Environmental Chemistry for a Sustainable World

K M Gothandam · Shivendu Ranjan
Nandita Dasgupta · Eric Lichtfouse
Editors

Nanoscience and Biotechnology for Environmental Applications

Springer

Environmental Chemistry for a Sustainable World

Volume 22

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Editors

Nanoscience and Biotechnology for Environmental Applications

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Editors
K M Gothandam
School of Bio Sciences & Technology
Vellore Institute of Technology
Vellore, Tamil Nadu, India

Nandita Dasgupta
Department of Biotechnology,
Institute of Engineering and Technology
Dr. A.P.J. Abdul Kalam Technical University
Lucknow, Uttar Pradesh, India

Shivendu Ranjan
DST-Centre for Policy Research
Lucknow, Uttar Pradesh, India

Eric Lichtfouse
Aix-Marseille University
CEREGE, CNRS, IRD, INRA, Coll France
Aix en Provence, France

ISSN 2213-7114 ISSN 2213-7122 (electronic)
Environmental Chemistry for a Sustainable World
ISBN 978-3-319-97921-2 ISBN 978-3-319-97922-9 (eBook)
https://doi.org/10.1007/978-3-319-97922-9

Library of Congress Control Number: 2018968289

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Dedicated to all real sufferers for the lack of a clean environment

Preface

Water pollution is a major issue for health and food security in the context of increasing industrialization and urbanization. As a consequence, novel remediation technologies are developing fast. In particular, pollution issues can be solved by environmental biotechnologies, which include bioremediation, nanobiotechnology, biosensors and enzyme degradation. This book is the second of several volumes on Environmental Biotechnology, which are published in series Environmental Chemistry for a Sustainable World.

In the first chapter, Escudero-Oñate and Ferrando-Climent review the use of microalgae to remove contaminants with special attention to pharmaceuticals. Chapter 2 by Sezgin presents management strategies for solid textile waste. In Chap. 3, Palanisamy et al. explain the environmental benefits of biopolymers. Yadav et al. detail in Chap. 4 the applications of microbes for environment and health with special focus on metagenomics. Then, Sreedharan and Rao review microbial- and enzyme-mediated degradation of azo dyes in Chap. 5 (Fig. 1). Chiral drug synthesis using epoxide hydrolase as green catalyst is reviewed in Chap. 6 by Saini and Sareen. Chapter 7, by Selvarajan et al., discusses lactose intolerance, nano-immobilization and application of β-galactosidase. Dhanasekaran et al. review

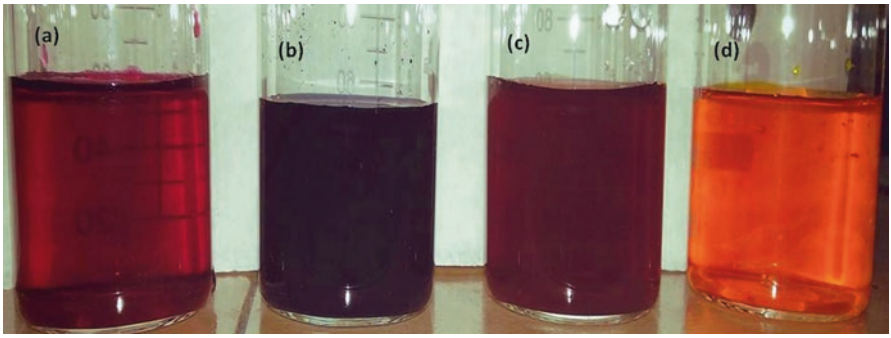

Fig. 1 Four different azo dyes which are used in almost all textile industries of India: (**a**) reactive red 195A, (**b**) reactive blue 198, (**c**) reactive brown F3B, and (**d**) reactive yellow 145

microbes responsible for bad water odour and taste in Chap. 8. Nanoremediation techniques are presented by Avipsha et al. in Chap. 9.

Thanks for reading

Vellore, Tamil Nadu, India — K M Gothandam
Lucknow, Uttar Pradesh, India — Shivendu Ranjan
Aix en Provence, France — Nandita Dasgupta
Eric Lichtfouse

Contents

Contributors

Kokati Venkata Bhaskara Rao Molecular and Microbiology Research Laboratory, Department of Biomedical Sciences, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

Saravanan Chandraleka Department of Chemistry, Urumu Dhanalakshmi College, Tiruchirappalli, Tamil Nadu, India

Dharumadurai Dhanasekaran Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

Carlos Escudero-Oñate Norwegian Institute for Water Research (NIVA), Oslo, Norway

Laura Ferrando-Climent Tracer Technology Fluid Flow and Environmental Technology, Institute for Energy Technology, Kjeller, Norway

Aravind Jeyaseelan College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

Merve Kucukali-Ozturk Textile Technologies and Design Faculty, Department of Textile Engineering, Istanbul Technical University, Istanbul, Turkey

Selvanathan Latha Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Kamaraj Murugesan College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

Ashutosh Mani Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

Vaithilingam Mohanasrinivasan School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Anbazagan Nivetha School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Kanmani Palanisamy Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, India

Suresh Babu Palanisamy College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

Priya Saini Department of Biochemistry, Panjab University, Chandigarh, India

Dipti Sareen Department of Biochemistry, Panjab University, Chandigarh, India

Avipsha Sarkar Department of Biotechnology, School of BioScience and Technology, VIT University, Vellore, Tamilnadu, India

Ethiraj Selvarajan School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Department of Genetic Engineering, School of Bioengineering, SRM Institute of Science & Technology, Kattankulathur, Chennai, Tamil Nadu, India

Shampa Sen Department of Biotechnology, School of BioScience and Technology, VIT University, Vellore, Tamilnadu, India

Sombuddha Sengupta Department of Biotechnology, School of BioScience and Technology, VIT University, Vellore, Tamilnadu, India

Hande Sezgin Textile Technologies and Design Faculty, Department of Textile Engineering, Istanbul Technical University, Istanbul, Turkey

Nand Kumar Singh Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

Swati Singh Center of Bioinformatics, University of Allahabad, Allahabad, India

Govindhan Sivaranjani Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Veena Sreedharan Molecular and Microbiology Research Laboratory, Department of Biomedical Sciences, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

Chandrasekaran Subathra Devi School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Alok Kumar Yadav Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

Birendra Singh Yadav Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

Ipek Yalcin-Enis Textile Technologies and Design Faculty, Department of Textile Engineering, Istanbul Technical University, Istanbul, Turkey

About the Editors

Dr. Gothandam K M is currently working as dean and professor at the School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India. His main area of research is plant biotechnology and environmental biotechnology. He has published many scientific research and review articles in international peer-reviewed journals and also refereed many journals of high impact factor. http://orcid.org/0000-0003-1576-5324

Dr. Shivendu Ranjan has completed his B.Tech. and Ph.D. in biotechnology from VIT University, Vellore, India, and has expertise in nano(bio)technology. He was elected as a Fellow (FLS) of the oldest active biological society started in 1778, The Linnean Society (London) and elected Fellow of Bose Scientific Society (FBSS). He is currently working as a Scientist at DST-Centre for Policy Research, Lucknow, which is funded by the Ministry of Science and Technology, Govt of India. He is also serving as a Senior Research Associate (Adjunct) at the Faculty of Engineering and Built Environment, University of Johannesburg, Johannesburg, South Africa. He also worked as the Head at the Research and Technology Development at E-Spin Nanotech Pvt. Ltd., SIDBI Incubation Center, Indian Institute of Technology, Kanpur, India. After joining E-Spin Nanotech, IIT Kanpur, he has success-

fully developed prototypes for many products and has applied two patents. Dr. Ranjan has taken lead in the DSIR Certification for R&D Unit of E-Spin Nanotech Pvt Ltd. He is also the Founder-Director of Xpert Arena Technological Services Pvt Ltd., and Advisor of many companies – Eckovation Solutions Pvt Ltd. (IIT Delhi-based start-up), Chaperon Biotech Pvt Ltd (IIT Kanpur based start-up), Kyntox Biotech India Pvt Ltd., and Xcellogen Biotech Pvt Ltd. Dr. Shivendu is also a Reviewer of Iran National Science Foundation (INSF), Tehran, Iran and a Jury at Venture Cup, Denmark, from past 3 consecutive years. He had founded and drafted the concept for the first edition of the "VIT Bio Summit" in 2012, and the same has been continued till date by the university.

His research interests are multidisciplinary and include micro/nanobiotechnology, nano-toxicology, environmental nanotechnology, nanomedicine, and nanoemulsions. His extended areas of interest are science policy, science diplomacy, technology readiness analysis, technology dissemination, policy implementation. He is the Associate Editor of *Environmental Chemistry Letters* – a Springer journal of 3.2 impact factor – and an Editorial Board Member in *Biotechnology and Biotechnological Equipment* (Taylor and Francis, USA). He is a Research Topic Editor in *Frontiers in Pharmacology* (FrontiersIn, USA, journal of 3.83 Impact Factor). He is serving as the executive editor of a journal in iMed Press, USA, and also serving as an editorial board member and referee for reputed international peer-reviewed journals. He has published sixteen edited books and one authored book in Springer, Switzerland, and two with CRC Press, USA. He has recently finished his contract of three volumes of book in Elsevier, two volumes in CRC Press, and one with Wiley and RSC (UK). He has published many scientific articles in international peer-reviewed journals and has authored many book chapters as well as review articles. He has bagged several awards and recognition from different national as well as international organizations. Website: https://sites.google.com/view/shivenduranjan

Nandita Dasgupta has a working experience in micro/nanoscience and is currently working as an Assistant Professor at the Department of Biotechnology, Institute of Engineering and Technology, Lucknow, India. She was elected as a Fellow (FLS) of the oldest active biological society started in 1778, The Linnean Society (London) and elected Fellow of Bose Scientific Society (FBSS). She is also serving as a Senior Research Associate (Adjunct) at the Faculty of Engineering and Built Environment, University of Johannesburg, Johannesburg, South Africa. Earlier at LV Prasad Eye Institute, Bhubaneswar, India, she has worked on mesenchymal stem cell-derived exosomes for the treatment of uveitis. She has an exposure of working at university, research institutes, and industries including VIT University, Vellore, Tamil Nadu, India; CSIR-Central Food Technological Research Institute, Mysore, India; and Uttar Pradesh Drugs and Pharmaceutical Co. Ltd., Lucknow, India and Indian Institute of Food Processing Technology (IIFPT), Thanjavur, Ministry of Food Processing Industries, Government of India. At IIFPT, Thanjavur, she was involved in a project funded by a leading pharmaceutical company, Dr. Reddy's Laboratories, and have successfully engineered micro-vehicles for model drug molecules. Her areas of interest include micro/nanomaterial fabrication and its applications in various fields – medicine, food, environment, agriculture, biomedical.

She has published six edited books and one authored book in Springer, Switzerland, and two with CRC Press, USA. She has finished a contract for three book volumes in Elsevier, one volume with Wiley, two book volumes in CRC Press, and one volume in RSC (UK). She has authored many chapters and also published many scientific articles in international peer-reviewed journals. She has received the Certificate for "Outstanding Contribution" in Reviewing from Elsevier, Netherlands. She has also been nominated for Advisory Panel for prototype establishment of Elsevier Inc., Netherlands. She is the Associate Editor of *Environmental Chemistry Letters* – a Springer journal of 3.2 impact factor – and also serving as the editorial board member and referee for reputed international peer-reviewed journals. She has received several awards and recognitions from different national and international organizations. Google Scholar: https://scholar.google.co.in/citations?user=u4p3mNkAAAAJ

Eric Lichtfouse, born in 1960, PhD in organic chemistry at Strasbourg University, is a geochemist working on carbon sequestration and climate change at the European Centre of Research and School in Environmental Geosciences[1]. He has invented the ^{13}C-dating method allowing to measure the dynamics of soil organic molecules[2]. He has published about 100 articles in organic synthesis, petroleum geochemistry, environmental chemistry, food chemistry, soil science and phytoremediation[3]. He is chief editor and founder of the journal *Environmental Chemistry Letters*[4], the book series *Sustainable Agriculture Reviews*[5] and *Environmental Chemistry for a Sustainable World*[6] and the magazine *Publier La Science*[7]. His book, *Scientific Writing for Impact Factor Journals*[8], contains the micro-article[9], a new tool to identify the novelty of experimental results. He is also a triathlete who got the bronze medal in age groups at the World ITU Cross Triathlon Championships. He has qualified for the Word Ironman, Ironman 70.3 and XTerra Championships, finished 5 times in the top 3 of French Triathlon and Duathlon Championships and completed 17 ironman competitions. Further details are available on LinkedIn and ResearchGate.

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11. https://fr.linkedin.com/in/ericlichtfouse
12. https://www.researchgate.net/profile/Eric_Lichtfouse
13. http://www.researcherid.com/rid/F-4759-2011
14. http://orcid.org/0000-0002-8535-8073

Chapter 1
Microalgae for Biodiesel Production and Pharmaceutical Removal from Water

Carlos Escudero-Oñate and Laura Ferrando-Climent

Contents

Abstract During the past 20 years, the presence of pharmaceutical active compounds in water bodies has been gaining increasing attention, and nowadays there is broad acknowledgement that they should be considered an emerging environmental problem. The existing scientific literature clearly points out that pharmaceuticals enter the environment and provoke adverse effects. The main source of these compounds is wastewater, since, after intake, the pharmaceutical compounds are absorbed, metabolized, and finally excreted into the sewerage system. Although wastewater is normally collected and delivered to treatment plants, it has been demonstrated that the regular treatments applied in such facilities are not completely effective for removal of a variety of pharmaceuticals, which are subsequently introduced into the environment.

C. Escudero-Oñate (✉)
Norwegian Institute for Water Research (NIVA), Oslo, Norway
e-mail: carlos.escudero@niva.no

L. Ferrando-Climent
Tracer Technology Department, Fluid Flow and Environmental Technology, Institute for Energy Technology, Kjeller, Norway

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_1

Microalgae can play a relevant role in remediation of wastewater. In addition to their well-known capacity to remove organic carbon, nutrients, and even heavy metals from water, microalgae have recently been revealed to have significant potential to remove pharmaceutical compounds from polluted effluents. Microalgae offer a further benefit to close the mass-to-energy loop, since their content of carbohydrates and oils allows them to be considered as a potential feedstock for the production of biofuels.

In this chapter, the authors review the potential of microalgae to remove contaminants of emerging concern from water, paying special attention to pharmaceutical compounds. The immobilization techniques that can be used to facilitate the harvesting process and valorization of the biomass for the production of biodiesel are also assessed.

1.1 Introduction

During recent decades, pharmaceutical compounds have become a concerning group of emerging pollutants, according to a large number of studies (Aga 2008; Barceló and Petrovic 2007, 2008; Boxall et al. 2012; Farré et al. 2008; Fatta-Kassinos 2010; Verlicchi et al. 2010, 2012, 2014). The fact that large amounts of these drugs reach the environment via urban sewerage systems is evidence that conventional wastewater treatment plants are inefficient for their removal. An overview of the life cycle of pharmaceutical products and their potential to enter the environment during the manufacturer-to-end-user chain is presented in Fig. 1.1. Pharmaceutical compounds are designed to have target effects in the human body, but it is still not very well known how these substances can affect other organisms

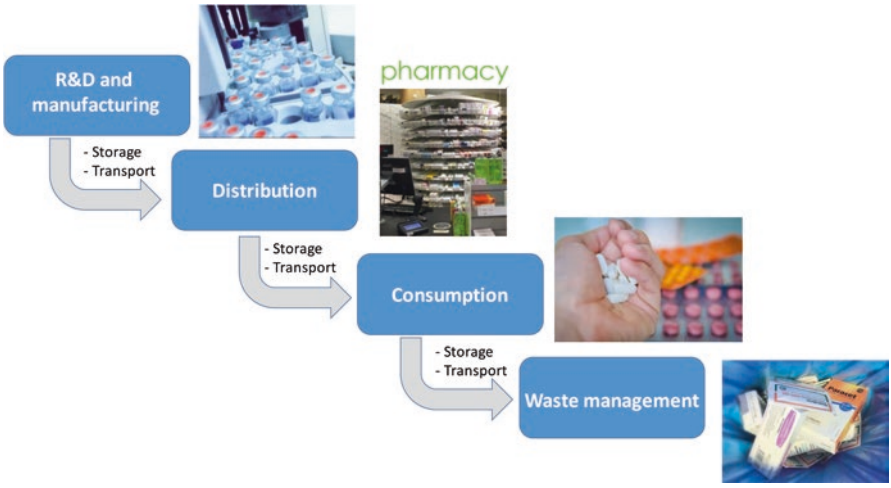


Fig. 1.1 Overview of the life cycle of pharmaceutical products, showing the multiple operations that might release pharmaceuticals into the environment.

in the natural environment, as well as having indirect impacts on human health. Unfortunately, the concentrations of pharmaceutical compounds in urban wastewater are increasing because of the aging of populations, increases in population density (Le Corre et al. 2012), and, in some geographical areas, water scarcity associated with climate change (Osorio et al. 2012; Petrovic et al. 2012). As contaminants of emerging concern, pharmaceutical active compounds have attracted the attention of the scientific community all over the world.

In most cases, these compounds are unregulated pollutants, which may become candidates for future regulation, depending on their potential incidence in the environment and toxic effects. To date, there are just a few regulations in force that deal with the discharge of this kind of micropollutants into the environment. Efforts are currently being made to set new policies to address the issue of increasing occurrence of pharmaceutical active compounds in the environment and to create a framework for controlling the release of these substances. In directive 2008/105/EC under the decision (EU) 2015/495 of March 20, 2015, the European Union (EU) has recently established a watch list of substances to be monitored throughout the EU; it establishes that substances that are found to pose a significant risk should be considered for inclusion in a priority substances list (European Commission 2015). In the watch list it is possible to find some macrolide antibiotics (such as erythromycin) and the nonsteroidal anti-inflammatory drug (NSAID) diclofenac. A critical assessment of the environmental fate and the effects of pharmaceutical active compounds will contribute to the future enforcement of regulations, as well as providing a set of best management practices related to water quality.

Conventional aerated activated-sludge biodegradation treatment—used in almost all urban sewerage systems—has demonstrated poor efficiency or even absolute inefficiency for removal of most of these contaminants (Ferrando-Climent et al. 2012; Gagnon and Lajeunesse 2008; Kümmerer et al. 1997; Lishman et al. 2006; Onesios et al. 2009; Paxeus 2004; Verlicchi et al. 2013; Zhang et al. 2013). Other technologies involving oxidation processes (e.g., ozonization, peroxone, and Fenton processes) (Klavarioti et al. 2009), advanced biological processes (e.g., fungal degradation) (Cruz-Morató et al. 2012, 2013; Ferrando-Climent et al. 2015; Jelic et al. 2012; Marco-Urrea et al. 2009, 2010a, 2010b), membranes (e.g., nanofiltration, ultrafiltration, and reverse osmosis), and membrane bioreactors (Dolar et al. 2012; Dolar and Košutić 2013; Garcia-Galan et al. 2010; Kovalova et al. 2012; Reif Lopez 2011; Sipma et al. 2010) have also been extensively evaluated for the removal of micropollutants. However, most of these technologies have exhibited important drawbacks that hinder their deployment for large-scale use in real scenarios. This has been the case for ozonation and membrane-based techniques, for example. Other processes, such as the Fenton process or fungal biodegradation, have also failed in the context of real application because they require large volumes of acid to adjust the pH of the raw wastewater to the optimal operative levels for these techniques.

Conversely, photosynthetic microorganisms, such as microalgae, have been shown to be a potential alternative for the removal of micropollutants, organic compounds, and inorganic compounds from polluted effluents (de-Bashan and Bashan

2010; Kumar et al. 2011; Subashchandrabose et al. 2011; Sun et al. 2011; Yan et al. 2014). For instance, recent studies have demonstrated the ability of cyanobacteria to successfully degrade very persistent pollutants such as benzo[α]pyrene (Yan et al. 2014). These microorganisms have huge—but just barely explored—biotechnological prospects for the removal of micropollutants from water. Among their main benefits are the fact that many strains of these microorganisms can live and grow optimally at normal wastewater pH values (about 8–8.5), so there is no need to perform expensive pH regulation through addition of acids or bases. Furthermore, a consortium of different microalgae could provide a photosynthetic system that generates oxygen (an electron acceptor), which is a key factor in the oxidative degradation of pollutants by autotrophic bacteria (Subashchandrabose et al. 2011).

The use of these kinds of microorganisms in wastewater treatment exhibits another set of advantages: (i) for their growth, they require nitrogen and phosphorus, so they take it up from water, contributing to the minimization of the discharge of nutrients into the environment; and (ii) the biomass can be valorized in subsequent processes to produce biofuels.

In this chapter, we review different aspects of the culturing and immobilization of microalgae, and the use of these special groups of microorganisms for the removal of pharmaceutical compounds and other contaminants of emerging concern from water. The valorization of the obtained biomass for the production of biodiesel is also discussed.

1.2 Use of Microalgae for Water Treatment

1.2.1 Cultivation of Microalgae

The conditions in which microalgae are cultivated strongly affect their chemical composition and growth characteristics. The growth of these kinds of microorganisms is influenced by a variety of factors, both biotic and abiotic. Biotic factors include the presence of pathogens (such as bacteria, fungi, and viruses) and competition from other microalgae, whereas abiotic factors include light (quality and quantity), temperature, pH, salinity, qualitative and quantitative profiles of nutrients, dissolved oxygen concentration, and presence of toxic compounds (Gonçalves et al. 2017). Another parameter that strongly influences microalgal growth is temperature (Davison 1991), and the optimal growing temperature is very dependent on the strain. When microalgae are grown in large-scale equipment, other parameters such as the residence time, liquid and gas flow/exchange rates, harvesting, presence of preferred flow paths and short circuits, agitation and mixing, presence of shear forces, and formation of shadows should also be considered.

Use of different energy sources (light or organic) and carbon sources (inorganic or organic) is recognized as a key factor that significantly influences relevant parameters such as the lipid accumulation of microalgae (Chen et al. 2011). Cultivation

conditions may be classified into four general categories: (i) photoautotrophic, (ii) heterotrophic, (iii) mixotrophic, and (iv) photoheterotrophic (Yeh and Chang 2012). Phototrophic (or autotrophic) cultivation occurs when microalgae use light as an energy source and inorganic carbon (such as bicarbonate and CO_2) as a carbon source to form chemical energy through the photosynthesis process. A major advantage of the autotrophic nutritional mode is the production of valuable products, such as algal lipids, at the expense of CO_2. The photosynthesis process takes place in the chloroplasts and is dominated by light and dark reactions (Venkata Mohan et al. 2015). This is the most widely used cultivation mode in industrial environments for scale-up of outdoor systems. Phototrophic cultivation can be carried out in open ponds or closed photobioreactors. In the case of cultures that can be easily contaminated, closed photobioreactors are the preferred choice, while open systems are preferable for microalgae that can live in extreme environments (i.e., high pH or salinity) or that exhibit very fast growth (Amaro et al. 2012; Huang et al. 2010).

A feasible alternative for phototrophic cultures, but restricted to a few microalgal species, is the use of their heterotrophic growth capacity in the absence of light, replacing the fixation of atmospheric CO_2 by organic carbon sources dissolved in culture media (Perez-Garcia et al. 2011). In heterotrophic cultivation, the microalgae make use of organic molecules as primary energy and carbon sources, facilitating high biomass productivity and providing economic feasibility for large-scale production (Perez-Garcia et al. 2011).

