, metabolomics and food supply, nutrition and diet research progress series. Nova Science Publishers Inc., New York, pp 365–384

Araki T, Lu Z, Morishita T (1998) Optimization of parameters for isolation of protoplasts from *Gracilaria verrucosa* (Rhodophyta). J Mar Biotechnol 6:193–197

Armisen R (1995) World-wide use and importance of *Gracilaria*. J Appl Phycol 7(3):231. https://doi.org/10.1007/BF00003998

Armisen R, Galatas F (2009) Agar. In: Phillips GO, Williams PA (eds) Handbook of hydrocolloids, 2nd edn. Woodhead Publ., UK, pp 82–107. https://doi.org/10.1533/9781845695873.82

Baghel RS, Trivedi N, Gupta V, Neori A, Reddy CRK, Lali A, Jha B (2015) Biorefining of marine macroalgal biomass for production of biofuel and commodity chemicals. Green Chem 17 (4):2436–2443. https://doi.org/10.1039/C4GC02532F

Baghel RS, Trivedi N, Reddy CRK (2016) A simple process for recovery of a stream of products from marine macroalgal biomass. Bioresour Technol 203:160–165. https://doi.org/10.1016/j.biortech.2015.12.051

Barbeyron T, Michel G, Potin P, Henrissat B, Kloareg B (2000) iota-Carrageenases constitute a novel family of glycoside hydrolases, unrelated to that of kappa-carrageenases. J Biol Chem

Barbeyron T, Flament D, Michel G, Potin P, Kloareg B (2001) The sulphated-galactan hydrolases, agarases and carrageenases: structural biology and molecular evolution. Cah Biol Mar 42(1/2):169–183

Bixler HJ, Porse H (2011) A decade of change in the seaweed hydrocolloids industry. J Appl Phycol 23(3):321–335. https://doi.org/10.1007/s10811-010-9529-3

Blanco-Pascual N, Alemán A, Gómez-Guillén MC, Montero MP (2014) Enzyme-assisted extraction of κ/ι-hybrid carrageenan from *Mastocarpus stellatus* for obtaining bioactive ingredients and their application for edible active film development. Food Funct 5(2):319–329

Boonstra AS (2015) The macroalgae-based biorefinery-a comprehensive review and a prospective study of future macroalgae-based biorefinery systems. Master's thesis

Boulho R, Marty C, Freile-Pelegrín Y, Robledo D, Bourgougnon N, Bedoux G (2017) Antiherpetic (HSV-1) activity of carrageenans from the red seaweed *Solieria chordalis* (Rhodophyta, Gigartinales) extracted by microwave-assisted extraction (MAE). J Appl Phycol 29(5):2219–2228. https://doi.org/10.1007/s10811-017-1192-5

Campo VL, Kawano DF, da Silva Jr DB, Carvalho I (2009) Carrageenans: biological properties, chemical modifications and structural analysis—a review. Carbohydr Polym 77(2):167–180. https://doi.org/10.1016/j.carbpol.2009.01.020

Cappello G, Marzio L (2005) Rifaximin in patients with lactose intolerance. Dig Liver Dis 37 (5):316–319. https://doi.org/10.1016/j.dld.2004.12.007

Cardoso MS, G Carvalho L, J Silva P, S Rodrigues M, R Pereira O, Pereira L (2014). Bioproducts from seaweeds: a review with special focus on the Iberian Peninsula. Curr Organ Chem 18 (7):896–917. https://doi.org/10.2174/138527281807140515154116

Cervantes-Cisneros DE, Arguello-Esparza D, Cabello-Galindo A, Picazo B, Aguilar CN, Ruiz HA, Rodríguez-Jasso RM (2017). Hydrothermal processes for extraction of macroalgae high value-added compounds. In: Hydrothermal process in biorefineries. Springer, Cham, pp 461–481. https://doi.org/10.1007/978-3-319-56457-9_20

Cesário MT, da Fonseca MR, Marques MM, de Almeida MCMD (2018) Marine algal carbohydrates as carbon sources for the production of biochemicals and biomaterials. Biotechnol Adv 798–817. https://doi.org/10.1016/j.biotechadv.2018.02.006