The mixotrophic growth regime is a variant of that used for heterotrophic growth, where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operate concurrently (Perez-Garcia et al. 2011). Some strains of microalgae can grow under a mixotrophic regime combining autotrophic and heterotrophic mechanisms by assimilating available organic compounds, as well as atmospheric CO_2, as a carbon source (Venkata Mohan et al. 2015). Although photoautotrophic and heterotrophic cultivation of microalgae are the most common growth modes, growth in mixotrophic conditions may pose advantages in some applications, since these microorganisms can then garner the benefits of autotrophic and heterotrophic cultivation (Zhan et al. 2016).

1.2.2 Immobilization of Microalgae

One of the most important challenges related to the use of microalgae in the detoxification of polluted effluents is connected to their separation from the effluent prior to discharge. Microalgae do not normally sink quantitatively just by gravity, and then harvesting operations are required. Regardless of whether the water treatment is performed in a shallow open pond (where the culture is circulated by a paddle wheel) or in a more sophisticated photobioreactor system, harvesting of the produced biomass is a major issue. According to Jacob-Lopes et al. (2015), just the recovery of microalgae from the liquid stream accounts for about 20–30% of the total costs.

There are a large number of potential technologies available for the recovery of microalgae. Among them, those most widely used are based on physical operations such as filtration, gravity sedimentation, centrifugation, flotation (Boonma et al. 2015; Chen et al. 2011), electrocoagulation, electroflotation, and polymer coagulation. However, the use of these methods is not considered cost effective for wastewater treatment (de-Bashan and Bashan 2010). Chemical methods, such as those based on chemical flocculation, provide an economical solution to harvest microalgal biomass efficiently (Lam and Lee 2012a). Nevertheless, these methods require the addition of relatively large amounts of chemicals to the water and thus are not considered environmentally friendly practices (Chen et al. 2011). The use of chemical coagulants and flocculants (such as combinations of aluminum or iron salts and polyelectrolytes) could potentially contaminate the harvested microalgal biomass and have adverse effects on the product quality for further valorization of the biomass (Azma et al. 2011; Harun et al. 2010). The problems connected to the separation of microalgae from wastewater are attributed mainly to their small size (Henderson et al. 2008), their tendency to exhibit neutral buoyancy, and the fact that autotrophic cultures are relatively dilute (with biomass concentrations between 1 and 8 g L^{-1}) (Pulz 2001).

Each algal species presents unique challenges due to the array of sizes, shapes, densities, and cell surface properties encountered. In addition to the characteristics of the microalgae themselves, when a harvesting operation is being developed in a large-sized reactor, special attention should be paid to its configuration. No individual technique can be applied to fit all reactors' configurations. A successful and cost-effective harvesting process should therefore consider the characteristics of the reactors, paying special attention to the fluid dynamics characteristics. A low-cost, energy-efficient method with high recovery efficiency is then required. To overcome the drawbacks of harvesting operations, some researchers have developed and explored immobilization techniques. Biomass immobilization consists of attachment or entrapment of biomass on a support, normally of a polymeric nature. Mallick (2002) categorized the immobilization methods into six main groups: covalent coupling, affinity immobilization, adsorption, confinement in a liquid–liquid emulsion, capture behind a semipermeable membrane, and entrapment. The technique widely used to immobilize microalgal cells is based on the gel entrapment method (Lam and Lee 2012a). If the obtained material is going to be used in wastewater treatment, the entrapment materials need to meet a set of characteristics: they should be phototransparent, insoluble, nontoxic, and they should exhibit good mechanical and chemical stability, with high diffusivity.

Immobilization of microalgae for wastewater treatment provides a set of advantages in comparison with the conventional suspension technique. The most relevant ones are: (i) flexibility in photobioreactor design; (ii) increased reaction rates, arising from higher cell density; (iii) enhanced operational stability; (iv) avoidance of cell washouts; (v) easier cultivation, harvesting, and handling of the produced biomass; (vi) easy replacement of the algae; (vii) provision of shelter and protection of cell integrity from harsh environmental conditions such as salinity, metal toxicity,

variations in pH, and any product inhibition; and (viii) continuous utilization of algae in a nondestructive way (Eroglu et al. 2015).

Although synthetic polymers such as polyacrylamide, polyurethane, and polyvinyl might be used, the most widely employed immobilization methods are based on biopolymers such as agar, alginate, and carrageenan. Their environmentally friendliness, low toxicity, and high transparency justifies this selection (Lam and Lee 2012a; Moreno-Garrido 2008). Several authors have reported the use of immobilized strains of microalgae for the removal of pollutants from water, but the research has been focused mainly on the removal of nutrients and, to a lesser extent, heavy metals. It is worth noting that despite the availability of scientific literature regarding the removal of nutrients and heavy metals using immobilized strains of microalgae, there is a scarcity of reports that tackle the use of these microorganisms in the removal of contaminants of emerging concern, such as pharmaceutical compounds. An overview of the different immobilizations strategies applied to algal species and the target pollutants is presented in Table 1.1.

1.3 Use of Microalgae for Removal of Pharmaceuticals from Wastewater

Very few studies have reported the removal of pharmaceutical active compounds using microalgae-based technology, and most of the works are very recent. However, the existing literature on microalgae-based treatments points out that they have huge potential for the detoxification of wastewater (Norvill et al. 2016; Sullivan Graham et al. 2017). Most of the studies performed to date have been devoted to those groups of pharmaceuticals that cause concern because of their large consumption and their well-known potential effects on living organisms, as is the case for antibiotics (because of generation of antibiotic resistance) and hormones (because of their inherent endocrine disruptor effects).

Guo and coauthors (Guo et al. 2016) explored the removal of the antibiotic 7-amino cephalosporanic acid from wastewater in a batch reactor. The authors studied three different strains of microalgae—*Chlorella* sp. Cha-01, *Chlamydomonas* sp. Tai-03, and *Mychonastes* sp. YL-02—isolated from Southern Taiwan. For the cultivation of the microorganisms, culture medium BG-11 was employed. The researchers exposed the microorganisms to a relatively high antibiotic concentration (between 25 and 150 mg L^{-1}), which was very different from levels reported in the environment. The authors assessed the attenuation in the concentration of the antibiotics achieved by (i) hydrolysis, (ii) photolysis, and (iii) adsorption onto the three strains of microalgae. In this paper, hydrolysis and photolysis were observed to play a very relevant role in the dissipation of the antibiotics in the water. Adsorption was found to take place at a first and very fast step, since equilibrium was achieved after approximately 10 min of exposure. The researchers found that the relatively high concentration of the antibiotics did not severely influence the lipid biosynthesis of

Table 1.1 Immobilization matrices, microalgal strains, and pollutants targeted. To date, the research has mainly targeted nutrients

Immobilization matrix	Microalgal strains	Target pollutants	Reference
N and P species			
Alginate beads	*Chlorella vulgaris*	Ammonium, phosphate	Tam and Wong (2000)
	Nannochloropsis sp., *Scenedesmus intermedius*	Total phosphorous, total nitrogen	Jiménez-Pérez et al. (2004)
	Chlorella vulgaris and *Azospirillum brasilense* (coimmobilization)	Ammonium, phosphate	de-Bashan et al. (2002)
	Chlorella sorokiniana and *Azospirillum brasilense* (coimmobilization)	Phosphate	Hernandez et al. (2006)
Carragenan beads	*Spirulina maxima*	Total phosphorus, ammonium	Cañizares et al. (1993)
	Scenedesmus acutus, *Scenedesmus obliquus*	Ammonium, phosphate	Chevalier and de la Noüe (1985)
Agar beads	*Chlorella vulgaris*, cyanobacterium *Anabaena doliolum*	Phosphate, nitrate, nitrite	Mallick and Rai (1994)
Alginate beads			
Carragenan beads			
Chitosan beads			
Chitosan beads	*Scenedesmus* sp.	Phosphate, nitrate	Fierro et al. (2008)
Flat-surface alginate screens	*Scenedesmus bicellularis*	Ammonium, phosphate	Kaya et al. (1995)
Alginate beads			
Filter paper	*Trentepohlia aurea*	Ammonium, nitrate, nitrite	Abe et al. (2003)
Polyvinyl foams	*Scenedesmus obliquus*	Nitrate	Urrutia et al. (1995)
Polyurethane foams			
Alginate beads	*Chlorella vulgaris*, *Chlorella kessleri*, *Scenedesmus quadricauda*	Ammonium, phosphate	Travieso et al. (1996)
Carrageenan beads			
Polystyrene foams			
Polyurethane foams			
Chitosan nanofibers	*Chlorella vulgaris*	Nitrate	Eroglu et al. (2012)
Graphene nanosheets			Wahid et al. (2013a, b)
Graphene oxide nanosheets			Wahid et al. (2013a, b)
Heavy metals			
Polysulfone	*Phormidium laminosum*	Cu(II), Fe(II), Ni(II), Zn(II)	Blanco et al. (1999)
Epoxy resin			

(continued)

Table 1.1 (continued)

Immobilization matrix	Microalgal strains	Target pollutants	Reference
Alginate beads	*Chlorella* sp.	Cu(II), Zn(II)	Wan Maznah et al. (2012)
	Chlamydomonas sp.		
Alginate beads	*Chlamydomonas reinhardtii*	Hg(II), Cd(II), Pb(II)	Bayramoğlu et al. (2006)
Alginate beads	*Chlorella salina*	Co(II), Zn(II), Mn(II)	Garnham et al. (1992)

Adapted from Eroglu et al. (2015), with permission

the microalgae, and they proposed valorization of the biomass for the production of biodiesel. This decision was rationally supported by the high concentration of lipids in the microalgae. In the case of the strain *Chlamydomonas* sp. Tai-03, the authors reported a lipid concentration of about 45% (w/w) after 312 h of exposure to the antibiotic (initial concentration 100 mg L^{-1}) (Guo et al. 2016). In another study dealing with antibiotics, *Chlorella vulgaris* was evaluated for the removal of tetracycline in a high-rate algal pond configuration (de Godos et al. 2012). The researchers showed that the antibiotic was completely photodegraded in the wastewater in less than 45 h and that sorption onto biomass played an important role in the elimination of tetracycline.

Besides removal of antibiotics, removal of hormones has been assessed using microalgae-based processes. In this regard, Homs-Diaz and coauthors tested *Selenastrum capricornutum* and *Chlamydomonas reinhardtii* for possible biodegradation of the hormones β-estradiol and 17α-ethinylestradiol (Hom-Diaz et al. 2015). β-Estradiol was almost completely removed from water by treatment with *S. capricornutum* (88–100% removal), while 17α-ethinylestradiol removal was in the range 60–95%, depending on the culture conditions. The decay in the concentrations of both hormones in *S. capricornutum* cultures was attributed to biodegradation, although sorption onto the biomass was also found to take place, contributing to the overall removal of the hormones from the water. On the other hand, β-estradiol and 17α-ethinylestradiol were completely removed in those experiments performed with *Chlamydomonas reinhardtii,* but sorption was identified as the main removal mechanism. In the same study, the removal of other endocrine disruptors such as bisphenol A (BPA) was also tracked. The authors pointed out that BPA could be effectively removed from water using this microalgae-based approach. It is important to highlight that two transformation products were tentatively identified by the authors. However, the endocrine disruptor effect of the treated effluent was not tracked to assess the detoxification achieved by this process.

In 2016, Solé and Matamoros (2016) published a paper in which they thoroughly assessed the removal of six endocrine-disrupting compounds from treated wastewater, using free and immobilized strains of microalgae. The compounds they assessed were BPA, 17-α-ethinylestradiol, 4-octylphenol, bisphenol AF, bisphenol F, and 2,4-dichlorophenol. The experiments were performed in a batch reactor. The

researchers obtained the microalgal consortium from a high-rate algal pond treating urban wastewater, and they reported that the main microalgal populations were *Chlorella* sp. and *Nitzschia acicularis*. They observed that although the inoculum they used also contained bacteria, the microalgae accounted for over 90% of the total biomass. The paper did not describe the addition of any culture media; the microorganisms were allowed to acclimate to the growth conditions through exposure to secondary-treated wastewater. In this study, the microalgal biomass was immobilized. The matrix chosen for the immobilization was calcium alginate gel, following a procedure based on dropwise addition of the microalgae–sodium alginate suspension into a fixing solution of 2% $CaCl_2$. When the exposure experiments were performed, a general decay in the concentration of all of the micropollutants was observed in both cases (i.e., with use of entrapped and nonentrapped microalgae), but also in control assays (a control assay without beads and another with just calcium alginate beads). The compounds chosen by the researchers then seemed to undergo hydrolytic and/or photolytic reactions in their experimental conditions. In this study an environmentally relevant concentration was employed, as the reactors were spiked with a mixture of the six endocrine-disrupting compounds to reach an initial concentration of 100 μg L^{-1}. The authors reported different kinetic effects in the removal of the endocrine-disrupting compounds when the entrapped and nonentrapped biomasses were compared. While in the case of bisphenol AF, bisphenol F, and 2,4-dichlorophenol the entrapment of the biomass in calcium alginate enhanced the removal rate in comparison with the rates observed in the nonentrapped biomass reactors, the presence of free microalgae was found to increase the removal kinetics in the case of BPA, 17-α-ethinylestradiol, and 4-octylphenol in comparison with those observed in the control reactors.

In their latest study, the same researchers studied the removal of a representative group of emerging contaminants, including pharmaceutical active compounds and other chemicals such as pesticides and plasticizers (Matamoros et al. 2016). The authors assessed the removal from water in batch reactors using a mixture of *Chlorella* sp. and *Scenedesmus* sp. (obtained from a pond used to treat urban wastewater). The compounds targeted were caffeine, ibuprofen, galaxolide, tributyl phosphate, 4-octylphenol, tris(2-chloroethyl) phosphate, and carbamazepine. The kinetic experiments were performed with simulation of an environmentally relevant concentration of the compounds (5 μg L^{-1}) in water composed of 25% wastewater (effluent obtained after primary treatment) plus 75% groundwater. In this way, the researchers simulated the composition expected in the mixing liquor of a high-rate algal pond. In this study, the different potential contributions to the removal of the pollutants from the water were split into volatilization, photodegradation, spontaneous degradation, biodegradation enhanced by microalgae, and a direct microalgal effect. The findings indicated that 4-octylphenol, tributyl phosphate, and galaxolide were 99% removed by volatilization. Photodegradation was not found to be relevant for any of the studied compounds. It is worth noting that the researchers performed the assays under continuous gentle air bubbling, so volatilization was expected to play a major role in the removal of these compounds from the water with relatively high vapor pressure. Biodegradation was the key process affecting the degradation

of caffeine and ibuprofen (99% and 95%, respectively), and biodegradation enhanced by microalgae was observed only in the case of caffeine (40%). Carbamazepine and tris(2-chloroethyl) phosphate were regarded as recalcitrant, and their concentrations remained almost unaltered in any of the experiments during the 10-day time span. Finally, Escapa et al. (2016) performed studies of removal of high concentrations of paracetamol and salicylic acid, using *Chlorella sorokiniana* biomass, and reported higher removal efficiency in the case of salicylic acid (93–98%) than that observed for paracetamol (41–69%) (Escapa et al. 2016). It should be noted that the biomass growth was observed to be significantly stimulated by the presence of these pharmaceuticals.

In addition to the aforementioned studies regarding pharmaceuticals, it is well known that microalgae can remove inorganic nutrients (nitrogen and phosphorous) from wastewater, playing a very important role in the improvement of the overall water quality before discharge. This fact, combined with the increasingly stringent regulations regarding release of nutrients from wastewater treatment plants, has prompted researchers to develop microalgae-based alternatives for the abatement of eutrophication. Within this scope, some authors have proposed the upgrading of regular wastewater treatment plants with microalgae as a tertiary stage (Arbib et al. 2014; González et al. 1997).

A conventional wastewater treatment scheme is presented in Fig. 1.2. First, primary treatment is performed and consists of (i) a screening chamber to get rid of large solids from the wastewater, (ii) grit chambers to remove the grit, and (iii) settling tanks to allow particles to sink at the bottom. After the primary treatment, removal of most of the settleable and floating material is achieved. The obtained sludge is then sent to digester plants and the water flows on to the secondary treatment.

The secondary treatment of wastewater is designed to biologically degrade the different organic matter, in most instances using aerobic biological processes. During the activated-sludge process, the primary effluent flows into an aeration tank, where it is mixed with microorganisms. The aeration tank injects a steady supply of air into the wastewater, ensuring that the microorganisms have an adequate supply of oxygen to break down the organic matter that remains in the effluent. The effluent then flows into secondary settling tanks, where a fraction of the sludge is recirculated into the biological reactor and the remainder is sent for sludge treatment.

After the secondary treatment, a tertiary treatment using microalgae offers an interesting alternative for upgraded wastewater treatment, since it provides final polishing of the effluent prior to discharge, coupled with the production of potentially valuable biomass, which can be used for several purposes (Abdel-Raouf et al. 2012) such as the production of biofuels. Arbib et al. (2014) explored the advantages of microalgae-based treatments over conventional treatment and summarized them as follows: (i) nitrogen and phosphorous are removed from the water and can be converted into biomass without any external source of organic carbon; (ii) the effluent discharged into receiving water bodies is oxygenated; and (iii) high-value products can be extracted from the biomass that is generated.

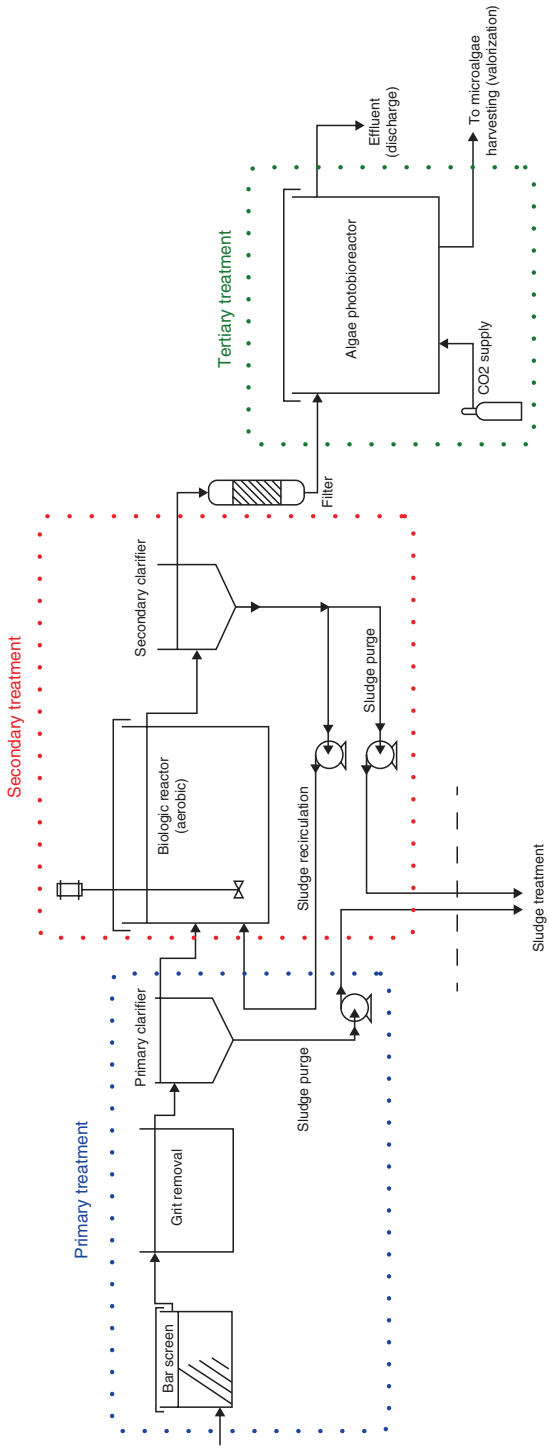


Fig. 1.2 Layout of a conventional wastewater treatment plant upgraded with tertiary treatment based on a microalgal bioreactor attached after the filter of the secondary clarifier. Such tertiary treatment increases the water quality prior to discharge and provides valuable sludge

In addition to the aforementioned advantages, it should also be noted that water treatment alternatives based on the use of photosynthetic microorganisms may become a relevant strategy for the abatement of climate change. These kinds of microorganisms exhibit much higher growth rates than those of terrestrial plants, leading to a CO_2 conversion performance 10–50 times greater than that observed in terrestrial plants (Arbib et al. 2014; Li et al. 2008). As was mentioned earlier, microalgae-based technology offers a challenging—but, at the same time, promising—scenario to develop improved water treatment schemes. The process should produce a cleaner water effluent than that provided by regular wastewater treatment plant schemes, and biomass with good valorization potential—for example, to obtain liquid biofuels.

1.4 Processing of Algal Biomass into Liquid Biofuels

Society uses fossil resources to produce fuels for transportation and heating, and for use as precursors of the vast quantity of essential petrochemical products. The use of fossil resources has major limitations, as the future availability and price of oil are both uncertain. In addition to this, the use of fossil resources as fuels contributes to the production of greenhouse gases and is a major factor in climate change. Reducing societal reliance on fossil fuel by replacing it with carbon sourced from biomass offers the potential to address these issues (Cichocka et al. 2011). Biomass-derived biofuels provide a new path to reduce petroleum reliance, with a concomitant decrease in the emissions of greenhouse gases.

As was mentioned earlier, microalgae have considerable potential to be used in the treatment of wastewater. Once they have fulfilled that role, they can be continuously harvested from the outlet effluent and processed using different schemes to yield liquid fuels such as biodiesel and bioethanol. These two renewable fuels have attracted most of the attention as candidates to replace fossil-based transport fuels. While biodiesel from microalgae is produced from their lipid fraction, bioethanol is produced from the carbohydrates present in the cells.

The EU has adopted important targets regarding the contribution of bioenergy in various sectors of the economy (e.g., road transport), and a general increasing trend in the production of liquid biofuels has been observed in recent years (Fontaras et al. 2012). The growing interest in liquid biofuels in the European Union can be observed in Figs. 1.3 and 1.4, where the production of biodiesel and biogasoline (expressed in 1000 tonnes of oil equivalents (ktoe)) in the period of 2004–2015 in the EU and in the individual member states is plotted. Those countries whose production values were zero or unknown during that time span have been excluded. The data presented in Figs. 1.3 and 1.4 were retrieved from the Eurostat database (Eurostat 2017).