Chauhan PS, Saxena A (2016) Bacterial carrageenases: an overview of production and biotechnological applications. 3 Biotech 6(2):146. https://doi.org/10.1007/s13205-016-0461-3

Chen YW, Lee HV, Juan JC, Phang SM (2016) Production of new cellulose nanomaterial from red algae marine biomass *Gelidium elegans*. Carbohydr Polym 151:1210–1219. https://doi.org/10.1016/j.carbpol.2016.06.083

Chew KW, Show PL, Yap YJ, Juan JC, Phang SM, Ling TC, Chang JS (2018) Sonication and grinding pre-treatments on *Gelidium amansii* seaweed for the extraction and characterization of Agarose. Front Environ Sci Eng 12(4):2. https://doi.org/10.1007/s11783-018-1040-0

Chirapart A, Praiboon J (2018) Comparison of the photosynthetic efficiency, agar yield, and properties of *Gracilaria salicornia* (Gracilariales, Rhodophyta) with and without adelphoparasite. J Appl Phycol 30(1):149–157. https://doi.org/10.1007/s10811-017-1222-3

Clarke JH, Davidson K, Rixon JE, Halstead JR, Fransen MP, Gilbert HJ, Hazlewood GP (2000) A comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase. Appl Microbiol Biotechnol 53(6):661–667. https://doi.org/10.1007/s002530000344

Courtois J (2009) Oligosaccharides from land plants and algae: production and applications in therapeutics and biotechnology. Curr Opin Microbiol 12(3):261–273. https://doi.org/10.1016/j.mib.2009.04.007

Cumashi A, Ushakova NA, Preobrazhenskaya ME, D'incecco A, Piccoli A, Totani L, Usov AI (2007) A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. Glycobiology 17 (5):541–552. https://doi.org/10.1093/glycob/cwm014

Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. Water Res 37(18):4311–4330. https://doi.org/10.1016/S0043-1354(03)00293-8

de Borba Gurpilhares D, Moreira TR, da Luz Bueno J, Cinelli LP, Mazzola PG, Pessoa A, Sette LD (2016) Algae's sulfated polysaccharides modifications: potential use of microbial enzymes. Process Biochem 51(8):989–998. https://doi.org/10.1016/j.procbio.2016.04.020

Diharmi A, Fardiaz D, Andarwulan N, Heruwati ES (2017) Chemical and physical characteristics of carrageenan extracted from *Eucheuma spinosum* harvested from three different Indonesian coastal sea regions. Phycol Res 65(3):256–261. https://doi.org/10.1111/pre.12178

Draget KI (2009) Alginates. In: Phillips GO, Williams PA (eds) Handbook of hydrocolloids. CRC Press and Woodhead Publishing, Boca Ratón, pp 807–825. https://doi.org/10.1533/9781845695873.807

Ekborg NA, Taylor LE, Longmire AG, Henrissat B, Weiner RM, Hutcheson SW (2006) Genomic and proteomic analyses of the agarolytic system expressed by *Saccharophagus degradans* 2-40. Appl Environ Microbiol 72(5):3396–3405. https://doi.org/10.1128/AEM.72.5.3396-3405.2006

Ermakova S, Men'shova R, Vishchuk O, Kim SM, Um BH, Isakov V (2013) Water-soluble polysaccharides from the brown alga *Eiseniabicyclis*: structural characteristics and antitumor activity. Algal Res 2:51–58

Fu XT, Lin H, Kim SM (2008) Purification and characterization of a novel β-agarase, AgaA34, from *Agarivoransalbus* YKW-34. Appl Microbiol Biotechnol 78(2):265–273. https://doi.org/10.1007/s00253-007-1303-3

Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. Int J Syst Evol Microbiol 45 (4):755–761. https://doi.org/10.1099/00207713-45-4-755

Gold P (1992) Use of a novel agarose gel-digesting enzyme for easy and rapid purification of PCR-amplified DNA for sequencing. Biotechniques 13(1):132–134

Gómez-Ordóñez E (2013) Nutritional evaluation and biological properties of edible marine algae. In vitro and in vivo studies (Evaluación nutricional y propiedades biológicas de algas marinas comestibles. Estudios in vitro e in vivo). Thesis: Universidad Complutense de Madrid, Spain