As may be observed, there is growing interest in the development of production of biodiesel and biogasoline in the EU. Although in 2004 the combined biodiesel production of the 28 EU countries did not reach 2000 ktoe, in 2015, just 11 years

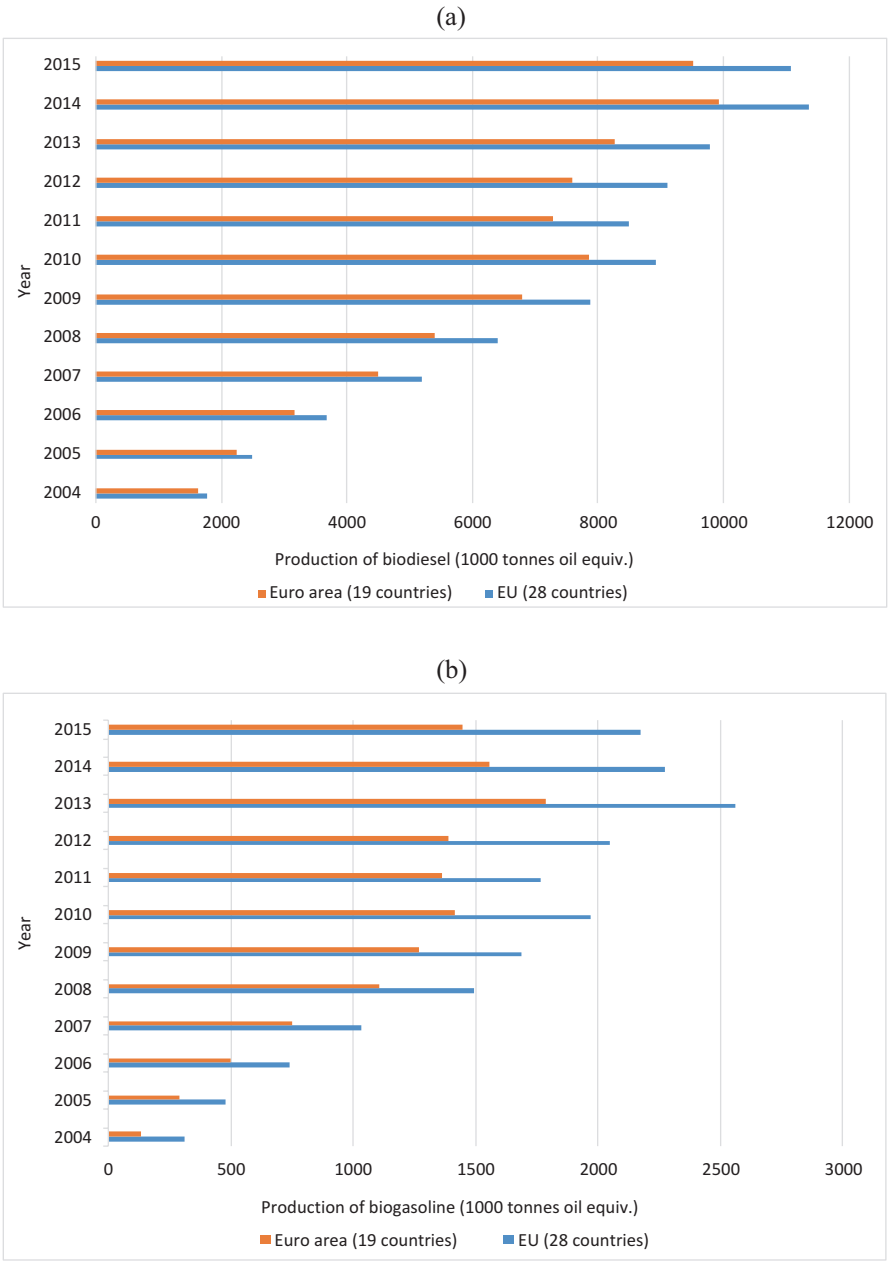


Fig. 1.3 Production of biodiesel (**a**) and biogasoline (**b**) in the European Union (*EU*; *orange* denotes the 19 eurozone countries; *blue* denotes the 28 EU countries) in the period of 2004–2015 (Eurostat 2017). The data are expressed in 1000 tonnes of oil equivalents (*ktoe*). The figures show an overall growing trend in the production of these biofuels in Europe

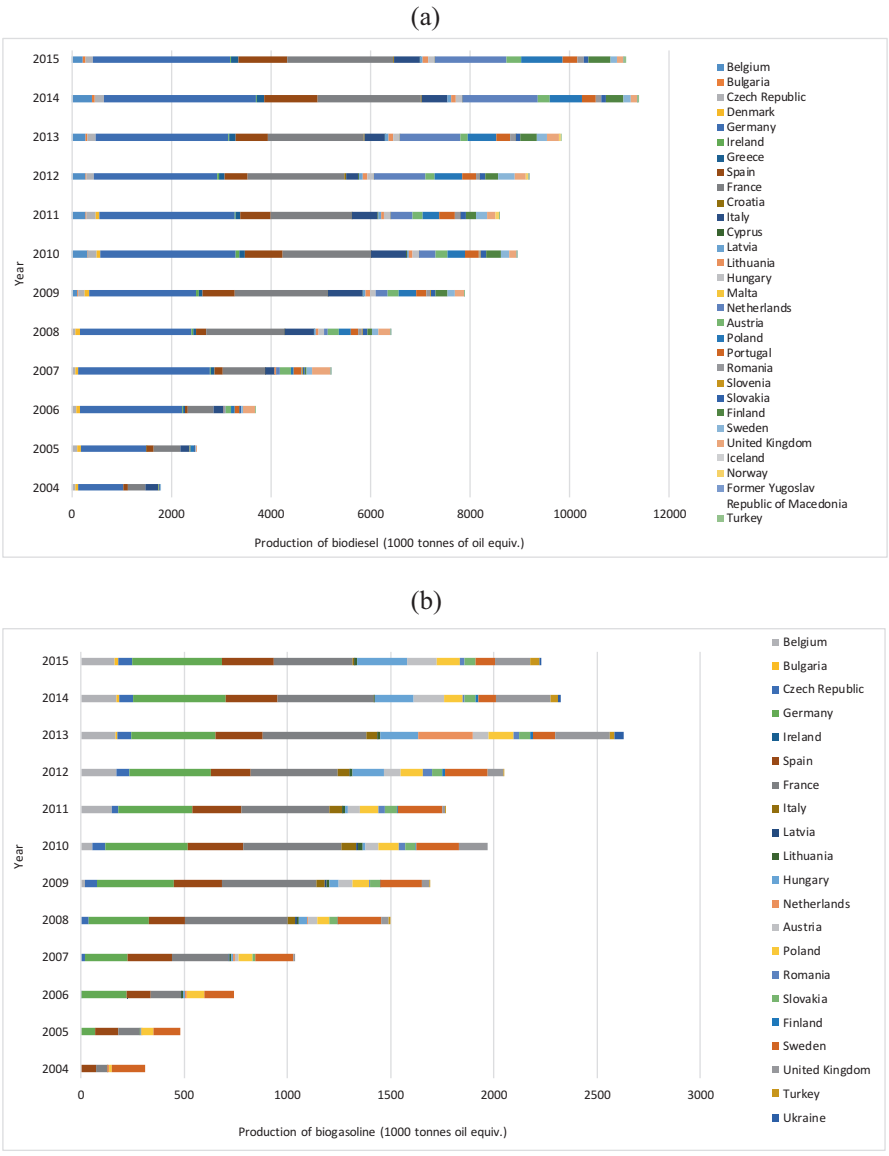


Fig. 1.4 Production of biodiesel (**a**) and biogasoline (**b**) in individual European Union (*EU*) countries in the period of 2004–2015 (Eurostat 2017). The data are expressed in 1000 tonnes of oil equivalents (*ktoe*)

later, the overall production got close to 12,000 ktoe. In the case of biogasoline, the production was about 400 ktoe in 2004 and exhibited a generally increasing trend until 2013, when it reached about 2500 ktoe. Between that year and 2015 the production of this type of biofuel decreased. It is clear that in the EU, of the two liquid

biofuels assessed, biodiesel has attracted most of the attention from producers. The data presented in Fig. 1.4 show the production of biodiesel and biogasoline by country and their evolution in the period of 2004–2015. The data reveal that of the 28 EU countries, Germany, France, Spain, Italy, and the Netherlands were the largest producers of biodiesel. When it comes to biogasoline, Germany, Spain, and France were the hot spots that concentrated the largest production of this type of biofuel.

Biodiesel has become more attractive recently because of its environmental benefits, increases in petroleum prices, and uncertainties concerning petroleum availability (Bozbas 2008). At present this biofuel seems to be the most promising candidate to fully replace fossil fuels in the near future.

1.4.1 Production of Biodiesel

Nowadays, biodiesel is produced mainly from vegetable oils, whose origin derives mostly from food crops. This fact raises non-negligible ethical issues such as land use competition between fuel and food. In addition to this, because of the increasing demand for these oil crops, their market price has increased, raising the costs related to the production of biodiesel. Alternative feedstocks then need to be developed to provide a solution to this problem (Yen et al. 2013). Among the different alternatives to use of crops as oil sources are microalgae. Microalgae have higher growth rates than plants and a large surface to volume ratio, making them excellent collectors of light and giving them good mass transfer properties. These organisms have been shown to be very effective in the production and accumulation of lipids in their structures (Tsukahara and Sawayama 2005) and offer the following advantages as sources of biodiesel feedstocks (Um and Kim 2009): (i) the growth of microalgae is extremely fast compared with that of terrestrial plants, and the biomass can be doubled within 24 h; (ii) the oil content of microalgal biomass can reach over 50% of the dry cell weight; (iii) the oil yield by cultivated area is larger than that of oilseed crops; (iv) microalgae are aquatic microorganisms and thus do not compete for the land needed for agricultural crops; (v) the production of microalgae does not compete with human food production; (vi) microalgae are able to grow under conditions that are not suitable for conventional crops; (vii) microalgae can convert CO_2 into biomass and may contribute to the reduction of CO_2 concentrations in the atmosphere, as the production of 1 kg of dry algal biomass utilizes about 1.83 kg of CO_2 (Chisti 2007); and (viii) biofuels produced from microalgae do not contain sulfur, are nontoxic, and are highly biodegradable.

Biodiesel derived from microalgae seems to be the only renewable biofuel that has the potential to completely displace petroleum-derived transport fuels without provoking adverse effects in the supply of food and other crop products. Most productive oil crops, such as oil palm, do not come close to microalgae in being able to sustainably provide the necessary amounts of biodiesel. Similarly, bioethanol produced from sugarcane is no match for microalgal biodiesel. Several recent studies have demonstrated the viability of industrial processes for the production of bio-

diesel, with some of them suggesting an anaerobic digestion process after extraction of lipid from algal biomass for further recovery of biogas (Ramos Tercero et al. 2014; Santander et al. 2014; Sawaengsak et al. 2014).

For algal biodiesel to be an acceptable substitute for fossil fuels, its properties must match or exceed these established in the International Biodiesel Standard for Vehicles (EN14214) (Brennan and Owende 2010). One of the major drawbacks of biodiesel obtained from algal oil is that it contains a high proportion of polyunsaturated fatty acids when compared with vegetable oils, making it more susceptible to oxidation in storage and therefore limiting its utilization (Chisti 2007). Despite this, algal biodiesel has been found to show physical and chemical properties similar to those of petroleum diesel and first-generation biodiesel from oil crops, and it compares favorably with the international standard EN14214 (Brennan and Owende 2010). To overcome the drawback of an excessive number of unsaturations in the aliphatic chains, a potential solution could be based on partial catalytic hydrogenation of the oil (Jang et al. 2005). Such a process would contribute to reducing the degree of unsaturation to yield a product with improved stability.

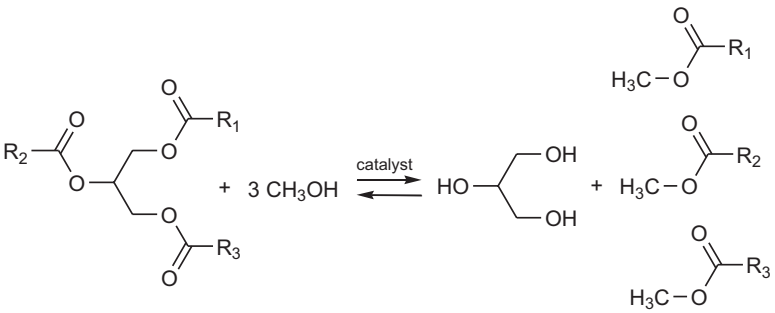
When it comes to the assessment of biomass for biofuel production, there are two key parameters: the lipid content (the percentage of lipid per dry weight of biomass) and the lipid productivity (the amount of lipid produced per liter of working volume per day). Both the lipid content and the biomass production rate should be considered simultaneously to ensure efficient microalgal lipid production. The suitability of the fatty acid for the production of biodiesel is also strongly dependent on the number of carbon units it contains. Fatty acid chains containing from 14 to 20 carbon units are considered suitable for biodiesel production (Yen et al. 2013). The amount of lipids and the profile of the fatty acids varies enormously from one strain to another. In Table 1.2 a summary of the lipid content of several microalgal strains is presented. In addition to the strain, the growth conditions can severely affect the biosynthesis and accumulation of lipids.

Lipids can be extracted to yield oil similar to that obtained from land-based oilseed crops, and the obtained algal oil can be converted to biodiesel through the same methods that are applied to vegetable oil. The most common method employed in oil extraction from microalgal biomass is based on solvent extraction using hexane, ethanol, methanol, and a methanol–chloroform mixture (Lam and Lee 2012b). In this solid–liquid extraction process, diffusion is always the rate-limiting factor in the overall mechanism. This factor becomes critical in the case of microalgae, since the cell wall further hinders the solvent from diffusing into the inner cell for lipid extraction. Therefore, a cell disruption method can be introduced to enhance solvent diffusion efficiency and consequently increase the microalgal lipid recovery rate. Widely employed techniques to disrupt microalgal cell walls are autoclaving, bead beating, use of ultrasound or microwave power, and osmotic shock (Lam and Lee 2012b).

Obtaining biodiesel from microalgal oil involves a methanolysis reaction to break the ester bonds of the fatty acids and the three alcohol groups of glycerol to yield fatty acid methyl esters (FAMEs). The reaction is defined as transesterification, since the glycerol ester is transformed into a methoxy ester and glycerol is released as a by-product. The transesterification reaction is shown in Scheme 1.1.

Table 1.2 Lipid content in several strains of microalgae. The oil content is highly strain dependent and ranges from 12% to 77%

Microalgal strain	Oil content (% of dry weight)	Reference
Botryococcus braunii	25–75	Chisti (2007)
Chlorella sp.	28–32	Chisti (2007)
Crypthecodinium cohnii	20	Chisti (2007)
Cylindrotheca sp.	16–37	Chisti (2007)
Dunaliella primolecta	23	Chisti (2007)
Isochrysis sp.	25–33	Chisti (2007)
Monallanthus salina	>20	Chisti (2007)
Nannochloris sp.	20–35	Chisti (2007)
Nannochloropsis sp.	31–68	Chisti (2007)
Neochloris oleoabundans	35–54	Chisti (2007)
Nitzschia sp.	45–47	Chisti (2007)
Phaeodactylum tricornutum	20–30	Chisti (2007)
Schizochytrium sp.	50–77	Chisti (2007)
Tetraselmis sueica	15–23	Chisti (2007)
Chlamydomonas sp. Tai-03	28.6	Tan et al. (2016)
Scenedesmus sp.	43.0	Griffiths et al. (2012)
Nannochloropsis sp.	35.0	Griffiths et al. (2012)
Chlorella vulgaris (UTEX 395)	57.0	Griffiths et al. (2012)
Ankistrodesmus falcatus (UTEX 242)	12.0	Griffiths et al. (2012)
Chlorella sorokiniana CY1	61.0	Chen et al. (2013)



Scheme 1.1 Transesterification reaction to produce fatty acid methyl esters (*FAMEs*) from triglycerides. The reaction takes place with addition of a catalyst, which can be either an acid or a base

In this reaction, each mole of triglyceride consumes 3 moles of alcohol to produce 1 mole of glycerol and 3 moles of methyl esters. In an industrial process, usually 6 moles of methanol are used for each mole of triglyceride to ensure that the reaction is driven in the direction of methyl esters (Fukuda et al. 2001; Yen et al. 2013). This reaction requires either acid or alkaline catalysis. Reactions using alkaline catalysis are faster than those using acid catalysis. However, alkaline catalysis

has a major drawback connected to saponification when free fatty acids are present in the triglyceride raw material (Yen et al. 2013). To overcome the problems of low reaction rates (when using acid catalysis) and saponification (when using alkaline catalysis in a feed with a high content of free acids), some authors have proposed the use of a two-stage process involving initial conversion of free fatty acids to their methyl esters under acid-catalyzed conditions followed by an alkali-catalyzed step to enhance the yield of the overall process and allow the use of low-grade feedstock, which normally would not have been used (Behzadi and Farid 2007; Goff et al. 2004; Zhang et al. 2003).

Another method that has recently attracted attention is direct production of biodiesel using raw microalgae as the feedstock. The process is defined as in situ transesterification and involves exposing the oil-bearing biomass to a mixture of a solvent and a catalyst. The solvent plays a double role in this process, acting as (i) an extractant of the oil from the biomass and (ii) a reactant in the transesterification reaction (Lam and Lee 2012b). A very important drawback of such a process is the presence of moisture in the feedstock. It has been found that water can inhibit the performance of the transesterification reaction because of the presence of lateral reactions. It then becomes necessary to include a thorough biomass drying step prior to an in situ transesterification reaction (Lam and Lee 2012b). A summary of the methods used for the production of biodiesel from microalgae is presented in Table 1.3.

Despite the existing knowledge regarding the production of biodiesel from microalgae, there is general agreement in the scientific community regarding the necessity for improvement in the cultivation, harvesting, and processing of the biomass to make the overall process economically viable. Coupling of large-scale production of microalgae with wastewater treatment could positively contribute to increasing the economic feasibility of the production of biodiesel.

1.5 Conclusion and Future Perspectives

Microalgae offer great potential as the next generation of advanced water treatments for the removal of low but relevant concentrations of pharmaceutical compounds and other organic micropollutants. Some strains have revealed excellent performance in the removal of a variety of compounds such as analgesics, antibiotics, and endocrine-disrupting compounds. Microalgae-based wastewater treatment technology also offers an alternative for the abatement of eutrophication, since microalgae consume inorganic nitrogen and phosphorus species during their growth.

Despite their potential as a low-cost and environmentally friendly water treatment, the practical application of microalgae in real wastewater treatment scenarios has remained limited to date. One of the most important factors that hinders the use of microalgae-based wastewater treatment technologies is the cost related to harvesting of the biomass. A potential solution to overcome this issue could be based on the use of entrapped microalgal biomass. The robustness of microalgae in sus-

Table 1.3 Methods employed and yields obtained in the production of biodiesel from different microalgal strains. Acid, alkaline, and enzymatic transesterification result in high biodiesel production yields

Species	Method	Catalyst	Temperature (°C)	Time (min)	Yield (weight %)	Reference
Chlorella protothecoides	Transesterification	H_2SO_4	30	240	80	Xu et al. (2006)
Botryococcus braunii	Liquefaction	Na_2CO_3	300	120	–	Dote et al. (1994)
Dunaliella tertiolecta	Liquefaction	–	340	60	37	Minowa et al. (1995)
Nannochloropsis oculata	Transesterification	CaO/Al_2O_3 catalyst	50	240	97.5	Umdu et al. (2009)
Chlorella protothecoides	Transesterification	75% *Candida* sp. lipase	38	720	98.15	Cheng et al. (2009)
Chlorella protothecoides	Transesterification	30% immobilized lipase	38	720	98.15	Cheng et al. (2009)
Chlorella minutissima	Transesterification	Sodium methylate	110	300	82	Tang et al. (2011)
Schizochytrium sp.	In situ transesterification	–	70	120	30	Levine et al. (2011)
Neochloris oleoabundans						Levine et al. (2011)

Adapted from Bahadar and Bilal Khan (2013), with permission

taining their metabolic functions after undergoing immobilization procedures, and the capacity for producing microalgae entrapped in phototransparent gels with good water permeability, open up challenging and almost unexplored paths for the development of new water treatment schemes, avoiding the major drawback of biomass harvesting.

The search for alternative fuels for transport is one of the main energy issues worldwide, and biofuels (especially biodiesel) represent such an alternative. In this context, biodiesel derived from microalgae can play a key role in diminishing society's reliance on fossil fuels for transport. Biomass derived from microalgae has been demonstrated to be a valuable feedstock for the production of biodiesel because of its high lipid biosynthetic capacity. Use of entrapped microalgae for tertiary wastewater treatment could help to dramatically decrease the cost of biomass harvesting and contribute to increasing the economic viability of an overall integrative process, including water treatment and biomass valorization through conversion into biodiesel.

Acknowledgements This research was financially supported through the NordForsk Nordic Center of Excellence NordAqua program (project number 82845).

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Chapter 2
Risks and Management of Textile Waste

Ipek Yalcin-Enis, Merve Kucukali-Ozturk, and Hande Sezgin

Contents

Abstract World textile production has been consistently increasing in recent years. Global population growth and rising living standards have caused an increase in textile demands as a natural consequence of basic needs and have also resulted in overconsumption as a consequence of fast fashion trends. A World Bank study has predicted a 70% global increase in municipal solid waste by 2025, which means that the expected waste volume will rise from today's 1.3 billion tonnes to 2.2 billion tonnes per year. Solid waste dumping is a crucial risk, especially for developing

I. Yalcin-Enis · M. Kucukali-Ozturk · H. Sezgin (✉)
Textile Technologies and Design Faculty, Department of Textile Engineering,
Istanbul Technical University, Istanbul, Turkey
e-mail: ipekyalcin@itu.edu.tr; kucukalim@itu.edu.tr; sezginh@itu.edu.tr

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_2

countries. Insufficient collection and thoughtless disposal of solid waste causes land and air pollution and creates risks to human health and the environment. Thus, the management of textile waste has gained importance, and developing nations should spend a major part of their municipal revenues on waste management.

In this chapter we review the risks of textile waste and waste management strategies from various aspects. The general outline of this review includes three main topics: (i) the types of textile waste, (ii) the top five strategies for waste management, and (iii) utilization of textile waste in novel product designs. Textile waste can be divided into three groups: production waste, preconsumer waste, and postconsumer waste. Although 35% of the initial input is lost before the product reaches the consumer, the main risk pertains to postproduction waste when a 2-year lifetime for clothing is taken into consideration as a consequence of fast fashion trends. Moreover, the management of textile waste is a formidable problem. The overall guiding principles for waste management, from the most to the least environmentally favored, are reduction, reuse, recycling, energy recovery, and disposal of waste. Unfortunately, huge amounts of textile waste are landfilled just because of thoughtless types of acquisition. However, 45% of postconsumer textile waste can be worn as secondhand clothing, 30% of it can be cut up and used as industrial rags, 20% of it can be biodegraded after landfilling, and only the remaining 5% of it will be unusable. Since waste generation is not adequately controlled, utilization of this waste is gaining importance; thus, both designers and engineers are studying ways of making new products from this waste. These promising solutions are discussed in the latter part of this review.

2.1 Introduction

According to forecasts, the world's population will reach 8.2 billion in 2025, with a current annual growth rate of 1%. Nearly half of this population is now living in urban areas. In developing countries, the rate of urbanization is higher, with growing industrialization, which can result in increases in energy consumption and waste generation (Ouda et al. 2016). A World Bank study has predicted a 70% global increase in municipal solid waste by 2025, which means that the estimated waste volume will rise from today's 1.3 billion tonnes per year to 2.2 billion tonnes per year by 2025. Moreover, the amount of waste in developing countries is predicted to more than double (Girl 2015). Fossil fuels are still mostly used in energy supply although they pollute the environment and their use causes climate change. Therefore, renewable energy sources have become crucial in recent times (Ouda et al. 2016). Since solid waste is increasing day by day, solid waste management has become a worldwide environmental matter. In general, awareness of organization and planning in waste management has not yet reached a satisfactory level since the available information about current regulations is deficient and there are also financial limitations in many developing countries (Tıinmaz and Demir 2006).

The received wisdom is that textiles are a necessity in human beings' lives. However, with overconsumption of textile products, scarcity of raw materials and future environmental damage come into prominence (Torstensson 2011). The textile industry comprises many production steps such as fiber harvesting, cleaning, spinning, fabric formation, dyeing, and processing with different treatments. Every step brings about environmental hazards (Torstensson 2011). The textile industry creates large volumes of fibrous waste. Therefore, the utilization of this waste for development of fiber-reinforced composites is also gaining importance and people have become more focused on it in recent times (Umar et al. 2017). Unlike primary textiles, recycled textiles are mostly used in low-grade applications such as insulation and seat-filling materials in automobiles, building materials, and upholstery materials because of their low quality indexes (Lu and Hamouda 2014). Although textile production is moving away from the USA and Europe, the utilization of textile waste still maintains its importance in those parts of the world. Since they now have less industrial textile waste (production waste), the USA and Europe are mostly focused on utilization of postconsumer waste (Altun 2016).