Gong F, Lu QS, You Y, Gao HW, Bao GQ, Gao X, Tian SG, Zhang Y-P (2005) Preparation of transfusable human universal red blood cell with recombinant α-galactosidase. J Exp Hematol 2

Greer CW, Yaphe W (1984) Enzymatic analysis of carrageenans: structure of carrageenan from *Eucheuma nudum*. In: Eleventh international seaweed symposium. Springer, Dordrecht, pp 563–567. https://doi.org/10.1007/978-94-009-6560-7_116

Griffiths M, Harrison ST, Smit M, Maharajh D (2016) Major commercial products from micro- and macroalgae. In: Algae biotechnology. Springer, Cham, pp 269–300. https://doi.org/10.1007/978-3-319-12334-9_14

Guibet M, Colin S, Barbeyron T, Genicot S, Kloareg B, Michel G, Helbert W (2007) Degradation of λ-carrageenan by *Pseudoalteromonas carrageenovora* λ-carrageenase: a new family of glycoside hydrolases unrelated to κ-and ι-carrageenases. Biochem J 404(1):105–114. https://doi.org/10.1042//BJ20061359

Gupta S, Abu-Ghannam N (2011) Bioactive potential and possible health effects of edible brown seaweeds. Trends Food Sci Technol 22:315–326. https://doi.org/10.1016/j.tifs.2011.03.011

Hamer GK, Bhattacharjee SS, Yaphe W (1977) Analysis of the enzymic hydrolysis products of agarose by ^{13}C-nmr spectroscopy. Carbohydr Res 54(1):C7-C10. https://doi.org/10.1016/s0008-6215(00)80567-4

Harun R, Yip JW, Thiruvenkadam S, Ghani WA, Cherrington T, Danquah MK (2014) Algal biomass conversion to bioethanol–a step by step assessment. Biotechnol J 9(1):73–86. https://doi.org/10.1002/biot.201200353

Hayashi L, Bulboa C, Kradolfer P, Soriano G, Robledo D (2014) Cultivation of red seaweeds: a Latin American perspective. J Appl Phycol 26(2):719–727. https://doi.org/10.1007/s10811-013-0143-z

Hilliou L, Larotonda FDS, Abreu P, Ramos AM, Sereno AM, Gonçalves MP (2006) Effect of extraction parameters on the chemical structure and gel properties of κ/ι-hybrid carrageenans obtained from *Mastocarpus stellatus*. Biomol Eng 23(4):201–208. https://doi.org/10.1016/j.bioeng.2006.04.003

Hu Z, Lin BK, Xu Y, Zhong MQ, Liu GM (2009) Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. HZ105. J Appl Microbiol 106(1):181–190. https://doi.org/10.1111/j.1365-2672.2008.03990.x

Huang Q, Zhang H, Song H, Wang Y, An F (2017) Rheological properties of a polysaccharide with highly sulfated groups extracted from *Gracialaria greville*. J Food Process Eng 40(6). https://doi.org/10.1111/jfpe.12564

Husain Q (2010) β-Galactosidases and their potential applications: a review. Crit Rev Biotechnol 30(1):41–62. https://doi.org/10.3109/07388550903330497

Jiang R, Ingle KN, Golberg A (2016) Macroalgae (seaweed) for liquid transportation biofuel production: what is next? Algal Res 14:48–57. https://doi.org/10.1016/j.algal.2016.01.001

Jiao G, Yu G, Zhang J, Ewart HS (2011) Chemical structures and bioactivities of sulfated polysaccharides from marine algae. Mar Drugs 9(2):196–223. https://doi.org/10.3390/md9020196

John RP, Anisha GS, Nampoothiri KM, Pandey A (2011) Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour Technol 102(1):186–193. https://doi.org/10.1016/j.biortech.2010.06.139

Jouanneau D, Boulenguer P, Mazoyer J, Helbert W (2010) Enzymatic degradation of hybrid ι-/ν-carrageenan by *Alteromonas fortis* ι-carrageenase. Carbohyd Res 345(7):934–940. https://doi.org/10.1016/j.carres.2010.02.014