The life cycle of textile materials is getting shorter day by day because of continuous changes in fashion markets, in association with low prices (Lu and Hamouda 2014). The fashion industry has a huge influence on the global and human resources needed for both production and consumption of products. Moreover, the increasing interest in fast fashion trends has caused reductions in the production times, prices, and life-spans of fashion items. Thus, an overconsumption problem arises (Lawless and Medvedev 2016). Waste recycling is a very important issue to save natural resources and help minimize climate change (Umar et al. 2017). Since textiles are almost 100% recyclable, everything in the textile and apparel industries should be utilized (Hawley 2006). With increasing environmental awareness, the need to optimize solid waste management is becoming significant. Therefore, the textile and apparel industries are making efforts to decrease postconsumer textile waste disposal (Domina and Koch 1997).

2.2 Textile Waste

Things that people do not need anymore and want to get rid of can be defined as waste (Nielsen and Schmidt 2014). Different types of waste can be classified as solids, liquids, or gases, according to their physical state. Different types of solid waste can be classified according to their original use (packaging waste, textile waste, food waste, etc.), materials (glass, paper, etc.), physical properties (combustible, compostable, recyclable, etc.), origin (domestic, commercial, agricultural, industrial, etc.), and safety level (hazardous or nonhazardous). Household waste and commercial waste together can be classified as municipal solid waste (McDougall et al. 2008). The world's annual waste generation amounts to 7–10 billion tonnes in total, approximately 2 billion tonnes of which is municipal solid waste (International

Solid Waste Association 2015). Hence, it is a fact that unnecessary consumption is a part of everyday life, resulting in huge volumes of solid waste (Costa et al. 2017).

Although textiles are fundamentally used to protect the body from cold, heat, and light, and to preserve modesty, they have become a reflection of personality, wealth, or interest in fashion. Nowadays, because of technological improvements, textiles are used in a wide range of applications rather than only for fabrication of garments (Gulich 2006). From the sourcing of raw materials to textile production, garment manufacturing, and distribution to retail stores, the textile industries generate huge amounts of waste, which occupy a large place in the municipal solid waste category (Karaosman et al. 2017).

Global population growth and increasing demand for new products have led to irrepressible textile production and consumption (Zamani 2014; Barot and Sinha 2015). One of the most important reasons for textile waste generation is the idea, created by the fashion industry, that people need new products each season (Zamani 2014). Large amounts of production and postconsumer fiber waste have been amassed with the growth of the world's population and rising living standards (Lu and Hamouda 2014). It is predicted that global fiber consumption will reach 110 million tonnes in the year 2020 (Voncina 2016).

The idea of recycling textile materials arose during the Industrial Revolution in the UK in the 1700s and 1800s (Gardetti and Torres 2013). The importance of reusing or recycling textile waste becomes more prominent when it is considered that for the production of one T-shirt and one pair of cotton jeans, 2720 liters and 10,850 liters of water, respectively, are needed (Ringler and Zhu 2015). However, it has been seen that recycling of textile products falls behind recycling of other materials. While 15–20% of textile materials are recycled, 80% of steel, 65% of paper, and 30% of plastics are recycled (Voncina 2016). Textile waste can mainly be categorized into three groups: production waste, preconsumer waste, and postconsumer waste (Fig. 2.1).

2.2.1 Production Waste

Production waste is composed of fibers, yarns, fabric scraps, and apparel cuttings generated by fiber producers, textile mills, and fabric and apparel manufacturers (Domina and Koch 1997). The types of waste can vary depending on the manufacturing steps used where the waste is generated (Wang 2010). Especially in the manufacturing sector, fabric cutoffs and fabric roll ends constitute a large amount of waste (Gardetti and Torres 2013). Additionally, fabric defects that occur during manufacturing generate production waste, which results in tremendous costs to organizations. It is a fact that the total cost of defects is often a significant percentage of the total manufacturing cost in most organizations. Moreover, reworking, replacement production, and inspection incur wasteful handling time and effort (Silva 2012). The carpet sector also generates a lot of waste (mostly composed of a single fiber type) but has devoted significant effort to carpet waste collection and

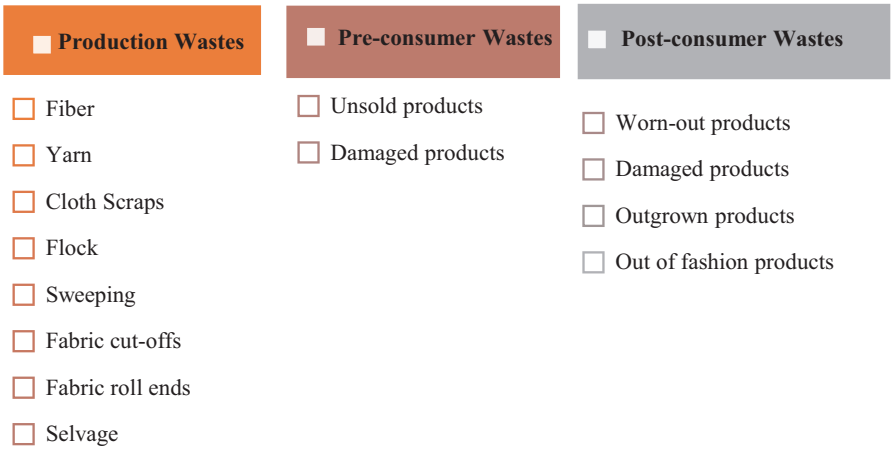

Fig. 2.1 Different types of waste. Solid textile waste can be divided into three categories: production waste, preconsumer waste, and postconsumer waste. Production waste comprises waste from several textile manufacturing steps, preconsumer waste can be unsold/damaged products in stores, and postconsumer waste consists of products that the owners no longer want to use

recycling (Wang 2010). There are three ways to dispose of production waste: (i) it can enter the solid waste stream and end up in landfills or waste incinerators; (ii) it can be converted into energy to power the manufacturing process; or (iii) it can be sold to a textile waste recycler, who may process it into fibers that can be made into new recycled fabrics, apparel, or nonapparel items (Domina and Koch 1997).

2.2.2 Preconsumer Waste

Preconsumer waste consists of products that are manufactured with design mistakes, fabric faults, or the wrong colors being produced for sale and consumption (Ekström 2014). In other words, preconsumer waste consists of unsold and damaged products in the retail sector (Gardetti and Torres 2013). Preconsumer waste is not completely valueless for the retailer because it can be sold to an outlet, jobber, or consolidator. Preconsumer waste can be mainly disposed of in four ways: (i) it can be sent directly to the companies' own outlets; (ii) it can be sold to other outlets, jobbers, or consolidators, who in turn resell the merchandise to other outlet stores; (iii) it can be sent directly to nonprofit organizations if retailers neither have their own clearance centers nor sell this waste to jobbers; or (iv) it can be sent directly to landfill by retailers. However, this last option is the least used one, since most preconsumer waste still has some resale value (Domina and Koch 1997). Companies have different suggested solutions for these products. For instance, H&M sells these products in its own outlets, while Marks & Spencer directs these products to

charities (Gardetti and Torres 2013). Approximately 65% of the initial input is delivered to consumers as new clothing, while 35% of the initial input becomes waste during the production and preconsumer stages (Karaosman et al. 2017).

2.2.3 Postconsumer Waste

Postconsumer waste consists of any types of garments or household articles made from fabricated textiles that the owner no longer needs and decides to discard. Consumers may discard these articles when they are worn out, damaged, outgrown, or out of fashion. The volume of postconsumer waste is very large and is comparable with the rate of fiber consumption (Wang 2010). Although a part of this postconsumer waste is given to charities or passed on to friends and family members, most of it is deposited into the trash and ends up in municipal landfills (Hawley 2006). The amount of postconsumer waste is very large in comparison with other waste types (Wang 2010). It has been estimated that the volumes of postconsumer textile waste that go to landfills are 10.5 million tonnes per year in the USA, 350,000 tonnes per year in the UK, and 287,000 tonnes per year in Turkey (Karaosman et al. 2017). Since an item of clothing has approximately a 2-year lifetime, postconsumer waste should be collected for acquisition purposes (Karaosman et al. 2017).

2.2.3.1 Fast Fashion Trends

The apparel industry is currently dominated by fast fashion, resulting in overconsumption, where consumers buy more than they need (Pookulangara and Shephard 2013). Therefore, beyond consumer need, the desire for fashionable goods contributes to consumption in greater volumes (Hawley 2006).

Fast fashion can be defined as providing the newest fashionable products that respond quickly to consumers' demands. In contrast to the standard 6-month time to market in the apparel industry, fast fashion involves only a few weeks of time in the product development process from the design to the finished product. Of the pioneer fast fashion retailers, Topshop has reduced its time to market to 6–9 weeks while H&M's time to market is only 3 weeks. Besides a reduced time to market, the fast fashion industry offers a large number of different styles of clothing. For instance, Zara produces 12,000 styles selected from 40,000 styles created by 200 in-house designers annually. Since the fast fashion industry offers products at low prices, the volumes of postconsumer textile waste that consumers throw away after wearing them several times are increasing day by day (Lee 2017). Moreover, consumers are more likely to throw away inexpensive clothes than expensive ones, as the latter would give them feelings of guilt (Strähle and Hauk 2017).

In compliance with the fact that fashion relies on new materials to replace old ones, there is a strong relationship between the fashion system and waste (Binotto

and Payne 2017). In other words, it can be said that the end of fashion is the beginning of waste (Torstensson 2011). When a textile product is thrown away as post-consumer waste in a landfill, all of the materials and energy used during its manufacturing—as well as the carbon emissions from transport of the product along the supply chain and the labor input throughout these stages—are wasted (Binotto and Payne 2017; Strähle and Matthaei 2017).

2.2.3.2 Slow Fashion Trends

In recent years, the existence of fast fashion has encouraged the growth of the slow fashion movement. Rather than focusing on time, the slow fashion movement is based on a philosophy of awareness of designers', buyers', retailers', and consumers' respective needs and the impacts of fashion on workers, consumers, and ecosystems (Pookulangara and Shephard 2013). In contrast to fast fashion, slow fashion focuses on reducing the number of trends and seasons, and this maximizes the production quality to improve the value of garments (Ozdamar Ertekin and Atik 2015). To sustain the slow fashion movement, three approaches have been defined: (i) emphasis on local design and production, which encourages local producers and creates cultural diversity; (ii) creation of a transparent production system by elimination of generic designer or brand names and improved relations between producers and consumers; and (iii) improvement in the understanding of textile articles from raw materials to end products, by raised awareness of the hidden realities of material sourcing, production stages, working conditions, distances traveled for distribution, and so on (Clark 2008). H&M's sustainability report emphasizes transparency by stating that H&M was the first fashion retailer to make its supplier list public (in 2013) and continues to collect more in-depth product information to share with customers and other stakeholders (H&M Group 2016).

2.3 Textile Waste Management

The management of municipal solid waste has reached a critical phase, owing to the lack of suitable facilities to treat and dispose of huge amounts of the waste generated in metropolitan cities (Sharholy et al. 2008). Most countries are trying to decrease the amount of disposal in landfills and increase the amount of recycling. For instance, the European Union (EU) has tasked member countries with reusing or recycling 50% of their municipal waste by 2020 (Fortuna and Diyamandoglu 2017). In the waste hierarchy (see Fig. 2.2), prevention constitutes the first stage; reuse, recycling, production of energy from waste, and landfilling come after it.

The purpose of the circular economy is to extend the life of materials and promote recycling to maximize material service per resource input while reducing environmental impacts and resource usage (Tisserant et al. 2017). Furthermore, the

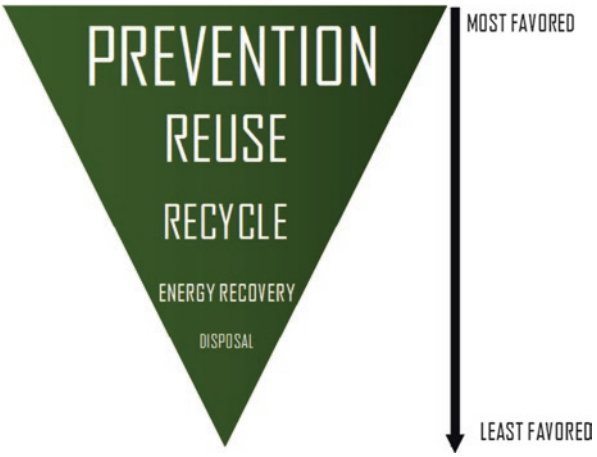

Fig. 2.2 The textile waste management hierarchy ranks the various management strategies from the most to the least environmentally preferred. The hierarchy emphasizes prevention, reuse, and recycling as key to sustainable materials management. At the top of the five stages of the hierarchy is waste prevention, which is the best option

circular economy promotes collection of products and their recovery in the same product chain (Fortuna and Diyamandoglu 2017). The 3R (reduce, reuse, and recycle) approach to waste management has been established internationally as one of the fundamental concepts of the circular economy for a sustainable society (Yano and Sakai 2016; Tisserant et al. 2017). Among leading fashion retailers, H&M, Levi Strauss & Co., and Marks & Spencer are brands that pay attention to the circular economy (H&M Group 2016; Levi Strauss & Co. 2015; Marks & Spencer 2016).

2.3.1 Disposal (Landfilling)

Disposal of solid waste is the least favored waste management method, in which the last destination of waste is a landfill site. Countries try to manage waste with other options. However, there is still a huge amount of waste that ends its life in a landfill even though it could be recycled (Bhuiya 2017). In the USA, only 15% of postconsumer textile waste was recycled or donated in 2009, while the rest of it (85%) was landfilled. Thus, of the USA's total textile production weight of 25.46 billion pounds in 2009, 21 billion pounds was subsequently landfilled. Since the USA's projected textile production weight in 2019 is 35.4 billion pounds, efficient use of landfill capacity has become an important issue (Lee 2017). On the other hand, if appropriate acquisition techniques are used, 45% of postconsumer textile waste can be worn as secondhand clothing, 30% of it can be cut up and used as industrial rags, 20% of it can be biodegraded after landfilling, and only the remaining 5% of it will be unusable (Lee 2017). In landfills, synthetic textile waste does not decompose, while woolen garments do decompose but produce methane and carbon dioxide gases, contributing to global warming (Strähle and Hauk 2017). With the disposal of waste in landfills, it must be noted that methane emissions are more harmful than carbon dioxide emissions (Sotayo et al. 2015).

2.3.2 *Energy Recovery (Energy from Waste)*

Energy from waste, or waste to energy, is a process of generating energy in the form of heat and/or electricity from the treatment of waste. This process mostly produces energy by burning or from inflammable fuel elements such as methane, methanol, ethanol, or synthetic fuels (Klass 2000).

Energy can be recovered from waste by different techniques—mainly incineration, gasification, and anaerobic digestion (Murphy and McKeogh 2004).

Incineration is the combustion of waste to recover energy, in which the residual waste is burned at a high temperature and energy is recovered as electricity or heat. In some countries, textile waste is incinerated. The heat and power that are recovered from this process can be used instead of other sources of energy (Zamani 2014). Incineration decreases the amount of the waste by about 90%, depending on the degree of recovery and the composition of the materials. Incineration cannot end the need for landfilling, but it can reduce the amount of waste that is landfilled. Throughout the incineration process, flue gases (CO_2, H_2O, O_2, N_2) are generated, which are the main sources of fuel energy (Bosmans et al. 2013). In the past, incineration of waste posed a risk to the environment by creating toxic compounds during the process (Tammemagi 1999). Nowadays, however, this environmental risk can be eliminated if the incineration method is combined with energy recovery, control of the emissions, and use of a suitable method for disposal of the final waste (Bosmans et al. 2013).

Gasification is partial oxidation of organic substances at high temperatures (500–1800 °C) to produce a synthetic gas (Bosmans et al. 2013). The main advantage of gasification compared with incineration is higher electrical generation efficiency. This can be provided by use of combined cycle gas turbines in this method. On the other hand, these turbines decrease the temperature of the residual heat and thus reduce the thermal energy production. Gasification is preferably used for electricity production (Morris and Waldheim 1998).

Anaerobic digestion converts organic waste into a methane-rich biogas with use of microorganisms. The obtained biogas is burned to generate electricity and heat, or it is turned into biomethane (Nishio and Nakashimada 2007). The aim of anaerobic digestion is to convert organic waste into biogas—a renewable fuel further used for the production of green electricity or heat, or as a vehicle fuel. The digested substrate in anaerobic digestion can be used as a fertilizer in agriculture (Holm-Nielsen et al. 2009). Anaerobic digestion of animal fertilizer provides some environmental and agricultural advantages such as increased fertilizer quality of the manure, a considerable reduction in odors, inactivation of pathogens, and biogas production (Holm-Nielsen et al. 2009).

The cellulosic part of a waste textile such as cotton or viscose constitutes about 40% of the total waste textile. Waste cellulosic textiles can be used in biomass production, while waste cotton textiles are preferred for both biogas and methane production. Ethanol is also one of the products that can be produced from cotton-based waste textiles by use of enzymatic hydrolysis, followed by fermentation (Jeihanipour et al. 2010).

2.3.3 *Recycling*

The term "textile recycling" has come to the fore since the mid-1940s, when US charities and the textile industry started repurposing clothes, shoes, and accessories (Nodoushani et al. 2016). In daily life, all types of utilization of textiles are mostly included in the category of recycling. However, according to the quality of the final product, this category can be also subcategorized into "downcycling" and "upcycling." In the recycling process, the quality of the recycled final product is equal to that of the base or original product. Downcycling is a recovery process in which a waste material is reprocessed into raw material with a lower value than the original material. Downcycling can avoid dissipation of useful materials, decrease the usage of new raw materials and air, and decrease water pollution. Upcycling is a recovery process in which a waste material is reprocessed into a raw material with a higher value than the original material (Vats 2015). In other words, upcycling can be defined as transforming waste material into a new product of the same quality as—or of better quality than—the old one. The idea of upcycling products was introduced by William McDonough and Michael Braungart. They put forward the idea that there should be a process, unlike recycling, in which the final product has a value at least equal to that of the original product (Gardetti and Torres 2013). Turning an old curtain into a new garment or an old pair of jeans into a new bag can be examples of upcycling (Ekström 2014).

In the process of recycling postconsumer textile waste, recyclers are confronted with many toilsome operations such as sorting, separation, and processing (Strähle and Philipsen 2017). Ninety-seven percent of textile waste can be recycled (Briga-Sá et al. 2013). However, the recovery rate for textiles is only 15% (Wang 2006). Textile recycling can be classified into mechanical recycling, chemical recycling, thermal recycling, and a mix of these technologies. Mechanical recycling is the most preferred technique and can be used for recycling of a wide range of textile waste composition (Zonatti et al. 2016). Mechanical recycling is based on a technique that reduces textile materials to smaller pieces (Oliveux et al. 2015). Traditional mechanical recycling turns waste garments into yarns and fibers (by pulling the fabric apart), and then they are either processed into recycled yarn for textile applications or processed for other applications such as nonwoven products, carpet underlay, sound insulators, thermal insulators, phase change materials, geotextile materials, filtration material, and many others (Haule et al. 2016). In the most commonly used mechanical method, the fabric is shredded into small pieces (Zamani 2014). Textile waste items in the form of fabric should be separated by their composition and color prior to shredding in order to prepare the recycled fibers for use in yarns or nonwoven applications (Zonatti et al. 2016). These recycled fibers are mostly used as filling materials for mattresses or upholstery, and as insulation material. In another mechanical recycling method, after the textile waste is shredded into small pieces, it is turned into low-quality fiber for use in insulation

materials, napkins, carpet underlays, and disposable diapers. Another mechanical method transforms high-quality textile products into different types of products, and this process is a type of upcycling.

Although the mechanical method can be applied to all kinds of fibers, the chemical recycling method is applied to synthetic fibers and their blends. In the chemical process, fibers are separated chemically, degraded, and then repolymerized into new fibers (Zamani 2014). One of the world's most popular sports brands, Nike, carries out 100% recycling of postconsumer and defective athletic shoes. The three main materials in the shoes—consisting of the upper fabric, midsole foam, and outsole rubber—are separated by a chemical process and ground up to be used in new products in the field of sports surfaces, such as grind rubber, grind foam, and grind fluff (Strähle and Philipsen 2017).

Other fiber recycling approaches are melt processing, polymer depolymerization, and waste-to-energy conversion (Wang 2010). Large numbers of products are generated from reprocessed fibers that are respun into new yarns or fabricated into woven, knitted, or nonwoven fabrics such as upholstery materials, composites, garment linings, household items, furniture upholstery, insulation materials, automobile sound absorption materials, automobile carpeting, and toys (Bhatia et al. 2014). For instance, polyester fiber-to-fiber recycling can enhance the sustainability of the textile industry, since polyester fibers are of the first rank in world fiber production (Lu and Hamouda 2014).

Thermal processes include different types of pyrolysis, and fibers can be recovered by these techniques. However, it is not only valuable fiber products that are recovered; gases such as carbon dioxide, hydrogen, and methane are produced by volatilization of the resin, and the resin can become carbonized on the fibers. These processes occur at temperatures between 450 °C and 700 °C, depending on the type of resin. For example, polyester resins need lower temperatures, whereas higher temperatures are required for epoxides or thermoplastics (Oliveux et al. 2015).

Pyrolysis is a process in which textile fibers are heated and the molecules of the polymers begin to divide into smaller molecules. Fuels are also pyrolysis products. The types and amounts of the produced fuels vary depending on the textile type because of the variety of textiles and the polymers that are used. Moreover, the amounts of smoke and fumes that are produced and how strongly the textile burns play important roles in the fabric treatment. Pyrolysis results in a 74 wt.% (weight percent) loss of the total textile weight, of which 31.5 wt.% is a light liquid fraction, 42.5 wt.% is a heavy liquid fraction, 12.5 wt.% is solid residue, and 13.5 wt.% is noncondensable gases (Miranda et al. 2007). With use of thermal recycling by way of pyrolysis and activation, textile waste is reprocessed for the production of a higher-value activated carbon product. Acrylic textile fabric waste is one of the polymers that is widely used in the textile field for this process (Nahil and Williams 2010).

2.3.4 Reuse

Directive 2008/98/EC of the European Parliament and of the Council (2008) describes reuse as follows: "Reuse means any operation by which products or components that are not waste are used again for the same purpose for which they were conceived." Product reuse and environmentalism are interrelated. The reuse of products plays an important role in waste management by conserving resources, reducing negative environmental influences, and diminishing the burden on waste management systems (Fortuna and Diyamandoglu 2017). Even when the prestages of collection, sorting, and reselling of secondhand garments for reuse are taken into account, reuse of textile products (instead of production of the same products from unused material) can reduce energy consumption by 90–95% (Zamani 2014). Moreover, by reuse of 1 kg of a textile product instead of production of a new one, 6000 L of water, 3.6 kg of carbon dioxide, 0.3 kg of chemical fertilizer, and 0.2 kg of insecticides can be saved (Vats and Rissanen 2016). Therefore, reuse of a product promotes sustainable consumption in contrast to the idea of the throwaway society (Fortuna and Diyamandoglu 2017).

By lengthening product life, reuse delays the time when the product enters the municipal solid waste stream, prolongs the life of waste management facilities, and helps to avoid the cost of recycling. Therefore, consumers need to be encouraged to broaden their environmental awareness by reusing products (Domina and Koch 1999). H&M has started a global garment collection program in cooperation with I:CO. If the collected garments are of wearable quality, they are sold as secondhand clothes, otherwise they are processed for reuse as cleaning cloths (Strähle and Philipsen 2017).