Kadam SU, Tiwari BK, O'Donnell CP (2015) Extraction, structure and biofunctional activities of laminarin from brown algae. Int J Food Sci Technol 50:24–31. https://doi.org/10.1111/ijfs.12692

Kara S, Arda E, Kavzak B, Pekcan Ö (2006) Phase transitions of κ-carrageenan gels in various types of salts. J Appl Polym Sci 102(3):3008–3016. https://doi.org/10.1002/app.24662

Khambhaty Y, Mody K, Gandhi MR, Thampy S, Maiti P, Brahmbhatt H, Ghosh PK (2012) *Kappaphycus alvarezii* as a source of bioethanol. Bioresour Technol 103(1):180–185. https://doi.org/10.1016/j.biortech.2011.10.015

Kim HT, Lee S, Lee D, Kim HS, Bang WG, Kim KH, Choi IG (2010) Overexpression and molecular characterization of Aga50D from *Saccharophagus degradans* 2-40: an exo-type β-agarase producing neoagarobiose. Appl Microbiol Biotechnol 86(1):227–234. https://doi.org/10.1007/s00253-009-2256-5

Kim HM, Wi SG, Jung S, Song Y, Bae HJ (2015) Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. Bioresour Technol 175:128–134. https://doi.org/10.1016/j.biortech.2014.10.050

Koch R (1882) Die aetiologie der tuberculose. Berl Klin Wochnschr xix: 221–230. Milest Microbiol 1556:109. https://doi.org/10.25646/5088

Kravchenko AO, Belous OS, Glazunov VP, Ermak IM (2013) Comprehensive study of phycobiliproteins and sulfated polysaccharides from the red alga *Ahnfeltiopsis flabelliformis*. Chem Nat Compd 49(2):201–205. https://doi.org/10.1007/s10600-013-0562-8

Kumar S, Gupta R, Kumar G, Sahoo D, Kuhad RC (2013) Bioethanol production from *Gracilaria verrucosa*, a red alga, in a biorefinery approach. Bioresour Technol 135:150–156. https://doi.org/10.1016/j.biortech.2012.10.120

Kwon OM, Kim DH, Kim SK, Jeong GT (2016) Production of sugars from macro-algae *Gracilaria verrucosa* using combined process of citric acid-catalyzed pretreatment and enzymatic hydrolysis. Algal Res 13:293–297. https://doi.org/10.1016/j.algal.2015.12.011

Lakshmikanth M, Manohar S, Patnakar J, Vaishampayan P, Shouche Y, Lalitha J (2006) Optimization of culture conditions for the production of extracellular agarases from newly isolated *Pseudomonas aeruginosa* AG LSL-11. World J Microbiol Biotechnol 22(5):531–537. https://doi.org/10.1007/s11274-005-9068-2

LeBlanc JG, Garro MS, Silvestroni A, Connes C, Piard JC, Sesma F, Savoy de Giori G (2004) Reduction of α-galactooligosaccharides in soyamilk by Lactobacillus fermentum CRL 722: in vitro and in vivo evaluation of fermented soyamilk. J Appl Microbiol 97(4):876–881. https://doi.org/10.1111/j.1365-2672.2004.02389.x

Ledermann B, Aras M, Frankenberg-Dinkel N (2017) Biosynthesis of cyanobacterial light-harvesting pigments and their assembly into phycobiliproteins. In: Modern topics in the phototrophic prokaryotes. Springer, Cham, pp 305–340. https://doi.org/10.1007/978-3-319-51365-2_9

Levine B, Weisman S (2004) Enzyme replacement as an effective treatment for the common symptoms of complex carbohydrate intolerance. Nutr Clin Care: Off Publ Tufts Univ 7(2):75–81

Lii CY, Chen CH, Yeh AI, Lai VMF (1999) Preliminary study on the degradation kinetics of agarose and carrageenans by ultrasound. Food Hydrocoll 13(6):477–481. https://doi.org/10.1016/S0268-005X(99)00031-4

Mandal P, Pujol CA, Carlucci MJ, Chattopadhyay K, Damonte EB, Ray B (2008) Anti-herpetic activity of a sulfated xylomannan from *Scinaia hatei*. Phytochemistry 69(11):2193–2199. https://doi.org/10.1016/j.phytochem.2008.05.004

Masarin F, Cedeno FRP, Chavez EGS, Oliveira LE, Gelli VC, Monti R (2016) Chemical analysis and biorefinery of red algae *Kappaphycus alvarezii* for efficient production of glucose from residue of carrageenan extraction process. Biotechnol Biofuels 9(1):122. https://doi.org/10.1186/s13068-016-0535-9

Mayakrishnan V, Kannappan P, Abdullah N, Ahmed ABA (2013) Cardioprotective activity of polysaccharides derived from marine algae: an overview. Trends Food Sci Technol 30:98–104. https://doi.org/10.1016/j.tifs.2013.01.007

McDonald HC, Poulose AJ (2010) U.S. Patent Application No. 12/528,986

McHugh DJ (2003) A guide to the seaweed industry. Food and Agriculture Organization of the United Nations, Rome, pp 12–22

Michel G, Chantalat L, Duee E, Barbeyron T, Henrissat B, Kloareg B, Dideberg O (2001) The κ-carrageenase of *P. carrageenovora* features a tunnel-shaped active site: a novel insight in the evolution of Clan-B glycoside hydrolases. Structure 9(6):513–525. https://doi.org/10.1016/s0969-2126(01)00612-8

Michel G, Nyval-Collen P, Barbeyron T, Czjzek M, Helbert W (2006) Bioconversion of red seaweed galactans: a focus on bacterial agarases and carrageenases. Appl Microbiol Biotechnol 71(1):23–33. https://doi.org/10.1007/s00253-006-0377-7

Michelin M, Ruiz HA, Silva DP, Ruzene DS, Teixeira JA, Polizeli MLT (2015) Cellulose from lignocellulosic waste. In: Polysaccharides: Bioactivity and Biotechnology, pp 475–511. https://doi.org/10.1007/978-3-319-03751-6_52-1

Milledge JJ, Smith B, Dyer PW, Harvey P (2014) Macroalgae-derived biofuel: a review of methods of energy extraction from seaweed biomass. Energies 7(11):7194–7222. https://doi.org/10.1016/j.rser.2018.03.100

Mohamed S, Hashim SN, Rahman HA (2012) Seaweeds: a sustainable functional food for complementary and alternative therapy. Trends Food Sci Technol 23:83–96. https://doi.org/10.1016/j.tifs.2011.09.001

Mollah MZI, Khan MA, Khan RA (2009) Effect of gamma irradiated sodium alginate on red amaranth (*Amaranthus cruentus* L.) as growth promoter. Rad Phys and Chem 78(1):61–64. https://doi.org/10.1016/j.radphyschem.2008.08.002

Naumoff DG (2004) Phylogenetic analysis of α-galactosidases of the GH27 family. Mol Biol 38 (3):388–400. https://doi.org/10.1023/B:MBIL.0000032210.97006.de

Necas J, Motosikova L (2013) Carrageenan: a review. Vet Med 58(4). https://doi.org/10.17221/6758-vetmed

Nedumaran T, Arulbalachandran D (2015) Seaweeds: a promising source for sustainable development. In: Environmental sustainability. Springer, New Delhi, pp 65–88. https://doi.org/10.1007/978-81-322-2056-5_4

Öğretmen ÖY, Duyar HA (2018) The effect of different extraction methods and pre-treatments on agar yield and physico-chemical properties of *Gelidium latifolium* (*Gelidiaceae*, Rhodophyta) from Sinop Peninsula Coast of Black Sea, Turkey. J Appl Phycol 30(2):1355–1360. https://doi.org/10.1007/s10811-017-1292-2

Ohta Y, Hatada Y, Miyazaki M, Nogi Y, Ito S, Horikoshi K (2005) Purification and characterization of a novel α-agarase from a Thalassomonas sp. Curr Microbiol 50(4):212–216. https://doi.org/10.1007/s00284-004-4435-z

Pai VB, Khan SA (2002) Gelation and rheology of xanthan/enzyme-modified guar blends. Carbohydr Polym 49(2):207–216. https://doi.org/10.1016/s0144-8617(01)00328-9