2.3.4.1 Secondhand Clothing

Secondhand items are products taken into a new stage of usage without a change in the product design or perhaps only with some (optional) refurbishment. The useful life of a product and the product life cycle have different meanings. The useful life of a product is defined as the period between acquisition of a new product and the time when its performance is no longer considered satisfactory. The definition of the product life cycle, from the consumer's point of view, is the period of use between the purchase and the discarding or replacement of the product. In general, the life cycle of a product is shorter than its useful life because consumers regularly replace used items with new products. Because these textiles have completed their life cycle but can still serve a purpose, a market for secondhand products is created (Strähle and Matthaei 2017). In the 1960s and 1970s, the secondhand market was controlled by charity shops, but in the 1980s, profit-oriented secondhand shops appeared (Voncina 2016). There are many factors (such as inexpensiveness, uniqueness, and environmental issues) that direct people to use secondhand clothes instead of new

ones. As is known to all, "green" products, which support environmental sustainability, are mostly highly priced products and many people cannot afford to buy them. However, by purchasing secondhand clothes, people can reduce the number of new products, and this can be more beneficial to the environment (Xu et al. 2014).

2.3.4.2 The Vintage Clothing Trend

Although vintage clothing is mostly confused with secondhand clothing, there are differences in their definitions. Vintage clothing can be identified by the age of the clothing (generally manufactured between the 1920s and 1980s). Textiles produced before 1920 are defined as antique, whereas clothes made since the 1980s are classified as modern pieces (Strähle and Matthaei 2017). In one study, Cervellon et al. (2012) studied the motivations of female consumers to buy secondhand or vintage fashion clothes. Their results indicated that strong differences in customer profiles and motives exist. The findings of the study showed that buying vintage items creates nostalgia, while consumers feel unique by using these items. Moreover, a higher level of education results in willingness to purchase more vintage pieces. On the other hand, Williams and Paddock (2003) have claimed that economic motives are the main factors in shopping for secondhand clothes.

2.3.5 Prevention (Reduction)

Waste prevention can be defined as acquisition of awareness about the adverse effects of generated waste on the environment and on people, and the importance of waste reduction and reuse of products (Nielsen and Schmidt 2014). Directive 2008/98/EC of the European Parliament and of the Council (2008) describes prevention (reduction) as follows: "Prevention means measures taken before a substance, material or product has become waste, that reduces: (a) the quantity of waste, including through the reuse of products or the extension of the life span of products; (b) the adverse impacts of the generated waste on the environment and human health; or (c) the content of harmful substances in materials and products."

2.4 Utilization of Textile Waste

Rather than being an option, sustainability is a necessity today (Dissanayake et al. 2017). Since the market has to keep on going, reducing production is not a realistic solution to control waste, but reinvention of methodologies to reduce waste and conserve natural resources can be. Therefore, designers and engineers are taking on

the responsibility for making new products from industrial waste (Costa et al. 2017). In the following sections, some academic research and novel designers' works are described to point out new perspectives and solutions for textile waste.

2.4.1 Engineering Solutions

When the literature is examined, it is seen that textile waste is mostly used in the production of insulation materials for building structures. In one study, Patnaik et al. (2015) designed and produced nonwoven sound and thermal insulation mats from waste wool, recycled polyester (RPET) fibers, and a mixture of them. Waste wool fiber is a commonly preferred raw material source for thermal and sound insulation applications because its use and disposal stages consume less energy than those of other natural materials. The results of this study showed that mats made from mixed RPET and wool waste provided the best insulation and acoustic properties among all samples tested. Binici and Aksogan (2015) used cotton waste and fly ash together with cement and water in building material production and tested the insulation properties. The results indicated that the thermal conductivity coefficients of the composite structures were about 29% lower than those of conventional concrete structures. Building weight is an important factor in earthquakes, and earthquake damage can be reduced with a lower specific weight of concrete. In this study, it was observed that while conventional concrete blocks had a specific weight of 1200 kg/m^3, composite blocks had a basic weight of about 800 kg/m^3. Briga-Sá et al. (2013) designed thermal insulation materials for roof construction and internal walls by using polyester apparel cutting waste of different sizes and compared their thermal conductivity with that of conventional insulation materials. The fabric waste was consolidated by sewing. The results showed that the thermal conductivity of the samples was between 0.052 and 0.060 W/mK. The authors noted that materials with a thermal conductivity coefficient less than 0.1 W/mK can be regarded as thermal insulators. Jordeva et al. (2015) manufactured sound insulation materials for roof construction and internal walls from polyester apparel cutting waste. They reported that the resulting insulation material had similar sound absorption properties (54.7–74.7% at a frequency range of 250–2000 Hz) to those of commercially used materials.

Carpet waste is also a big problem for the environment because it degrades very slowly in landfills. Fibers recovered from carpet waste are reprocessed into textile products such as nonwoven products. Recycled fibers from used carpets can be used as concrete reinforcing material to improve the shrinkage and toughness properties of the material (Wang 2010). In one study, Pakravan and Memarian (2016) used needle felt carpet waste in lightweight polymer concrete as aggregate to study the effect of the carpet waste on the physical and mechanical properties of the concrete. They shredded the carpet waste into small pieces and added it to the concrete material. Their results indicated that addition of 2.5% carpet waste to the polymer concrete decreased the density of the concrete by 23%. It was also seen that the strain

capacity and toughness of the concrete were increased by addition of carpet waste. Moreover, the energy absorption capacity of the concrete was increased by 53–129%, depending on the waste content. However, it was observed that the flexural and compressive strength of the polymer concrete decreased as the amount of added carpet waste increased. Mohammadhosseini and Yatim (2017) used carpet fiber waste and palm oil fuel ash to enhance the physical, mechanical, and microstructural properties of concrete. The results showed that although the compressive strength of concrete samples was not improved by the addition of fiber waste, higher tensile, impact, and flexural strengths were achieved with its addition.

Textile fiber waste has also been used as reinforcement in composites or laminates to achieve desired mechanical properties in different application areas. It has the ability to improve the strength and rigidity of composites. Ramamoorthy et al. (2014) reused discarded cotton–polyester blend bed linen fabrics as reinforcement material in composite production with different processing parameters (compression temperature, time, and pressure). They used three different matrices: polyester from the fabric itself, soybean oil–based thermoset resin, and thermoplastic bicomponent fiber. The results showed that the best mechanical properties were achieved with the soybean oil–based composites reinforced with recycled cotton–polyester. Yalcin et al. (2013) used needle-punched polyester nonwoven selvage waste (cut pieces, fibers, and in particle form) as a reinforcement material for the production of composite structures. They preferred low-density polyethylene and polypropylene as matrix materials. They also reprocessed the particle form–reinforced composites to see the effects of reprocessing on the mechanical and thermal properties of the composite structures. The results suggested that the particle form–reinforced composites had better mechanical properties, while the composites made with the cut pieces and fiber had better thermal properties and lower densities. The authors stated that these composite structures could be used in applications where high-density chipboard or compacted panels are used. Umar et al. (2017) used cotton noil waste and knitting waste yarn to produce woven fabrics and then used those fabrics as reinforcement materials in the manufacturing of composite structures. Their mechanical test results (tensile, bending, and impact) revealed that while the tensile and bending strength of waste yarn–reinforced samples were lower than those of glass fiber–reinforced samples, the impact energy of the waste yarn–reinforced samples was greater. They noted that waste yarn–reinforced composites could be used in areas where mechanical stresses are low.

Araújo et al. (2017) reinforced a polypropylene matrix with untreated cotton waste and cotton waste treated with acetylation or silanization to obtain a composite material with high mechanical and improved thermal properties for the automotive industry. Scanning electron microscopy images demonstrated that the fibers were broken by chemical treatment and the thermal stability of the fibers decreased with acetylation treatment. However, it was shown that through reinforcement of the polypropylene matrix with treated and untreated cotton waste, higher storage modulus, Young's modulus, and tensile strength values were achieved in comparison with those of neat polypropylene. In another study, Liu et al. (2017) manufactured foamed concretes from flue gas desulfurization gypsum and textile fiber waste to

increase the energy efficiency of buildings. They used different amounts of textile fiber waste (1, 2, 3, 4, and 5 wt.%), and the results showed that samples reinforced with 3 wt.% textile fiber content had the best performance in terms of both compressive strength and density values.

Sezgin et al. (2012) manufactured cotton and E-glass waste–reinforced hybrid composite plates with different amounts of E-glass and cotton fiber by a compression molding technique. The mechanical performance of the hybrid composites was evaluated by Shore-D hardness, tensile strength, and impact testing. On the basis of the mechanical test results, the authors concluded that the hybrid composites could be used as a buffer material in the automotive industry. Yalcin et al. (2012) manufactured textile waste–reinforced composites for developing tea tray designs (Fig. 2.3). They used 100% cotton knitted and woven fabric waste as a reinforcement material, while polypropylene was used as a matrix material. The performance of the tea trays was investigated by a three-point bending test, staining test, water absorption test, and surface temperature test. The results showed that the textile waste–reinforced tea tray possessed the necessary properties to be used as a tea tray in daily life.

Vats and Rissanen (2016) aimed to upcycle textile waste from hospitals (e.g., blanket covers and bed sheets) for use in new products. These polyester–cotton and cotton waste textiles were characterized for their mass per unit area, breaking force, and polyester content. It was indicated that the minimum breaking force needed for upcycling of different types of products should be between 150 and 400 N, and the results showed that the breaking force for the hospital textile waste exceeded the minimum requirement. However, it was noted that the bed sheets and blanket covers showed greater loss of mechanical properties at the corners.

Another application for textile waste is conversion into useful chemicals. In one study, Sheikh et al. (2015) converted terry towel waste into carboxymethyl cellulose, used it as a thickener in textile printing, and compared it with standard carboxymethyl cellulose by measuring the color value, bending length, and fastness to washing, crocking, and light. The results indicated that the pseudoplastic and shear

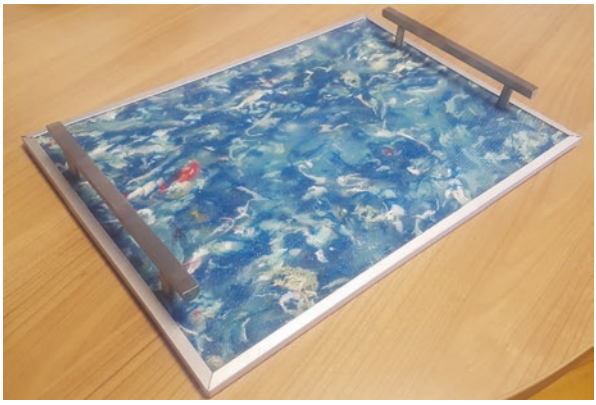

Fig. 2.3 Textile waste–reinforced composite tea tray created by Yalcin et al. (2012). This tray, composed of 100% cotton knitted and woven fabric production waste and a polypropylene matrix, is produced by a compression molding technique. (Reproduced from Yalcin et al. (2012), with permission)

thinning behavior, fastness properties, and color strength of the printed samples were similar to those of the currently used carboxymethyl cellulose.

In another study, Koç et al. (2016) obtained methyl cellulose from cotton towel waste. They characterized the structure of the methyl cellulose by analytical and spectroscopic methods and then analyzed the effect of the methyl cellulose on the hydration time of cement paste. They reported that the hydration start time was postponed by increasing the amount of methyl cellulose in the cement paste, which could provide higher-quality cement paste. In their study, Barot and Sinha (2015) chemically recycled postconsumer polyester clothing into bis(2-hydroxyethyl) terephthalate monomers, which could be further utilized industrially for various applications. Nahil and Williams (2010) thermally recycled acrylic textile waste by way of pyrolysis and activation to produce a higher-value activated carbon product. The results of Fourier transform infrared spectroscopy analysis showed that aromatic ring formation with nitrogen in the char structure occurred at high temperatures. Thus, after the recycling process, acrylic textile waste could be physically activated to produce microporous activated carbon with a large surface area.

Jeihanipour et al. (2010) used an eco-friendly solvent for cellulose N-methyl morpholine-N-oxide for separation and pretreatment of the cellulose. This solvent was mixed with blended textile fibers at 120 °C and at atmospheric pressure to dissolve the cellulose and separate it from the undissolved noncellulosic fibers. The cellulose was then either hydrolyzed by cellulose enzymes, followed by fermentation, to produce ethanol or digested directly to produce biogas. This process produced remarkable increases in the enzymatic hydrolysis rate and the biogas production rate. Moreover, during 3 days of digestion there was a 30% yield of methane from the N-methyl morpholine-N-oxide–treated cotton and viscose fibers, while untreated fibers produced only 0.02% and 1.91% of their theoretical yield over the same time period. In other research, Haule et al. (2016) studied the dissolution of purified cotton waste garments in N-methyl morpholine-N-oxide solution and then they spun them into new fibers. The molecular and mechanical properties of these fibers were analyzed and compared with those of standard lyocell fibers. In terms of molecular properties, the fibers spun from cotton waste garments had higher molecular weight and specific gravity than the standard lyocell fibers, with greater tensile properties and improved wet strength recovery. Gholamzad et al. (2014) applied an alkaline pretreatment to textile waste in order to enhance ethanol production from the cellulose part of a polyester–cotton textile and recovery of the polyester. The pretreatment was applied by using different alkaline solutions. The results of this study showed that all of the pretreatments provided an increase in the enzymatic hydrolysis yield to over 88%, while it was only 46.3% for the untreated textile. The maximum yield of ethanol production, which was 70%, was achieved after pretreatment with sodium hydroxide–urea at −20 °C. Moreover, alkaline pretreatment followed by hydrolysis provided recovery of 98% of the polyester without any significant change in properties.

Fig. 2.4 Biocomposite furniture set designed by Bernardita Marambio, a Chilean designer. The chairs and table are made with Demodé®, a new material that utilizes what would otherwise be wasted textiles from factories in Santiago, Chile, for use by consumers. The particle board is made with 100% biodegradable starch-based bioresin, which gives structural strength and is eco-friendly. (Costa et al. 2017) (Courtesy of Bernardita Marambio)

2.4.2 Design Perspectives

In addition to academic studies focusing on utilization of textile waste, designers are also working on this subject. Kushwaha and Swami (2016) have developed 30 different upcycled products (cushion covers, table mats, holders and folders, handbags, wallets, yokes, collars, earrings, and necklaces) from leather scraps to increase the value of leather waste. Bernardita Marambio, a Chilean designer, has used cotton textile waste together with a 100% biodegradable adhesive made with starch to design novel value-added furniture, including chairs and a table (Fig. 2.4). The designer's aim was to draw attention to the large amount of textile waste in landfills (Costa et al. 2017).

In one study, Kim (2014) designed 29 different high value–added upcycled luxury handbags for the Dubai fashion market by using preconsumer leather and fabric waste. The upcycled items were produced to sell at Harvey Nichols and Bloomingdale's in Dubai.

As is known, babies grow so fast that they can wear their clothes for only a very short time. Cara Sheppard, a Canadian crafter, launched an initiative in 2015, designing keepsake animal toys for families from their babies' old clothes (Fig. 2.5). Through this initiative, she not only recycles babies' textile waste but also preserves an adorable memory for their families (Keepsakes 2015).

Fig. 2.5 Keepsake Memory owl and turtle—upcycled from old fabric such as sleepwear, hospital blankets, or baby clothes—created by Cara Sheppard, a Canadian crafter. By creating these toys, she not only recycles babies' textile waste but also preserves an adorable memory for their families. (Keepsakes 2015) (Courtesy of Cara Sheppard)

Fig. 2.6 Plant sculptures created from upcycled textile waste by Wendy Moyer, a textile sculptor. She transforms textile waste into lush soft plant sculptures by using hand sewing and heat together to create the final shape, and she describes her technique as "fire sculpting" (Moyer) (Courtesy of Wendy Moyer)

Wendy Moyer, an American textile sculptor living in the artists' community of San Miguel de Allende in Central Mexico, upcycles fabrics from their original purposes to create impressive new objects (Fig. 2.6). She utilizes natural and synthetic fabric waste, transforming them into lush soft plant sculptures. She uses both hand sewing and heat together to create the final shape, and she describes her technique as "fire sculpting" (Moyer).

Fig. 2.7 Upcycled-Saree Collection Furniture created by Avni Sejpal, a Mumbai-based designer, who upcycles old sarees that have holes, stains, or tears into poufs, ottomans, stools, and benches. With this collection, she became the winner of the A'Design Award & Competition in the category of projects and green design in 2015. (A'Design Award & Competition 2015) (Courtesy of Avni Sejpal)

Avni Sejpal, a Mumbai-based designer, has created a collection called Upcycled-Saree Collection Furniture. She upcycles old sarees (bright and vivid draped garments worn by Indian women) that have holes, stains, or tears into poufs, ottomans, stools, and benches (Fig. 2.7). All of the products are handcrafted, and the wooden stools are manufactured with mango or acacia wood. With this collection, she became the winner of A'Design Award & Competition in the category of projects and green design in 2015. (A'Design Award & Competition).

2.5 Conclusion

For the global textile industry, one of the biggest challenges is the scarcity of resources, while the demand is ever increasing. With the overconsumption of resources and the preponderance of fast fashion trends in the textile industry, the generated textile waste volumes increase correspondingly day by day. In addition to production and preconsumer waste, postconsumer waste constitutes a huge proportion of the textile waste category generated by consumers captured by fast fashion movements. Although landfilling is the least favored option in the textile management hierarchy, vast amounts of textile waste are disposed of in landfills every day. However, it should be taken into consideration that during waste disposal, all of the materials, the consumed energy, the carbon emissions during the transport of the

goods along the supply chain, and the labor input are also wasted. Furthermore, money is wasted. Therefore, besides energy recovery from textile waste, recycling and reuse of this waste should be encouraged in order to decrease the environmental impacts and energy consumption, for a more livable world. On the other hand, the first priority for management should be the prevention option, which should be assisted by creating environmental awareness to minimize the amount of solid waste going to landfills.

In this chapter we have highlighted the importance of waste management and shown the pros and cons of different waste management options. After describing the textile waste types, we have analyzed every step of the waste management hierarchy at length. We have also described engineering solutions for textile waste by referring to technical information on alternative usage and designers' work, including novel and value-added products/items created using different upcycling techniques, in which the designers take responsibility for creating public awareness of this issue. Through this review, the ever-growing risk of textile waste that is disposed of in landfills has been brought to light by discussion of management options in every aspect and ways of utilization from different perspectives. Moreover, it is hoped that the enriched content of this work may help to create awareness not only among those who produce, distribute, and sell these items, but also among consumers, by encouraging them to consider the history of textile items before buying, while using, and after consuming them.

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Chapter 3
Biopolymer Technologies for Environmental Applications

Kanmani Palanisamy, Aravind Jeyaseelan, Kamaraj Murugesan, and Suresh Babu Palanisamy

Contents

K. Palanisamy
Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, India

A. Jeyaseelan (✉) · K. Murugesan · S. B. Palanisamy
College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_3

Abstract Pollution of water resources resulting from effluent discharge has been a long-standing environmental concern. Traditional methods of wastewater treatment are often ineffective in meeting the required standards and are not cost-effective. Use of biopolymers as adsorbents and natural flocculants in wastewater treatment is thus gaining prominence. Production from nonrenewable resources and their resistance to biodegradation are issues that deter us from relying on conventional petrochemical-based polymers. In this context, biopolymers derived from natural sources are emerging as sustainable and safe alternatives. Especially, cellulose and chitosan have attracted a great deal of attention.

Here, we reviewed the potential environmental benefits of other equally resourceful biopolymers such as tannin, pectin, agar, alginate, acrylamide, carrageenan, starch, dextran, polylactic acid, and polyhydroxyalkanoates. The major points are as follows: (1) Biopolymers function as useful adsorbents for the removal of organic as well as inorganic pollutants encompassing fluorides, nitrates, phosphates, heavy metal hydrocarbons, dyes, and pesticides. For such applications, biopolymer-based hydrogels and nanocomposite films have been experimented with. (2) The coagulating-flocculating abilities of biopolymers result in enhanced effluent clarification. (3) Biopolymers have been relied upon for pro-environment initiatives in the agricultural and construction sectors. Soil strengthening, anti-desertification, and sealing of concrete leaks are a few instances where they could play a lead role. (4) The far-reaching applications of these compounds extend to making catalysts for hydrogen generation and proton-conducting membranes for electrochemical devices. Recent developments on these fronts, their techno-economic feasibilities, and future prospects have been focused in this review.

3.1 Introduction

Environmental pollution and the ensuing degeneration in the condition of our natural resources are issues that could not be ignored for the continued sustenance of life on earth. Discharge of industrial effluents keeps afflicting the quality of our water resources, and hence measures to ensure adequate effluent treatment are direly necessitated. Wastewater treatment has routinely entailed adsorption, membrane separation, ion exchange, coagulation-flocculation, floatation, evaporation, and electrochemical methods (Yargıç et al. 2015). Among these, adsorption using commercially available activated carbon has been widely practiced, especially for heavy metal and dye removal. This is neither environmentally friendly nor economically sound, owing to its energy-intensive and energy-expensive nature. In this context, natural adsorbents based on biopolymers are gaining popularity as cost-effective alternatives (Karnib et al. 2014).

Biopolymers are those obtained from plant, animal, or microbial sources and are composed of polysaccharides, proteins, glycolipids, lipopolysaccharides, or poly-

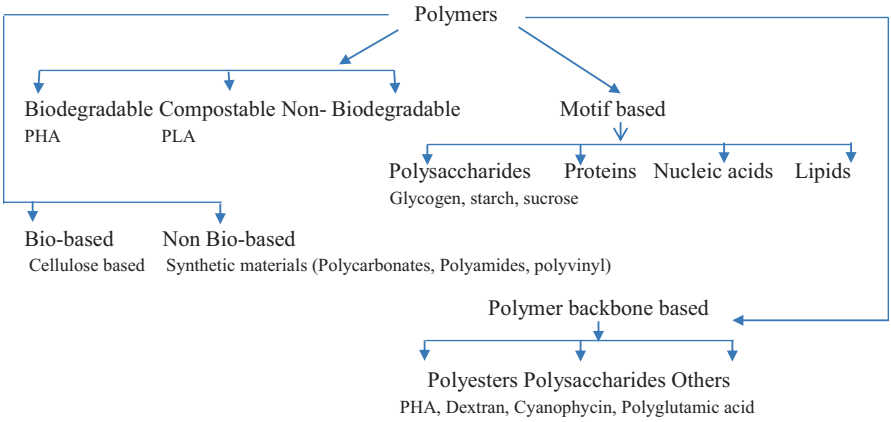


Fig. 3.1 Classification of polymers. Polymers could be either bio-based or synthetic. They might be biodegradable or non-biodegradable in nature, with biodegradability being the trait of most biopolymers. Motif-based and polymer backbone-based classifications also exist

hydroxyalkanoates. They have recently attracted a lot of attention owing to the pressing problems emanating from the use of petroleum-based polymers, and these naturally derived substances could be exploited for a range of environmental applications (Kalia and Avérous 2011). The general classification of polymers is provided in Fig. 3.1.

Biopolymer performance could be improved upon stabilization of the structure using cross-linking agents. This could be accomplished using formaldehyde, glutaraldehyde, glyoxal, or epichlorohydrin (Crini and Badot 2008). Alternatively, biocomposites could be prepared, wherein, either the matrix or the reinforcement is comprised of a biopolymer (Varghese and Das 2015). In such applications, the biopolymers are combined with tree gum, montmorillonite clay, or zinc oxide nanoparticles (Khan et al. 2017; Singh et al. 2010), with nanobiocomposites being recently acclaimed as the material of choice for pollutant elimination (Kuang et al. 2013; Li et al. 2010).