Parker W, Bruno D, Platt JL (1995) Xenoreactive natural antibodies in the world of natural antibodies: typical or unique? Transpl Immunol 3(3):181–191. https://doi.org/10.1016/0966-3274(95)80024-7

Pedersen G, Hagen HA, Asferg L, Sorensen E (1995) U.S. Patent No. 5,405,414. Washington, DC: U.S. Patent and Trademark Office

Pereira L, Gheda SF, Ribeiro-claro PJA (2013) Analysis by vibrational spectroscopy of seaweed polysaccharides with potential use in food, pharmaceutical, and cosmetic industries. Int J Carbohydr Chem 1–7. https://doi.org/10.1155/2013/537202

Pereira-Pacheco F, Robledo D, Rodríguez-Carvajal L, Freile-Pelegrín Y (2007) Optimization of native agar extraction from *Hydropuntia cornea* from Yucatán, México. Bioresour Technol 98 (6):1278–1284. https://doi.org/10.1016/j.biortech.2006.05.016

Pérez-Riverol A, Piñón-Ramos A, Morier-Díaz LF, Acosta-Herrera B, Valdés-Iglesias O, del Barrio-Alonso G (2014) In vitro antiviral activity of aqueous extract from red seaweed *tricleocarpa fragilis* to influenza a virus. Rev Cuba Farm 48(2):316–328

Peters FPJ, Vermeulen A, Kho TL (2001) Anderson-Fabry's disease: α galactosidase deficiency. Lancet 357(9250):138–140. https://doi.org/10.1016/S0140-6736(00)03554-6

Pluvinage B, Hehemann JH, Boraston AB (2013) Substrate recognition and hydrolysis by a family 50 exo-β-agarase, Aga50D, from the marine bacterium *Saccharophagus degradans*. J Biol Chem 288(39):28078–28088. https://doi.org/10.1074/jbc.M113.491068

Préchoux A, Genicot S, Rogniaux H, Helbert W (2013) Controlling carrageenan structure using a novel formylglycine-dependent sulfatase, an endo-4S-iota-carrageenan sulfatase. Mar Biotechnol 15(3):265–274. https://doi.org/10.1007/s10126-012-9483-y

Quitral V, Morales C, Sepúlveda M, Schwartz MM (2012) Nutritional and health properties of seaweeds and its potential as a functional ingredient. Rev Chil Nutr 39:196–202

Ra CH, Choi JG, Kang CH, Sunwoo IY, Jeong GT, Kim SK (2015) Thermal acid hydrolysis pretreatment, enzymatic saccharification and ethanol fermentation from red seaweed, *Gracilaria verrucosa*. Microbiol Biotechnol Lett 43(1):9–15. https://doi.org/10.4014/mbl.1410.10007

Rafiquzzaman SM, Ahmed R, Lee JM, Noh G, Jo GA, Kong IS (2016) Improved methods for isolation of carrageenan from *Hypnea musciformis* and its antioxidant activity. J Appl Phycol 28(2):1265–1274. https://doi.org/10.1039/C3FO60310E
Rengasamy KR, Kulkarni MG, Stirk WA, Van Staden J (2014) Bioactive metabolites and value-added products from marine macroalgae. In: Seafood processing by-products. Springer, New York, pp 423–454. https://doi.org/10.1007/978-1-4614-9590-1_21
Rhein-Knudsen N, Ale MT, Meyer AS (2015) Seaweed hydrocolloid production: an update on enzyme assisted extraction and modification technologies. Mar Drugs 13(6):3340–3359. https://doi.org/10.3390/md13063340
Roldán IUM, Mitsuhara AT, Desajacomo JPM, de Oliveira LE, Gelli VC, Monti R, Masarin F (2017) Chemical, structural, and ultrastructural analysis of waste from the carrageenan and sugar-bioethanol processes for future bioenergy generation. Biomass Bioenergy 107:233–243. https://doi.org/10.1016/j.biombioe.2017.10.008
Rowe RC, Sheskey PJ, Quinn ME (2009) Handbook of pharmaceutical excipients, 6th edn. Pharmaceutical Press, London, p 637
Ruiz HA, Rodríguez-Jasso RM, Fernandes BD, Vicente AA, Teixeira JA (2013) Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: a review. Renew Sustain Energy Rev 21:35–51. https://doi.org/10.1016/j.rser.2012.11.069
Ruiz HA, Parajó JC, Teixeira JA (2015) Biorefinery strategies for macroalgae-based in bioethanol production. Energy Sci Technol, Energy Manag 12:349–361. https://doi.org/10.1016/j.radphyschem.2008.08.002
Ruíz HA, Rodríguez-Jasso RM, Aguedo M, Kadar S, (2015) Hydrothermal pretreatment of macroalgal biomass for biorefineries. In: Prokop A, Bajpai RK, Zappi ME (eds) Algal biorefineries, products and refinery design, vol 2, pp 467–491. https://doi.org/10.1007/978-3-319-20200-6_15
Schubert N, García-Mendoza E, Pacheco-Ruiz I (2006) Carotenoid composition of marine red algae. J Phycol 42(6):1208–1216. https://doi.org/10.1111/j.1529-8817.2006.00274.x
Şener N, Apar DK, Özbek B (2006) A modelling study on milk lactose hydrolysis and β-galactosidase stability under sonication. Process Biochem 41(7):1493–1500. https://doi.org/10.1016/j.procbio.2006.02.008
Seo YB, Lee YW, Lee CH, You HC (2010) Red algae and their use in papermaking. Bioresour Technol 101(7):2549–2553. https://doi.org/10.1016/j.biortech.2009.11.088
Shi CS, Sang YX, Sun GQ, Li TY, Gong ZS, Wang XH (2017) Characterization and bioactivities of a novel polysaccharide obtained from *Gracilariopsis lemaneiformis*. An Acad Bras Ciênc 89 (1):175–189. https://doi.org/10.1590/0001-3765201720160488
Sirajunnisa AR, Surendhiran D (2016) Algae–A quintessential and positive resource of bioethanol production: a comprehensive review. Renew Sustain Energy Rev 66:248–267. https://doi.org/10.1016/j.rser.2016.07.024
Smith J, Mountfort D, Falshaw R (2005) A zymogram method for detecting carrageenase activity. Anal Biochem 347(2):336–338. https://doi.org/10.1016/j.ab.2005.09.019
Sousa AM, Alves VD, Morais S, Delerue-Matos C, Gonçalves MP (2010) Agar extraction from integrated multitrophic aquacultured *Gracilaria vermiculophylla*: evaluation of a microwave-assisted process using response surface methodology. Bioresour Technol 101 (9):3258–3267. https://doi.org/10.1016/j.biortech.2009.12.061
Souza RB, Frota AF, Silva J, Alves C, Neugebauer AZ, Pinteus S, Benevides NMB (2018) In vitro activities of kappa-carrageenan isolated from red marine alga *Hypnea musciformis*: antimicrobial, anticancer and neuroprotective potential. Int J Biol Macromol 112:1248–1256. https://doi.org/10.1016/j.ijbiomac.2018.02.029
Sudhakar K, Mamat R, Samykano M, Azmi WH, Ishak WF, Yusaf T (2018) An overview of marine macroalgae as bioresource. Renew Sustain Energy Rev 91:165–179. https://doi.org/10.1016/j.rser.2018.03.100

Sugano Y, Matsumoto T, Kodama H, Noma M (1993) Cloning and sequencing of agaA, a unique agarase 0107 gene from a marine bacterium, *Vibrio* sp. strain JT0107. Appl Environ Microbiol 59(11):3750–3756

Sun Y, Yang B, Wu Y, Liu Y, Gu X, Zhang H, Wang Z (2015) Structural characterization and antioxidant activities of κ-carrageenan oligosaccharides degraded by different methods. Food Chem 178:311–318. https://doi.org/10.1016/j.foodchem.2015.01.105

The Sea Weed Site (2015) Information on Marine Algae. Available online: http://seaweed.ie/uses_general/industrialgums.php. Accessed on 18 May 2015