Prominent pollutants removed with the aid of biopolymer adsorbents include heavy metals and synthetic dyes. Apart from these, fluorides, nitrates, phosphates, perchlorates, hydrocarbons, pesticides, and other persistent organic pollutants have been tackled. Biopolymers also function as natural coagulants for wastewater clarification (Ferral-Pérez et al. 2016). Further, the applications extend to diverse sectors such as agriculture, construction, and electrochemical industries, where biopolymers are used as natural additives in soil strengthening and as proton-conducting electrolytes in conjunction with suitable dopants.

Studies on the utilization of cellulose and chitosan-based biopolymers, especially for the purposes of heavy metal and dye removal, are most rampant (Ahmad et al. 2015; Silva et al. 2016). The former is procured from reeds, stalks, grasses, and the woody parts of vegetation, while the latter is extracted from shellfish and subsequently deacetylated. Chitosan and cellulose have been the most sought-after

biopolymers on account of their status as the most abundantly available ones. The possible environmental applications of chitosan and cellulose-based biopolymers are listed in Table 3.1.

However, a plethora of other plant as well as microbial biopolymers are amenable to environmental applications, and their potential has been uncovered by many researchers (Table 3.2). These include pectin, inulin, starch, alginate, lignin, tannin, agar, guar gum, xanthan gum, polyhydroxyalkanoate, and polycaprolactone (Bacelo et al. 2016; Nevárez et al. 2011; Swain et al. 2013; Varghese and Das 2015; Vijaya et al. 2017; Zhang et al. 2014a, b). The structures of important biopolymers discussed in this article are presented in Fig. 3.2. Recent research on the environmental relevance of such diversified biopolymers has been reviewed in this article, shedding light on the promises they hold, challenges to be overcome, and the direction in which future work in this domain is to be steered.

3.2 Biopolymer-Based Adsorbents for Pollutant Removal

Biopolymers are useful adsorbents for heavy metals and inorganic nonmetallic pollutants. Several natural as well as synthetic organic chemicals in the environment could also be effectively managed using biopolymer-based adsorbents. Among these applications, heavy metal and dye removal have been extensively investigated. Recent research in such arenas has been reviewed in this section.

3.2.1 Synthetic Dyes

Increasing numbers of synthetic organic compounds are now being manufactured, and their entry into the environment poses several safety concerns. As a result of insufficient safety testing, the ill effects of many of these compounds come to the limelight only after prolonged consumer usage, and by the time they are banned, the damage is already done. Low toxicity of the parent compound and amenability to biodegradation are generally desired in any xenobiotic compound for it to be considered safe. Synthetic dyes and pesticides are often toxic and recalcitrant in nature, hence problematic.

Bioremediation of dye pollutants is conceived to be quite a challenging one, on account of their structural complexity and toxicity. As their persistence raises environmental and health concerns, activated carbon adsorption is practiced to facilitate removal from aqueous solutions. Biopolymers could instead be used as cost-effective adsorbents for dye removal. For instance, pectin thorium(IV) tungstomolybdate nanocomposite was effective in remediation of the dye malachite green (Sharma et al. 2016). Fe0 nanoparticles stabilized using cross-linked orange skin pectin were applied for the removal of acid yellow 17 from aqueous solution. Bidentate and monodentate interactions were the mechanisms responsible for binding the cross-

Table 3.1 Environmental applications of chitosan and cellulose-based biopolymers

S. no.	Biopolymer	Source	Application	References
1.	Chitosan	Commercial	Metal ion sensor	Fen et al. (2015)
2.	Chitosan-coated montmorillonite clay	Commercial	Tungsten (W) adsorption	Gecol et al. (2006)
3.	Chitosan	Commercial	Methanol fuel cells	Hasani-Sadrabadi et al. (2012)
4.	Polypyrrole-chitosan	Commercial	Fluoride ion adsorption	Karthikeyan et al. (2011)
5.	Chitosan	Commercial	Mn(II), Fe(II), Co(II), Cu(II), Ni(II), Cd(II), and Pb(II) adsorption	Krishnapriya and Kandaswamy (2010)
6.	Chitosan-gum ghatti-polylactic acid	Commercial	Dichlorvos removal	Sahithya et al. (2015)
7.	Modified chitosan	–	Cu(II), Ni(II), Co(II), and Zn(II)	Sousa et al. (2009)
8.	Chitosan nanoparticle	Commercial	Malathion detection	Vasimalai and John (2013)
9.	Chitin and chitosan	–	Hydrocarbon biodegradation	Xu et al. (2005)
10.	Chitosan and pectin	Crab shells, citric fruits	Divalent metal ion adsorption	Debbaudt et al. (2004)
11.	Chitosan-alginate membrane	–	Removal of glyphosate	Carneiro et al. (2015)
12.	Chitosan	Crab	Pb(II), Cu(II), and Cd(II) adsorption	Khan et al. (2011)
13.	Chitosan	Commercial	Water-resistant construction material	Aguilar et al. (2016)
14.	Chitosan-gum ghatti-polylactic acid	Commercial	Removal of monocrotophos	Sahithya et al. (2016)
15.	Modified chitosan	Commercial	Coagulation of bentonite particles	Chatterjee et al. (2009)
16.	Chitosan and mercaptobenzimidazole	Commercial	Adsorption of Pd(II)	Sharma et al. (2015)
17.	Chitosan-activated carbon	Commercial	Pd(II) and Pt(II) removal	Sharififard et al. (2012)
18.	Chitosan-ethyl acrylate	Snow crab shell	Removal of basic dyes	Sadeghi-Kiakhani et al. (2013)
19.	Chitosan	Chicken feathers	Removal of Pb(II)	Kumari and Sobha (2016)
20.	k-carrageenan and cellulose	Commercial	Dye-sensitized solar cell	Rudhziah et al. (2015)
21.	Carboxymethyl cellulose	Commercial	Electrochemical device	Samsudin et al. (2014)
22.	Cellulose	Commercial	Malachite green adsorption	Sekhar et al. (2009)

(continued)

Table 3.1 (continued)

S. no.	Biopolymer	Source	Application	References
23.	Modified cellulose	Okra plant	Cu(II), Zn(II), Cd(II), and Pb(II)	Singha and Guleria (2014)
24.	Magnetic amine-linked cellulose	Corn stalk	Nitrate adsorption	Song et al. (2016a)
25.	Magnetic amine-linked cellulose	Corn stalk	Anionic dye removal	Song et al. (2016b)
26.	Cellulose	Microbial	Cr(VI) removal	Sathvika et al. (2015)
27.	Cellulose	Alfalfa plant	Water retention and soil stabilization	Maghchiche et al. (2010)
28.	Cellulose nanocomposite	Commercial	CH_4-sensing	Aldalbahia et al. (2016)
29.	Cellulose, xylan, and chitin	Commercial	Cr(VI) removal	Lin and Wang (2012)

Heavy metal and dye adsorption constitute the most widespread applications of cellulose and chitosan. However, it encompasses a range of other uses including pesticide removal. Electrochemical applications and sensors based on these biopolymers are also possible

linked polymers to the nanoparticles. Adsorption was mainly mediated by the polymers, while dye reduction was facilitated by the nanoparticles (Rakhshaee 2011).

Alginate served as a biocompatible, eco-friendly, and low-cost adsorbent for the elimination of cationic textile dyes from colored aqueous solutions, with basic violet 16, basic red 18, and basic blue 41 being the dyes tested against this adsorbent (Mahmoodi et al. 2012). Modified Ca(II) and Zn(II) biopolymer (alginate) hydrogel beads were used for the removal of methylene blue, and the adsorption process was optimized (Kumar et al. 2015). In another instance, a heterogeneous biopolymer composed of grape marc and calcium alginate was formulated as a potential adsorbent for the removal of pigments from agro-industrial effluent (Perez-Ameneiro et al. 2014).

Gum olibanum, a glucuronoarabinogalactan polymer, could be used as a stabilizer in the synthesis of Pd(II) nanoparticles. These biogenic nanoparticles mediated the reduction of coomassie brilliant blue G-250, rhodamine B, and methylene blue. They exhibited excellent dye degradation activity, and the results demonstrate the possible application of biogenic Pd(II) nanoparticles as nanocatalysts in environmental remediation of wastewaters polluted with toxic and mutagenic dyes (Kora and Rastogi 2016).

In other applications concerning dye removal, the biopolymer–metal complex wool-Pd/CdS photocatalysts exhibited high activity for the degradation of rhodamine B under visible light irradiation. The wool-Pd could not only enhance the utilization rate of noble metal Pd(II) but also significantly improve dye degradation. It could be recycled and hence cost-effective (Wang et al. 2013). Bacteria-derived biopolymers are also beneficial as biodegradable and nontoxic adsorbents for dye removal. Poly(γ-glutamic acid) is one such example, and the polymer was shown to

Table 3.2 Environmental applications of key biopolymers discussed in the review

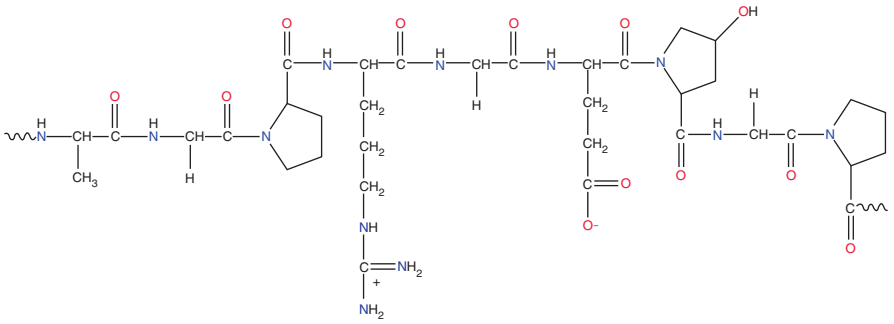
S. no.	Biopolymer	Source	Application	References
1.	Polyhydroxyalkanoate	Microbial	Biodegradable food packaging material	Fabra et al. (2014)
2.	Starch-glycerol	Cassava	Non-retrogradable eco-films	Seligra et al. (2016)
3.	Starch-albumin-glycerol	Corn, potato	Eco-friendly transparent packaging material	Gonzalez-Gutierrez et al. (2010)
4.	Hydrolyzed keratin	Feather	Eco-friendly packaging material	Dou et al. (2016)
5.	Gelatin and alginate	Commercial	Bi(III) sorption	Campos et al. (2008)
6.	Glucan, xanthan	Microbial	Soil treatment, anti-desertification	Chang et al. (2015c)
7.	Dextrin and starch	Commercial	Sorption of Cu(II), Fe(II), and Cr(VI)	Chauhan et al. (2006)
8.	Zein, gelatin, and guaiac resin	Commercial	Eco-friendly wood preservative	Croitoru et al. (2015)
9.	Poly(γ-glutamic acid)	Microbial	Dye sorption	Inbaraj et al. (2006)
10.	Guar gum	–	Phenol and phthalate removal	Kee et al. (2015)
11.	Glucan	Microbial	Soil strengthening	Chang and Cho (2012)
12.	Modified alginate-gelatin capsules	Commercial	Pd(II) sorption	Vincent et al. (2008)
13.	Guar gum-silver nanoparticles	Commercial	NH_3 detection	Pandey et al. (2012)
14.	Cationic inulin	Commercial	Algal biomass harvesting	Rahul et al. (2015)
15.	Lignin-based nanocomposite	Commercial	Water purification	Nevárez et al. (2011)
16.	Calcium alginate-grape marc	Winery industry waste	Dye removal	Perez-Ameneiro et al. (2014)
17.	Agar and carrageenan	Commercial	Biodegradable antimicrobial films	Kanmani and Rhim (2014)
18.	Gum kondagogu	Tree gum	Biosorption of Ni(II) and Cr(VI)	Vinod et al. (2010)
19.	Polycaprolactone-coated hydroxyapatite foams	–	Pb(II), Cu(II), and Cd(II) removal	Vila et al. (2011)
20.	Modified pectin	Orange skin	Removal of acid yellow 17	Rakhshaee (2011)
21.	Mussel-derived polydopamine with magnetic nanoparticles	Commercial	Multiple pollutants removal	Zhang et al. (2014a, b)

(continued)

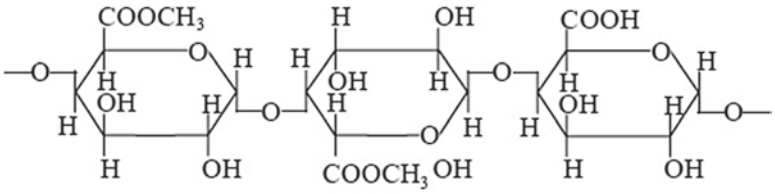
Table 3.2 (continued)

S. no.	Biopolymer	Source	Application	References
22.	Keratin	Chicken feathers	As(III) removal	Khosa and Ullah (2014)
23.	Xylan and lignin	Commercial	Bio oil production	Rutkowski (2011)
24.	Alginate hydrogels	Commercial	Recyclable catalyst for hydrogen generation	Ai et al. (2014)
25.	Lignosulfonate	Commercial	Bioleaching	Mu et al. (2016)
26.	Lavandin-coated biopolymer (maize starch)	Commercial	Biocide in ecological agriculture	Varona et al. (2010)
27.	Agar- and carrageenan-based film	Sea weed	Cr(VI) sensor	Farias et al. (2015)
28.	Sodium alginate	–	Dye removal	Mahmoodi et al. (2012)
29.	Glucuronoarabinogalactan with Pd(II) nanoparticles	Commercial	Dye degradation	Kora and Rastogi (2016)
30.	Gellan gum and agar gum	Commercial	Environmentally friendly construction materials	Chang et al. (2015b)
31.	Poly(γ-glutamic acid)	Microbial	Adsorption of basic brown 1	Inbaraj et al. (2008)
32.	Guar gum, xanthan gum, and locust bean gum	Guar beans, carob tree, and microbial source	Recovery of pulp fibers from paper mill effluent	Mukherjee et al. (2014)
33.	Poly(γ-glutamic acid)-based nanosystem	Commercial	Fe(III) removal	Bodnár et al. (2013)
34.	Xanthan gum–alginate binary biopolymer network	Commercial	Adsorptive removal of Co(II) and Ni(II)	Zhang et al. (2014a, b)
35.	Starch	Feather palm	Plasticizers	Sahari et al. (2012)

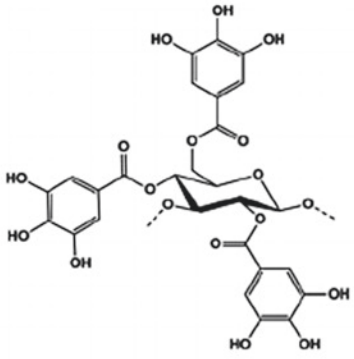
Environmental pollutants such as dyes, pesticides, and heavy metals could be tackled using a plethora of biopolymers. Food packaging materials made using these biodegradable polymers are gaining a lot of attention. Agriculture and construction sectors have also been benefitted from these eco-friendly substitutes



(a) Gelatin

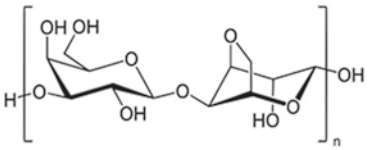


(b) Pectin galacturonan

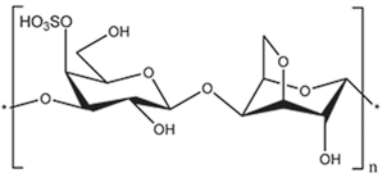


(c) Tannin

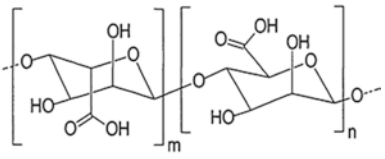
Fig. 3.2 The structures of a few important biopolymers other than cellulose and chitosan. A range of biopolymers obtained from plant, animal, as well as microbial sources are suitable for environmental applications. Gelatin (**a**), pectin (**b**), tannin (**c**), agar (**d**), carrageenan (**e**), alginate (**f**), lignin (**g**), keratin (**h**), dextran (**i**), polyhydroxyalkanoate (**j**), and poly(γ-glutamic acid) (**k**) are among them



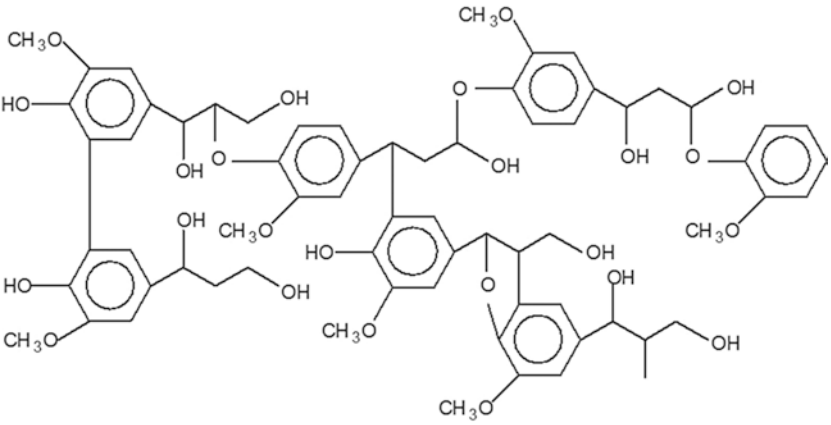
(d) Agar



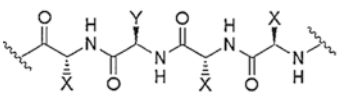
(e) Carrageenan



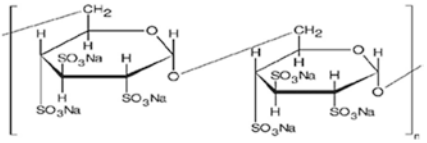
(f) Alginate



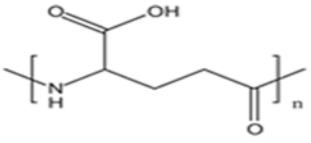
(g) Lignin



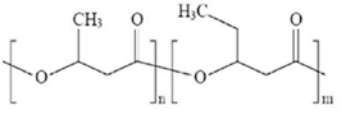
(h) Keratin



(i) Dextran



(k) Poly(γ-glutamic acid)



(j) Polyhydroxyalkanoate

Fig. 3.2 (continued)

be capable of removing the basic dyes auramine O, rhodamine B, and safranin O (Inbaraj et al. 2006). This bacterial polymeric substance also possessed adsorption efficiency for the dye basic brown, with the binding of dyes on poly(γ-glutamic acid) probably involving an ion-exchange mechanism in both cases (Inbaraj et al. 2008).

3.2.2 Pesticides and Other Persistent Organic Pollutants

Pesticides are xenobiotic organic compounds that are difficult to remove from the environment. Lindane is an organochlorine pesticide whose residues have been detected in drinking water sources. Viable methods are needed for the removal of this persistent organic pollutant, and iron-based nanomaterials are effective in the transformation of lindane. However, their use in the treatment of drinking water poses certain toxicity concerns. In an attempt to overcome such issues, FeS nanoparticles stabilized using a polymer from the Basidiomycetous fungus *Itajahia* sp. were applied for lindane degradation. As an improvisation of this method, residual lindane as well as the stabilizing polymer was completely degraded in a subsequent microbiological treatment. The latter process aggregated the FeS particles, leading to their easy removal by filtration, thereby nullifying the toxicity issues (Paknikar et al. 2005). This integration of nanotechnology and biotechnology holds promise as an efficient, cost-effective, and safe remediation technique for chlorinated pollutants in water sources.

Dichlorvos and monocrotophos are organophosphate insecticides whose mode of action is inhibition of cholinesterase activity. They do not display much affinity to soil organic particles and hence reach the groundwater in significant concentration. Biopolymer composites of gum ghatti and polylactic acid are useful in their removal. Montmorillonite(MMT)–CuO composites as a combination of MMT–CuO–Chitosan (Ch), MMT–CuO–Gum ghatti (Gg), and MMT–CuO polylactic acid (PLA) were utilized in the study and it was observed that all three components of the composite were involved in the adsorption process. Amines and alcohol were the main participating chemical groups (Sahithya et al. 2015, 2016). Thus, the biocomposites hold promise for the removal of organophosphate insecticides from aqueous environments.

Moving bed biofilm carriers made from polylactide-based products are convenient for the removal of bisphenol A (a compound used primarily in making polycarbonate plastic and epoxy resin), oseltamivir (an antiviral medication), and atrazine (an S-triazine class of herbicide). In a study using wastewater spiked with ^{14}C-labelled pollutants, cumulative evolution of $^{14}CO_2$ from samples incubated with freely moving carriers was far greater than in the control. Seeding the biofilm carriers with microbial strains enhanced the removal efficiency (Accinelli et al. 2012). Such biopolymer-based carriers inoculated with degradative microorganisms could be viewed as a novel concept in the remediation of xenobiotics.

3.2.3 Chlorinated Hydrocarbons and Oil Pollutants

Chlorinated hydrocarbons which require highly reducing conditions for biodegradation are dreaded for their recalcitrance and negative environmental impacts. Trichloroethylene removal from contaminated groundwater could be enhanced using xanthan gum-modified microscale zerovalent iron. The removal is attributed to both sorption and reduction processes, and a noteworthy feature of this application is that that the sorption capacity was lowered in the presence of xanthan gum, while the reduction capacity was significantly increased (Xin et al. 2015). This could be viewed as an effective in situ remediation strategy for groundwater contaminated with chlorinated hydrocarbons.

Oil-contaminated sites are important sources for polyhydroxyalkanoate producers such as *Pseudomonas* sp., which could be used for the bioremediation of crude oil pollutants (Goudarztalejerdi et al. 2015). Biopolymers could also be used as eco-friendly oil dispersants. The synergy between xanthan gum and silica nanoparticles was exploited for this purpose, and the oil-in-water emulsion thus stabilized had smaller droplet size, which is conducive for subsequent oil degradation. Xanthan gum favored the adsorption of silica nanoparticles at the interface and further enhanced the viscosity of the continuous phase, slowing down droplet coalescence significantly (Pi et al. 2016). These findings could pave way for the development of efficient, inexpensive, and environmentally suitable dispersants for dealing with oil spills in marine environments.

Oil sand process-affected water from bitumen extraction is quite toxic and is laden with naphthenic acids in addition to metals, hydrocarbons, and polyaromatic compounds. It is much corrosive in nature. A chemically modified keratin biopolymer was devised for the removal of naphthenic acids. Polyhedral oligomeric silsesquioxane nanocages and goethite dopant were used to unfold the keratin structure and improve functionality (Arshad et al. 2016).

3.2.4 Inorganic Nonmetallic Compounds

Biopolymer adsorbents are helpful in the removal of various inorganic nonmetallic pollutants including nitrates, phosphates, and fluorides. When drinking water contains fluoride level above the permissible limit, it causes adverse health effects. Biopolymers could be successfully applied for defluoridation. Alginate-based nanocomposites such as alginate-entrapped mixed metal oxide nanomaterials exhibit reliable performance in this regard (Swain et al. 2013). Aluminum cross-linked alginate beads could also be used as cheap and eco-friendly yet efficient adsorbents for the removal of fluoride from contaminated water (Kaygusuz et al. 2015). Another alginate-based adsorbent for fluoride removal is the chitosan-alginate bionanomaterial scaffold with in situ functionalized alumina hydroxide forming a scaffold-like structure and modified with silver nanoparticles (Kumar et al. 2016).