Usman A, Khalid S, Usman A, Hussain Z, Wang Y (2017) Algal polysaccharides, novel application, and outlook. In: Algae based polymers, blends, and composites, pp 115–153. https://doi.org/10.1016/b978-0-12-812360-7.00005-7

Usov AI (2011) Polysaccharides of the red algae. In: Advances in carbohydrate chemistry and biochemistry, vol 65. Academic Press, pp 115–217. https://doi.org/10.1016/b978-0-12-385520-6.00004-2

Vázquez-Delfín E, Robledo D, Freile-Pelegrín Y (2014) Microwave-assisted extraction of the Carrageenan from *Hypnea musciformis* (Cystocloniaceae, Rhodophyta). J Appl Phycol 26 (2):901–907. https://doi.org/10.1007/s10811-013-0090-8

Vega-Villasante FF, Del CortésLM C, Zúñiga LM, Jaime CB, Galindo LJ (2010) Small-scale culture of tilapia (*Oreochromis niloticus*), alimentary alternative for rural and peri-urban families in Mexico? REDVET-Rev Electrón Vet 11:1–15

Wang J, Mou H, Jiang X, Guan H (2006) Characterization of a novel β-agarase from marine *Alteromonas sp*. SY37–12 and its degrading products. Appl Microbiol Biotechnol 71(6):833

Wang W, Zhang P, Hao C, Zhang XE, Cui ZQ, Guan HS (2011) In vitro inhibitory effect of carrageenan oligosaccharide on influenza A H1N1 virus. Antivir Res 92(2):237–246. https://doi.org/10.1016/j.antiviral.2011.08.010

Web of science, Accessed 20 June 2018

Wei N, Quarterman J, Jin YS (2013) Marine macroalgae: an untapped resource for producing fuels and chemicals. Trends Biotechnol 31(2):70–77. https://doi.org/10.1016/j.tibtech.2012.10.009

Wu SC, Pan CL (2004) Preparation of algal oligosaccharide mixtures by bacterial agarases and their antioxidative properties. Fish Sci 70(6):1164–1173. https://doi.org/10.1111/j.1444-2906.2004.00919.x

Wu SC, Wen TN, Pan CL (2005) Algal oligosaccharide lysates prepared by two bacterial agarases stepwise hydrolyzed and their anti oxidative properties. Fish Sci 71(5):1149–1159. https://doi.org/10.1111/j.1444-2906.2005.01075.x

Yermak IM, Mischchenko NP, Davydova VN, Glazunov VP, Tarbeeva DV, Kravchenko AO, Sorokina IV (2017) Carrageenans-Sulfated Polysaccharides from Red Seaweeds as Matrices for the Inclusion of *Echinochrome*. Mar Drugs 15(11):337. https://doi.org/10.3390/md15110337

Yuan H, Song J (2005) Preparation, structural characterization and in vitro antitumor activity of kappa-carrageenan oligosaccharide fraction from *Kappaphycus striatum*. J Appl Phycol 17 (1):7–13. https://doi.org/10.1007/s10811-005-5513-8

Yuan H, Zhang W, Li X, Lü X, Li N, Gao X, Song J (2005) Preparation and in vitro antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. Carbohyd Res 340(4):685–692. https://doi.org/10.1016/j.carres.2004.12.026

Yuan H, Song J, Li X, Li N, Liu S (2011) Enhanced immunostimulatory and antitumor activity of different derivatives of κ-carrageenan oligosaccharides from *Kappaphycus striatum*. J Appl Phycol 23(1):59–65. https://doi.org/10.1007/s10811-010-9536-4

Zhao X, Jiao G, Yang Y, Li M, Li Q, Wang X, Yu G (2017) Structure and immunomodulatory activity of a sulfated agarose with pyruvate and xylose substitutes from *Polysiphonia senticulosa* Harvey. Carbohydr Polym 176:29–37. https://doi.org/10.1016/j.carbpol.2017.08.065

Zhu B, Ni F, Sun Y, Zhu X, Yin H, Yao Z, Du Y (2018) Insight into carrageenases: major review of sources, category, property, purification method, structure, and applications. In: Critical reviews in biotechnology, pp 1–16. https://doi.org/10.1080/07388551.2018.1472550