Nitrates are capable of causing deleterious health impacts such as methemoglobinemia, for instance, when they gain access to drinking water wells. Moreover, nitrates and phosphates affect coastal environments by stimulating eutrophication. Biopolymeric alginate and clinoptilolite-rich tuff pellets were explored for the removal of nitrates. This approach of biopolymer fabrication enhanced the adsorption capacity of native zeolite, resulting in a more efficient amphoteric tailored product. In this, the zeolite and biopolymer components were cross-linked using $FeCl_3$ and $CaCl_2$ (Chmielewska et al. 2010)

Biopolymers are also handy in applications concerning phosphate removal. *Arundo donax* Linn-based resin and activated carbon were applied for phosphate elimination from streams. The performance of the regenerated *Arundo donax* Linn-based resin was quite good, with 92–94% adsorption capacity, even after seven cycles of adsorption-desorption (Xu et al. 2015). In another study, the role of extracellular polymeric substances in enhanced biological phosphorus removal was investigated in phosphorus-accumulating granular sludge system. The results suggested that the polymeric substance played a critical role in facilitating accumulation and transfer of phosphorus between the cells and the bulk liquid (Li et al. 2015). Similarly, the extracellular polymeric substance produced by *Acinetobacter haemolyticus* MG606 was also capable of phosphate binding. Phosphate appeared to bind predominantly to the polysaccharide fraction of polymer and to a lesser extent to the protein fraction. Electrostatic interactions with amino groups and ligand exchange with hydroxyl groups were found to be the primary phosphate-binding mechanisms (Kaur and Ghosh 2015).

3.2.5 Heavy Metals

Heavy metal removal from water and wastewater is driven by high acute and chronic toxicity concerns, with a lot of research being carried out on this subject. Coagulation-flocculation, electrochemical methods, ion exchange, chemical precipitation, flotation, membrane filtration, and adsorption have been applied in order to deal with heavy metal contaminants, of which adsorption has taken the lead. The inexpensive and abundantly available biopolymers have lately gathered a lot of attention as eco-friendly adsorbents for heavy metal removal, a few of which are discussed here.

3.2.5.1 Lignin

Pb(II) is quite toxic, and treatment of polluted waters containing this metal above the permissible limit is a demanding task. Lignin, in spite of being the second most abundant natural polymer on earth, has been shunned owing to its low response rate and low adsorption capability for heavy metals. In an effort to explore its usage, lignin microspheres were prepared by an inverse suspension copolymerization method for the removal of Pb(II) from water. The surface area of the prepared

microspheres was 5.3 times as that of lignin and contained numerous amine functional groups. Quite promisingly, it showed fast response rate and high adsorption capacity for Pb(II), 137% and 280% greater than alkaline lignin and poplar lignin, respectively (Ge et al. 2016).

3.2.5.2 Poly(γ-glutamic acid)

Fe is an integral part of the natural environment and also an essential trace element. However, its presence could cause undesirable effects on aquatic environments. Chemical reactions mainly involve changes in Fe that are caused by an electron being transferred to it from other minerals. Because Fe(II) is more soluble than Fe(III), it can be released into solution and dramatically affect the chemistry and mineralogy of soils and surface waters.

Nanoparticle-enhanced ultrafiltration technique could be used for Fe(III) removal. Poly(γ-glutamic acid) linear biopolymer and its cross-linked nanoparticles were used to complex the metal ions by forming nano-sized spherical particles. These polymer-metal ion particles were then removed by membrane separation. Ultrafiltration techniques were studied with the aim of developing a nanoparticle-enhanced separation process for the efficient removal of Fe(III) ions (Bodnár et al. 2013). Poly(γ-glutamic acid) could thus bind and remove ferric ions to produce water with low concentration of the metal.

3.2.5.3 Keratin

Drinking water contamination by arsenic may occur as a result of the metal leaching into aquifers from natural geological sources or from mining and other industrial activities. It is known to inactivate key enzymes involved in cellular energy pathways and in DNA synthesis and repair. Chemically modified chicken feathers are valuable in removing this toxic element. They were treated with selective dopants such as poly(ethylene glycol), diglycidyl ether, poly(N-isopropylacrylamide), allyl alcohol, and trisilanolcyclohexyl polyhedral oligomeric silsesquioxane. The solubilized keratin was regenerated by precipitation at acidic pH. In this study, the allyl alcohol and polyhedral oligomeric silsesquioxane-treated biosorbents yielded the highest removal capacity for As(III) (Khosa and Ullah 2014).

3.2.5.4 Gum Kondagogu

Ni(II) is found in plating and metal-pickling wastewaters. It is known to cause essential metal imbalance, disrupt enzyme action, and contribute to oxidative stress. Water-insoluble Cr(III) is generally considered to be safe, but Cr(VI) is an established toxicant and carcinogen. An exudate tree gum "Gum kondagogu"

(*Cochlospermum gossypium*), a locally derived natural product from India, is effective in the adsorption of Ni(II) and total chromium (Vinod et al. 2010).

3.2.5.5 Alginate

Cd(II) is yet another toxic heavy metal and continued exposure to even low concentrations of it could result in kidney damage. Alginate is useful in the synthesis of photocatalysts for Cd(II) removal. For this, titania-polyvinyl alcohol-alginate beads were synthesized, and nano-sized TiO_2 powder photocatalysts were implanted in them. The beads displayed good Cd(II) removal efficiency and could be reused for at least five cycles. Mass transfer factor models indicated that resistance was mainly dependent on external mass transfer (Fulazzaky et al. 2017).

3.2.5.6 Polydopamine

Polydopamine polymer decorated with magnetic nanoparticles could be applied for the removal of multiple pollutants, including dyes and metal ions. Due to the catechol and amine groups, the polydopamine polymer provides multiple interactions to combine with pollutants. Methylene blue, tartrazine, Cu(II), Ag(II), and Hg(II) were among the targeted pollutants. Of these, Hg(II) especially is a highly toxic heavy metal capable of causing neurodegenerative effects (Zhang et al. 2014a). This opens up new possibilities for use of the polymer in environmental remediation.

3.2.5.7 Tannin

Tannin-based biosorbents find promising application in wastewater treatment and recovery of critical metals. These ubiquitous and inexpensive natural biopolymers could be easily extracted and converted into insoluble or immobilized matrices (tannin gels and tannin foams). Chemically modified forms of the adsorbent such as iron-loaded and amine-modified tannin gels could be produced with enhanced adsorption ability (Bacelo et al. 2016).

3.2.5.8 Hybrid Biopolymers

Hybrid NaA and xanthan gum-alginate composites made by controlling zeolite NaA dispersion into calcium ion cross-linked binary xanthan gum-alginate hydrogels are beneficial in Ni(II) and Co(II) biosorption. Pre-modification of the zeolite NaA by natural xanthan gum facilitated particle dispersion. Otherwise, xanthan gum moieties tend to interact with zeolite and prevent particle agglomeration. The

biocomposite possessed appreciable multidimensional features due to the combination of alginate and xanthan gum, and its operational stability was reinforced by the entrapped zeolites. Furthermore, a fixed bed column was used for removing the cations from simulated nuclear wastewater (Zhang et al. 2014b).

The demand for precious metals is now escalating, and hence low concentrations of such metals dissolved in wastewater need to be recovered. Biopolymers serve as eco-friendly and cost-effective adsorbents for this purpose. Immobilization of Cyphos IL-101 (tetradecyl(trihexyl)phosphonium chloride) in gelatin and sodium alginate capsules by ionotropic gelation in $CaCl_2$ solution was performed for Pd(II) recovery from acidic solutions. Pd(II) present in the form of $PdCl_2$ was probably bound to the resin through anion exchange with protonated phosphonium groups (ion pair formation). The presence of other anions decreased Pd(II) binding, while metals that do not form anionic complexes under the selected experimental conditions did not interfere with Pd(II) uptake (Vincent et al. 2008). An impregnated resin prepared by immobilization of Cyphos IL-101 into a composite biopolymer matrix comprised of gelatin and alginate for Bi(III) recovery from acidic solutions is another such example. The mechanism involved was ion exchange in this case as well (Campos et al. 2008).

3D-macroporous biopolymer-coated hydroxyapatite foams are potential devices for the treatment of Pb(II), Cd(II), and Cu(II) contamination of water. In an in vitro treatment system mimicking severe water contamination, the foams exhibited fast and effective metal ion immobilization into the hydroxyapatite structure. Coating of the foams with polycaprolactone and glutaraldehyde cross-linked gelatin improved their stability and integrity (Vila et al. 2011). These novel materials are promising advances in the development of easy-to-handle and low-cost water treatment methods, obviating the need for complex infrastructure.

Extraction of Cu(II), Fe(II), and Cr(VI) is feasible using the hydrogels of dextran and starch created with the monomers of acrylamide, N-isopropyl acrylamide, and 2-acrylamido-2-methylpropanesulfonic acid cross-linked with N,N-methylene bisacrylamide. The synthesized hydrogels had high water absorption capacity and possessed ion-exchange groups. Effect of functionalization on metal ion uptake was evaluated, and the results of this study are of value in the development of hydrogel-based technologies for metal ion sequestration and water purification (Chauhan et al. 2006).

3.2.5.9 Bacterial and Fungal Biopolymers

Considering the microbial biopolymers, extracellular polymer secreted by psychrotrophic *Pseudomonas fluorescens* BM07 when grown aerobically at 10.8 °C showed high ion-binding capacity with particular preference for uptake of Cd(II) and Hg(II) (45 and 70%, respectively). The percentage removal of other cations like Co(II), Zn(II), Ni(II), and Cu(II) was between 20 and 30%. Overall ion uptake by BM07 biopolymer showed a definite preference for larger over smaller cations. Carboxyl, amine, hydroxyl, and methoxyl functional groups were present in the secreted

biopolymer (Noghabi et al. 2007). Another example is the exopolymeric substance produced by a highly mercury-resistant strain of the yeast *Yarrowia* spp., which has Hg(II)-binding potential and is a promising biotechnological tool in designing bioreactors for treatment of Hg(II)-rich industrial wastewaters (Oyetibo et al. 2016).

3.2.5.10 Biopolymers from Activated Sludge

Quite interestingly, the biopolymers present in activated sludge could be extracted and suitably exploited in the removal of heavy metals. Biopolymers with different proportions of constituents were extracted and used in adsorption studies for Cu(II) ions after conditioning with cetyl trimethyl ammonium bromide and linear alkylbenzene sulfonate. The detergent cetyl trimethyl ammonium bromide increased the protein content, while linear alkylbenzene sulfonate was good at increasing the polysaccharide and nucleic acid contents (Zhou et al. 2016). The results signify that all biomass with high protein content could serve as adsorbents for metal ions.

3.2.5.11 Biopolymers for Heavy Metal Detection

Biopolymers could also be helpful in applications concerning heavy metal detection. The natural polysaccharides agar and carrageenan extracted from the cell wall of red algae were employed for making thin films using layer-by-layer self-assembly technique onto tin-doped indium oxide. The polysaccharides of interest were deposited in layers alternating with polyaniline. The presence of agar and carrageenan improved the electrochemical stability of the conducting polymer in an acid medium. Tin-doped indium oxide-agar-polyaniline system was the most promising one for detection of Cr(VI) (de Farias et al. 2015).

3.3 Biopolymer-Based Coagulants-Flocculants

Pre-treatment of wastewater using coagulants and flocculants augments the performance of primary sedimentation tank in an effluent treatment plant, resulting in greater biochemical oxygen demand and total suspended solids removal efficiency. Synthetic polyelectrolytes and metal cations predominantly used for this purpose could be replaced or supplemented with biopolymers, thereby enhancing the biodegradability of the resultant sludge, while simultaneously offering protection from the ill effects of residual alum.

When deliberating potential biopolymers for this application, ceric ion-induced polymethyl methacrylate grafting of oatmeal enhanced its flocculation characteristics of kaolin suspension and municipal wastewater. Comparatively, the parent biopolymer and the standard flocculant alum displayed inferior flocculating abilities in the “jar test” (Bharti et al. 2016). In another study involving oatmeal, microwave-

initiated (microwave irradiation alone) and microwave-assisted (using ceric ammonium nitrate) acrylamide grafting were attempted. The flocculation efficiencies of the synthesized copolymers were appreciable for attenuation of wastewater pollutant load. The performance of the grafted biopolymers, especially the microwave-assisted variant, was better than that of ungrafted oatmeal and alum (Bharti et al. 2015).

Tannin could also be a likely replacement for synthetic polyelectrolytes used as coagulants. It was more effective than AN913 as a coagulant aid and significantly reduced the required dose of alum (Özacar and Şengil 2003). Pectin is another useful biopolymer in wastewater treatment, and its efficacy was proven in reducing the turbidity of kaolin suspension (Ho et al. 2009).

Guar, locust bean gum, and *Opuntia* mucilage are beneficial in bringing down the pollution load of high-strength cosmetic industry wastewater, as reflected by lowered conductivity and turbidity values (Carpinteyro-Urban et al. 2012). The flocculating effects of guar gum are further useful in removing POPs such as phenol, 2,4-bis(1,1-dimethylethyl), and bis(2-ethylhexyl) phthalate from effluents. Bis(2-ethylhexyl) phthalate is the commonest member of phthalates which are used as plasticizers and is believed to cause endocrine disruption in males. 2,4-bis(1,1-dimethylethyl) phthalate used as an intermediate in the synthesis of UV stabilizers and antioxidants is toxic toward aquatic organisms. Upon comparison of the guar gum's removal efficiency with that of alum, numerous void spaces in the flocs produced by guar gum were observed, and hence it was more efficient in capturing the persistent organic pollutants (Kee et al. 2015). Thus, guar gum could be recommended as an alternative to chemical coagulants in treating persistent organic pollutants, due to its nontoxic and biodegradable characteristics. This natural polymer has also been utilized in the clarification of rubber mill wastewater (Mukherjee et al. 2013).

The flocculating abilities of biopolymers could be harnessed for recovery of value-added products from industrial effluents too. Guar gum serves as a green treatment option for pulp recycling. The floc settling velocities and sludge volume index obtained with this plant-derived polysaccharide were comparable to that of alum (Mukherjee et al. 2014). Among other applications that make use of the flocculating ability of biopolymers, cationic inulin was applied for harvesting the algal biomass of *Botryococcus* sp. for potential use in biodiesel production (Rahul et al. 2015).

Apart from the aforementioned plant-based substances, microbial biopolymers also possess useful flocculating activity. *Bacillus subtilis* isolated from palm oil mill wastewater was able to produce a biopolymer with high flocculating activity of kaolin suspension. Hybridization of the biopolymer with metal ions enhanced its flocculating activity. Biopolymer hybridized with divalent metal ions (Ca^{2+} or Mg^{2+}) displayed the highest flocculating activity compared to that of monovalent (K^{+}) and trivalent (Al^{3+}) ions (Khiew et al. 2016).

3.4 Biopolymers for Other Environmental Applications

3.4.1 Hydrogen Generation

In addition to water and wastewater treatment discussed thus far in the review, biopolymers also find their niche in a myriad of other environmentally relevant applications. Hydrogen generation is one such domain, and the use of lignocellulosic polymers as substrates for biohydrogen production is a pertinent area of research owing to their bountiful availability, renewable, and nonpolluting nature (Ren et al. 2016). However, since cellulosic polymers are not being considered in this review, developments and opportunities in this arena are not discussed. Nevertheless, there are notable instances of other biopolymer-based catalysts that are useful in the process, with alginate being the most conspicuous one. Cobalt grown in situ on macroscopic alginate hydrogels is a recyclable catalyst for hydrogen generation from the hydrolysis of $NaBH_4$. Considering the eco-friendly and inexpensive nature of alginate hydrogels and their superior catalytic activity, they hold promising application in hydrogen generation from the hydrolysis of borohydrides (Ai et al. 2014).

Alginate could also be used in the preparation of TiO_2 and Au-TiO_2 samples with high photocatalytic activity for hydrogen generation from water and methanol mixtures. The Au-TiO_2 sample prepared by the biopolymer templating method was approximately eight times more active in hydrogen generation using a solar simulator than an analogous sample prepared by means of the conventional deposition-precipitation method (Buaki-Sogo et al. 2013). Graphitic carbon sheets decorated with Mo_2C nanoparticles and prepared via biopolymer-derived solid-state reaction between $(NH_4)_6Mo_7O_{24}.4H_2O$ and sodium alginate at 900 °C under Ar showed substantial long-term durability in hydrogen generation (Cui et al. 2014). These results ascertain the usefulness of biopolymers in the preparation of catalysts for hydrogen generation.

3.4.2 Electrochemical Industry

The thermal, mechanical, and chemical stabilities of polymer electrolytes used in electrochemical devices such as fuel cells and batteries are of paramount importance. Electrolytes that make use of biopolymers have better environmental acceptability. Thus, a whole new range of toxicity-free, cost-effective, and easily processable proton-conducting membranes has emerged. I-carrageenan membranes doped with NH_4Br (Karthikeyan et al. 2017); tamarind seed polysaccharide with NH_4SCN as dopant (Premalatha et al. 2016); pectin doped with NH_4Cl and NH_4Br (Vijaya et al. 2017); and CMκ-carrageenan with ionic liquid 1-butyl-3-methylimidazolium chloride (Shamsudin et al. 2016) are among such examples. Poly(lactic-*co*-glycolic acid) is useful in promoting ionic conductivity in the active layer of light-emitting electrochemical cells (Zimmermann et al. 2016). Composite

of lignosulfonate-graphene hydrogel used as supercapacitor electrode material (Xiong et al. 2016) and natural lignin matrix employed as electrolyte in lithium-ion batteries (Gong et al. 2016) are other instances of biopolymer usage in electrochemical devices.

3.4.3 Soil Strengthening and Agriculture

Biopolymers are capable of enhancing soil characteristics. Xanthan gum interacts with the charged surfaces of clayey particles and form xanthan matrices that resemble a hard plastic. The strengthening effect of xanthan gum is dependent on type of soil, hydration level, xanthan gum content, and mixing method (Chang et al. 2015a). Guar gum and xanthan gum improve the mechanical properties of collapsible soil, resulting in an increased sheer strength (Ayeldeen et al. 2017). Climate change is leading to large-scale soil degradation and desertification. Treatment with even low concentrations of biopolymers could serve to boost interparticle cohesion, thereby reducing soil erosion. Such biopolymer treatment could be used to supplement anti-desertification strategies that are already in place (Chang et al. 2015c). These measures are greatly helpful in arid and semiarid locations.

As a dimension of biopolymer application in the agricultural sector, agrochemical formulations could be stabilized and their performance augmented by encapsulation in biopolymers. Lavandin (*Lavandula hybrida*) essential oil used as a natural biocide was encapsulated in n-octenyl succinic-modified starches, by removing the water from an oil-in-water emulsion stabilized using n-octenyl succinic starches as surfactants. A high-pressure precipitation technique of drying particles from gas-saturated solution was applied to perform the encapsulation. Oil losses due to its dissolution in supercritical CO_2 or emulsion destabilization were reduced by careful selection of the operating conditions (Varona et al. 2010).

3.4.4 Building Sector

Strengthening of soil is necessary for its use as a building material, for which cement is widely used, although it leads to greenhouse gas emissions. Biopolymers could be applied in lieu of cement, and several researchers have worked on it. The way biopolymers interact with the soil and strengthen it has been comprehensively reviewed (Chang et al. 2016). β-1,3/1,6-glucan-treated Korean residual soil hwangtoh exhibited increased compressive strength while simultaneously offering financial competitiveness and lower environmental impact than cement. The curing temperature of the polymer soil mixture was an ideal 60 °C (Chang and Cho 2012). Thermal gelation polymers are also quite amenable for soil strengthening purposes in land as well as waterfront constructions. Gellan gum and agar gum capable of hydrogen bonding were used in strengthening clayey and sandy soils. In case of

soils with significant fine contents, gellan gum was more preferable due to its interaction with soil fine particles, resulting in the formation of firm soil-biopolymer matrices (Chang et al. 2015b).

Exploring other ways in which biopolymers are applicable in the construction sector, they could be used as natural sealants for concrete cracks. In one such study, *Pseudomonas aeruginosa* strains 8821 and PAO1 capable of producing extracellular polymeric substances were genetically modified by incorporating the gene sequences of *Sporosarcina pasteurii* in order to confer calcium carbonate precipitation ability and enhance their performance as biosealants (Bergdale et al. 2012). These engineered strains provide exciting possibilities for future biosealants that could be applied in the environment. Biopolymers also minimize water-induced degradation and improve the mechanical properties of earthen constructions.

3.5 Conclusion

Looking beyond cellulose and chitosan, a diverse range of other plant and microbial biopolymers hold promising potential for environmental applications, as evidenced in this review. Production from renewable substrates and biodegradability are the desirable traits that have set them apart from synthetic polymers and justified the attention that they have received. Dye and heavy metal removal using polymer biosorbents has been a topic of intense research. However, larger size, limited internal porosity, lesser surface area, and the existence of internal diffusional resistance are certain factors that have deterred their full-fledged application. These too have been largely overcome with the novel routes of synthesis and functionalization of nanoscale adsorbents. Biopolymers find better acceptability than the toxic magnetic material-based adsorbents. Thus, they have carved a niche for themselves in this arena.

Applications of biopolymers are not limited to adsorption, and their use as coagulants-flocculants in wastewater treatment has been shown to improve biodegradation of the resultant sludge. Remediation of oil spills and elimination of recalcitrant pesticides are other domains in which biopolymers have been useful. They have also fared as effective tools in catalyzing hydrogen generation. Reinforcement of soil internal cohesion and prevention of soil erosion have been accomplished with the aid of biopolymers. They have replaced cement as eco-friendly soil-strengthening materials in the construction sector. Biopolymer-based electrolytes with excellent conducting properties have been successfully introduced in electrochemical devices. Such applications have offered an insight into the immense scope of biopolymers in pollution mitigation and environmental sustainability.

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Chapter 4
Methods in Metagenomics and Environmental Biotechnology

Birendra Singh Yadav, Alok Kumar Yadav, Swati Singh, Nand Kumar Singh, and Ashutosh Mani

Contents

Abstract Microbes are integral part of our environment. They have enormous industrial and medicinal applications. Even they play a crucial role during digestion where they are present in the form of gut flora. Genomic sequences are a prerequisite for molecular taxonomic characterization of novel microbes, and traditional

B. S. Yadav · A. K. Yadav · N. K. Singh · A. Mani (✉)
Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India
e-mail: amani@mnnit.ac.in

S. Singh
Center of Bioinformatics, University of Allahabad, Allahabad, India

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
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microbiology is dependent on clone cultures for DNA extraction of a specific microbe population. As a result vast varieties of species are missed since most of the microbes cannot be cultured in laboratory conditions. Metagenomics skips the requirement of culturing the microbes in lab as it studies genetic material which is directly taken from environmental samples.

Microorganisms are of great significance due to their applications in health, agriculture, and industry. Direct DNA sequencing of environmental samples has given opportunity to gather information about the microorganisms that were unexplored so far. Screening of useful bacteria that survive in different environmental conditions like heavily polluted soil, disease-affected tissues or cells, oil-contaminated water bodies, heavy metal-contaminated fields, etc. can be done easily by combining environmental and metagenomics approaches. The data obtained from environmental sample sequencing may be of great use in discovery of new drugs and antibiotics, new bacterial species, plant growth promoters, bioremediation as well as in many other industrial applications. The number of metagenomic studies has increased significantly in recent years, and it is believed that this trend will continue its pace due to huge applicability. This chapter also provides significant elaborations about methodology and tools, experimental design strategies, online resources, and databases applicable in metagenomic data analysis.

4.1 Introduction

During the evolution of life on earth, microbes have played an important role and have done much more for human beings for the sustenance and survival. As these microbes have adapted to the earth's environment, they are found everywhere, viz., on earth, inside earth, in water, and in air. To understand their impact on global ecology, it is most important to understand their diversity and life. According to estimates, about 99% of the microbes are not culturable in pure culture. It acts as the major debacle in understanding the microbial genetics and community ecology. These microbial communities are responsible for biological activities carried out in all environments including the ocean (DeLong 2005), soils, and human-associated habitats (Ravel et al. 2011). Although metagenomics is quite a young and emerging field, it has helped in understanding the microbial diversity which was not possible by using traditional and classical methods of microbiology. Metagenomics has emerged as the most powerful and reliable technique for genome analysis of the entire community of microbes overruling the need to isolate and culture individual microbial species (Arrial et al. 2009). It has wide potential in discovering novel enzymes for industrial applications, antibiotics against many harmful microbes for curing diseases, and organisms for experimental purposes.

Major metagenomics themes are (a) marker metagenomics that surveys microbial community structure by targeting the highly conserved 16S rRNA gene; (b) functional metagenomics that takes the total environmental DNA, from which it

infers the metabolic potential of the microbial community; and (c) identification of novel enzymes. Metagenomics uses two approaches: targeted metagenomics and shotgun sequencing. Targeted metagenomics is most commonly used to identify the phylogenetic diversity and the relative abundance in a given sample. This technique is mainly used to investigate the diversity of small subunit of rRNA (16S/18S rRNA) within a sample. It is often used to understand the impact of environmental contaminant that alters the microbial community structure. For conducting the study related to targeted metagenomics, the environmental DNA is extracted from the source, the particular gene of interest is amplified using PCR primers, and further these amplified results are sequenced using next-generation sequencing. Targeted metagenomics is useful in identifying the diversity of single gene of interest, but it is limited by the type of PCR primers used for the analysis (Shakya et al. 2013; Parada et al. 2016; Klindworth et al. 2013; Prosser 2015).

Similarly in shotgun metagenomics sequencing, the genomic complement of an environmental community is studied by using genome sequencing. Basically in this approach, the DNA is extracted from the environmental sample and fragmented to prepare sequencing libraries and further sequenced for the determination of total genomic content of that sample. Shotgun sequencing is often restricted by the depth of the sequencing.

Functional metagenomics has played a major role in understanding the role of microbial community in microbial ecology and global geochemical cycles. Furthermore it is a unique way to identify the novel enzymes from the environmental sample (Uchiyama and Miyazaki 2009). Therefore the functional metagenomics played major role in protein and nucleic acid database through addition of novel functional annotation. However major drawback of this technique includes a low hit rate of positive clones, low throughput, and time-consuming screening (Hosokawa et al. 2015).

Currently metagenomics is a powerful technique to have industrial applications in identification of novel biocatalysts, discovering novel antibiotics, and bioremediation. The application of metagenomics is increasing rapidly, and these are being listed below.

4.2 Application of Metagenomics and the Impact on Environmental Biotechnology

The new field of metagenomics is expected to bring fruitful result for the researchers working in the area of microbiology in mainly two ways: in first application it will provide knowledge about those bacteria which are still not cultivated so far (about 99% are uncultured in the pure culture). Secondly it will provide access to whole microbe community residing in variety of natural environment. Furthermore as we know that microbes are quite essential component of our life for the sustenance and these microbes play very crucial role in industries which are backbone

of our present economy planet. Direct access to the genetic makeup of microbes of the whole ecosystem community will provide new basis for fundamental research and new tool for application in environment, agriculture, human health, bio-industry, etc. (Fig. 4.1).

4.2.1 Industrial Enzymes

There is an increasing demand of novel enzymes for industrial applications, and metagenomics is playing an important role in providing these biomolecules (Lorenz et al. 2002; Schloss and Handelsman 2003) specially enzymes that are used in wide range of applications (Kirk et al. 2002). These are required in minute amount to synthesize huge amount of key molecules that are used in producing active pharmaceuticals as these are the major building block of those products (Patel et al. 1994). There are many industrial enzymes which have a very wide application in industries and act as their backbone like cellulases, xylanases, lipases, amylases, etc.

Cellulases have attracted industrialists due to their wide application and crucial enzyme activities that are inherited in various forms within them such as endoglucanases (EC 3.2.1.4), exoglycosidase, and β-glucosidases (EC 3.2.1.21). Today cellulase is the third most widely used enzyme in industries (Wilson 2009). Cellulases are mainly used in animal feed and improving the digestibility. Furthermore deinking of paper is another evolving application of this enzyme (Soni et al. 2008). Metagenomics has played a vital role in extracting cellulase from natural environments like compost soil, soil from cold region, rumen samples and many more. Even few workers have reported that cellulases are isolated from sugarcane soil and buffalo rumen (Alvarez et al. 2013; Duan et al. 2009).

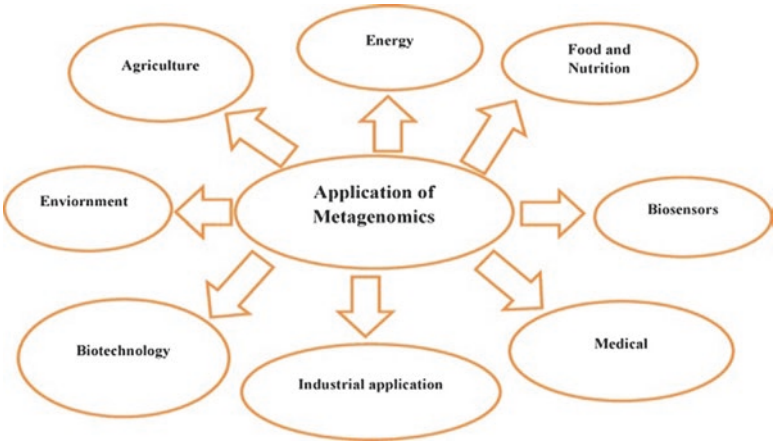

Fig. 4.1 Various aspects of applications of metagenomics (also known as environmental and community genomics) in different fields of biological science

Xylaneses are key enzymes that are widely used in degradation of xylan and are helpful in breaking of hemicellulose, regarded as essential component of cell wall. Xylaneses have wide spectrum of application in industries such as clarification of juices (Sharma 2012), detergents (Kumar et al. 2004), production of pharmacologically active polysaccharides for the antimicrobial agent use (Christakopoulos et al. 2001), antioxidants (Katapodis et al. 2003), and production of surfactants (Kashyap et al. 2014). Xylanases are produced by a wide range of microbes from different sources that have many application in industries. It is reported that xylaneses are present in insect gut that could be used for conversion of biomass into fermentable sugar which could be used for production of biofuels (Brennan et al. 2004; Lee et al. 2006; Jeong et al. 2012). This enzyme was reported in the saccharification of reed and could be used efficiently in the conversion of biomass to fermentable sugar for biofuel production (Wang et al. 2012).

Lipases are mainly triacylglycerol acylhydrolases that are actively involved in the conversion of triglycerides into diglycerides, monoglycerides, glycerols and fatty acids. Being resistant to varying environmental conditions like temperature, pH, organic solvent etc., they have great prospects in industries. It is widely found in many plant and animal sources and also reported in some microbes such as bacteria, fungus, and yeast, and these have varying application in oil industries, pharmaceutical industries, dairy industries etc. (Cardenas et al. 2001).

Amylases are mostly regarded as starch-degrading enzymes. They are quite abundant in plants, animals, and microbes. These have wide application in industries like food, fermentation, and pharma for hydrolysis of starch. AmyI3C6 commonly known as cold-adapted alpha amylase from the metagenomic libraries of cold and alkaline environment can be useful as it showed potent activity against two commercially known detergents. A novel amylase was isolated from a soil metagenome that showed 90% activity at low temperature which proved its potential for industrial exploitation (Sharma et al. 2010).

4.2.2 Bioactive Compounds and Antibiotics

Nowadays a major worldwide health-related problem involves treating infections which are resistant to antibiotics. These resistant microbes are able to cause severe mortality and impose a large budget on healthcare (Carlet et al. 2011). Earlier these antibiotics were used for treating human infection, but they became popular in agriculture and food industry as well as many other related sectors, thus finally imposing high impact on human health (Radhouani et al. 2014). In the current scenario, antibiotics are considered as the pillars of the modern medicine (Ball et al. 2013). This bacterial resistance against widely used common antibiotics has forced researchers to discover novel antibiotics against these microbial infectious diseases.

Today metagenomics is playing a very vital role in discovery of bioactive compounds and antibiotics. It is considered as an alternative way of isolating antibiotics

from environmental samples as well as to trace the mechanism of bacterial gene resistance. The combined approach of metagenomics and next-generation sequencing has paved way for success in study of antimicrobial resistance and microbial genomes (Forsberg et al. 2012; McGarvey et al. 2012). Generally, the bacterial gene resistance is mainly developed due to the horizontal gene transfer or spontaneous mutation in target gene (Hassan et al. 2012). The transfer of antibiotic resistance gene involves the mobility of genetic material to other bacterial species or the same group (Thomas and Nielsen 2005).

Metagenomics is putting effort to sort out the drug resistance genes in microorganisms against various class of antibiotics. Its another application is identification of bioactive molecules having antimicrobial properties (MacNeil et al. 2001; Gillespie et al. 2002; Lim et al. 2005). Today, antibiotic resistance of microbes is an alarming worldwide problem and emerging as a major threat (Čivljak et al. 2014) as these microbes are developing resistance against many traditional antibiotics, and on the other hand, many researchers are discovering many novel antimicrobial compounds from different environmental sources including microorganisms, plants, and animals likewise (Roy et al. 2013; de Souza Candido et al. 2014). It is reported that uncultivated soil microbes have potential of novel biomolecules which could be very well exploited in any biotechnological application (Wilson and Piel 2013). In this way we can conclude that these soil microbes can be an alternative source of bioactive molecules. Various active biomolecules which are identified by metagenomic approach include teicoplanin, friulimicin, azinomycin, rapamycin, borregomycin, etc.

4.2.3 Bioremediation

The process to degrade and detoxify environmental contaminants through microbe-mediated process is known as bioremediation (Chakraborty et al. 2012). It involves removal of biological and anthropogenic contaminants through natural process, so it is considered as the most effective approach (Lovley 2003). Bioremediation approaches can be classified into three main classes, (a) natural attenuation, (b) biostimulation, and (c) bioaugmentation.

In natural attenuation native organisms are used for detoxifying contaminants through using natural process. This process is quite effective in terms of cost, and no need of altering additives is required for this. In biostimulation the rate of bioremediation is increased through using native organisms but needs to remove some environmental constraints. This approach required addition of some nutrients to achieve fast rate of bioremediation. Sometimes this approach failes to achieve their faster rate of bioremediation by using native organism due to their inability to degrade contaminant of concern. To overcome this problem, some nonnative organisms or enzymes are added to enhance the rate of bioremediation which is known as bioaugmentation. This approach is considered as most invasive as nonnative organism. In some cases bioaugmentation is considered as most convenient mean of

remediation (Payne et al. 2011; Salanitro et al. 2000). The major drawback of bioaugmentation is that nonnative organism can't survive under the condition found in the contaminated ecosystem.

In the present scenario, metagenomic approach is widely used for environmental monitoring and bioremediation. Metagenomics approaches that are often used for monitoring the environmental microbes are targeted metagenomics or shotgun metagenomics. Targeted metagenomics is widely exploited to study the phylogenetic diversity and relative abundance of a particular gene in the environment. This approach is used to study the diversity of the rRNA sequence in the sample (16S/18S rRNA). It is often used to study the impact of environmental contaminant in microbial community structure. The major advantage of targeted metagenomics is that it provides the information about microbial community present in the set of sample and change in microbial diversity before and after perturbation.

Likewise in the shotgun metagenomics, the total genomic complement of the environmental community is probed by using genome sequencing. In this approach, environmental DNA is extracted and fragmented to prepare genomic libraries and further sequenced to determine the total genomic content. Using this approach potential of a microbial community can be identified. Recently metatranscriptomics and metaproteomics are being widely used to apply over environmental system. In metatranscriptomics ribonucleic acid (RNA) is extracted from the sample and converted to complementary deoxyribonucleic acid (cDNA) in a similar function as in metagenomics. The metaproteomics approach does not involve the nucleic acid sequencing but high-resolution mass spectrometry combined with enzymatic digest of the proteins and liquid chromatography (Hettich et al. 2013). Metaproteomics provides an information about the kind of protein present inside the environmental sample including posttranslational modification in proteins that may impact their activity.

Many industries are responsible for increased level of hydrocarbons in the environment due to the incomplete combustion of fossil fuel. Generation of these anthropogenic compounds into the environment results into the accumulation of large amount of aromatic hydrocarbons which leads to contamination of ecosystem (Jacques et al. 2007). Microorganisms are involved in many biogeochemical cycles and have potential of degradation of hydrocarbons (Alexander 1994). Metagenomics can be helpful in degradation of aromatic compounds by screening and identifying suitable organisms in a metagenomic library obtained from oil source (Sierra-García et al. 2014). Many genes and their pathways were identified for the degradation of phenol and aromatic compound by using metagenomic approach (Silva et al. 2013). Some bacterial population having capacity for the degradation of polycyclic aromatic compound (PAH) were isolated from cold environment by identifying their functional target (Marcos et al. 2006).

As we know that oil spillage has badly affected many parts of the natural marine ecosystem (National Academy of Science 2005) due to increased anthropogenic activity (Hazen et al. 2016; Atlas and Hazen 2011). In this context Deepwater Horizon oil spill is considered as the worst marine oil spill in the USA and considered as major threat for marine ecosystem biology (King et al. 2015). The first

application of metagenomics was to understand the mechanism behind the oil biodegradation in marine environment. The targeted metagenomics was applied to find out the microbial community in the surface water and reported as *Cycloclasticus*, *Alteromonas*, *Halomonas*, and *Pseudoalteromonas* (Redmond and Valentine 2012; Gutierrez et al. 2013). However they also reported that deep water is primarily composed of psychrophilic oil-degrading microbes related to *Oceanospirillales*, *Colwellia*, and *Cycloclasticus* (Hazen et al. 2010). Shotgun metagenomics approach was used for sample collected during Deepwater Horizon oil spill which revealed diverse group of genes responsible for chemotaxis and hydrocarbon degradation (Mason et al. 2012). The results of the single amplified genome showed genes involved in degradation of n-alkanes and cycloalkanes. Thus metagenomics sequencing approach helps in understanding the mechanism behind the oil degradation by microbial community in marine environment.

4.2.4 Applications in Agriculture

The productivity of agriculture is severely affected by presence of organic and inorganic anthropogenic pollutants that play a very significant role in abiotic stress. These kinds of abiotic stresses are responsible for reduction in crop yield. To improve the quality of such soil contaminated by anthropogenic pollutants, bioremediation is required. Microorganisms of soil metagenome are quite capable of producing biosurfactants which can remove many anthropogenic pollutants which may be either hydrocarbons or heavy metals (Sun et al. 2006). Biosurfactants are capable of removing hydrocarbons and heavy metals through the combination of soil washing and cleanup technology (Pacwa-Płociniczak et al. 2010; Liu et al. 2010a, b; Partovinia et al. 2010; Gottfried et al. 2010; Coppotelli et al. 2010; Kang et al. 2010). Some studies have revealed that biosurfactants isolated *Lactobacillus pentosus* had reduced the octane hydrocarbons from soil (Moldes et al. 2011). Some biosurfactant-producing species like *Burkholderia* isolated from oil-contaminated metagenome may act as a potential candidate for the reduction (bioremediation) of pesticides (Wattanaphon et al. 2008). Some studies have also revealed that biosurfactants are more efficient in removal of organic insoluble pollutant from soil than surfactants (Cameotra and Bollag 2003; Straube et al. 2003). The soil samples from such fields shall be subjected to metagenomics analysis, library preparation and subsequent analysis for identifying biosurfactant-producing microbes.

Besides application of biosurfactants for removal of many anthropogenic molecules which are either hydrocarbon or heavy metals, these may also be applicable in removal of plant pathogens due to their antimicrobial nature, thus promoting sustainable agriculture. Biosurfactants which are produced by rhizobacteria have antagonistic properties (Nihorimbere et al. 2011). For sustainable agriculture, biosurfactants and chemical surfactants are useful in controlling parasitism, antibiosis, competition, induced systemic resistance, and hypovirulence (Singh et al. 2007). In fact the application of surfactants in agriculture is mainly for enhancing the antago-

nistic activity of microbes and microbial products (Kim et al. 2004). Some studies have also revealed that these surfactants when applied in combination of certain fungus like *Myrothecium verrucaria* are found to be useful in the control of weed (Boyette et al. 2002).

Additionally, biosurfactants are also useful for inhibition of many phytopathogens. Biosurfactant isolated from *Pseudomonas* and *Bacillus* is reportedly used for the control of soft rot caused by *Pectobacterium* and *Dickeya* spp. and thus has been helpful in protection of economically valuable crops (Krzyzanowska et al. 2012). Many studies have reported that antipathogenic agents like rhamnolipids have the ability to kill zoospore of plant pathogens that are being resistant against many commercial pesticides (Sha et al. 2011, Kim et al. 2011). Some researchers have proposed that rhamnolipids also stimulate immunity in plants against various infectious agents (Vatsa et al. 2010). The lipopeptide biosurfactant of *Bacillus* origin was reported to inhibit growth of some phytopathogenic fungi like *Fusarium* spp., *Aspergillus* spp., and *Bipolaris sorokiniana*. Such biosurfactant of *Bacillus* origin can be very well exploited for their function as biocontrol agent (Velho et al. 2011). Surfactin isoform and this lipopeptide biosurfactant produced by *Brevibacillus brevis* strain HOB1 have reported potent antibacterial and antifungal properties which could be utilized for control of phytopathogens (Haddad 2008). *Pseudomonas fluorescens* biosurfactants are well reported for their antifungal property (Nielsen et al. 2002). Biosurfactants produced by the *Pseudomonas fluorescens* has potential in inhibition of certain fungal pathogens like *Pythium ultimum* (causes damping off and root rot of plants), *Fusarium oxysporum* (wilting in crop plants), and *Phytophthora cryptogea* (responsible for rotting of fruits and flowers) (Hultberg et al. 2008). Biosurfactants produced by *Bacillus subtilis* isolated from soil metagenome are found useful in the control of *Colletotrichum gloeosporioides* which is a causative agent of anthracnose on papaya leaves (Kim et al. 2010). A common plant pathogen *Pseudomonas aeruginosa* is found to be inhibited by the biosurfactants of staphylococcus of oil-contaminated soil metagenome (Eddouaouda et al. 2012). The abovementioned evidences support the claim that biosurfactants produced by many microbes could be very useful for control of various kinds of phytopathogens. Furthermore, these biosurfactants are emerging as an alternative source of commonly used pesticides and insecticides which are currently in agricultural practices. Metagenomics has great prospects in identifying many phytopathogens, plant growth-promoting microbes and biosurfactant-producing microbes as well.

4.2.5 Applications in Human Health

Human beings are always surrounded by microbes as they not only surface over them but also live within their body. The microbes which are residing inside the human flora are not fully characterized (less than 1%). Furthermore there are certain microbes in our environment which are causative agents of many infectious

diseases. These infectious microbes are mainly characterized by laboratory-based surveillance and syndromic surveillance which are strictly relying on the non-laboratory data. Detecting these causative agents of infectious diseases is failed in approximately 40% gastroenteritis cases and 60% in encephalitis cases when conventional approach is used (Finkbeiner et al. 2008; Ambrose et al. 2011).

The Human Microbiome Project enabled the scientific community to know about the sophisticated sequencing technologies and association of microbiome toward human health and disease (Peterson et al. 2009). Metagenomics has the potential to detect both known and novel microorganisms using culture-independent sequencing and analysis of all nucleic acids taken from the sample. The whole genome sequences of the pathogens can be detected using the advance bioinformatics tools which further help in drawing inferences about antibiotic resistance, virulence and evolution.

In the present scenario, metagenomics is playing a very crucial role in investigating novel species and strains (Wan et al. 2013; Mokili et al. 2013; Xu et al. 2011), outbreaks (Loman et al. 2013; Greninger et al. 2010), and complex diseases (Wang et al. 2012; Cho and Blaser 2012). As with the advancement of the next-generation sequencing and its cost-effectiveness, it could become an essential approach in investigation of infectious diseases at very low abundance and can be performed from clinical samples (Seth-Smith et al. 2013) or from single cells (McLean et al. 2013). The metagenomics approaches which are used for the detection of these infectious or pathogenic agents include deep amplicon sequencing and shotgun sequencing.

In deep amplicon sequencing, certain gene families are reported in every known member species in a particular taxonomic group. It employs the amplification of certain taxonomic markers such as rRNA genes. By using next-generation sequencing, many different amplicons in a sample can be sequenced, and the resulting sequences are compared with the reference standard to identify the species/genus associated with each sequence. The deep amplicon sequencing is capable of identifying the novel microorganisms. In the case of bacterial deep amplicon sequencing, they use specific primers that are specific to the conserved genes such as 16S, rRNA, chaperonin-60 (Links et al. 2012), and RNA polymerase (rpoB) (Wu et al. 2011). Likewise in protozoan and fungal deep amplicon sequencing approach, they only target 18S rRNA gene regions (Leng et al. 2011; Sirohi et al. 2012; Iliev et al. 2012). Major advantage of the deep amplicon sequencing lies in an enhancement of the assay's sensitivity for the microorganisms, with higher resolution. However the major drawback of this approach is the inaccurate estimation of the microbial community composition, which requires prior knowledge of pathogenic agent.

In shotgun metagenomics, all microbes are taken into account after sequencing all the nucleic acids extracted from a specimen. Extracted nucleic acids from the specimen are sequenced using next-generation approach, and their results are compared with their reference database. The database used in shotgun metagenomics are usually much larger than those used in deep amplicon sequencing and contain

all the known sequences as compared to the set of sequence from a single gene family. The major advantage of shotgun metagenomics over deep amplicon sequencing is that it is less biased and generates data that better reflect the sample's true population structure. Besides pathogen detection using shotgun metagenomics approach, it also has the potential to generate complete or nearly complete pathogen genome assemblies from the sample (Seth-Smith et al. 2013; McLean et al. 2013). These results provide an estimation of microbial phenotypes and microbial genotypes by determining the presence or absence of antimicrobial resistance and epidemic dynamics (Bertelli and Greub 2013).

Although metagenomics has immense potential to exploit genomics based information for identifying microbiomes that are relevant to the public health. Additionally it is of use in hospitals and healthcare facilities to identify unknown or novel pathogens as well as for characterization of normal and disease associated microbial communities. Through metagenomics approach, it became quite easier to identify the 78 species from the biofilm from the hospital sink with new bacterial phylum (McLean et al. 2013). Thus in the present scenario, metagenomics approach has proved itself as the most powerful tool for the detection of novel microorganisms.

4.2.6 Environmental Applications

Various kinds of microbes are living in our environments which are helpful in many ways. They play a very important role in decomposing dead material present in the environment and making it free from pollutants. There are certain microbes which are able to degrade oil whenever it spills over water surface. Many microbes also have the ability of cleaning the ground water. Here metagenomics may play very important role in identifying particular species which are concerned with water treatment purpose. Oil-consuming microbes that are present in sea are suitable examples of microbial bioremediation of water. Many other bacteria that are present in the soil have qualities of consuming heavy metals and may be helpful in reducing soil toxicity. Identification of these microbes is a major hurdle in further research and analysis in this regard. So this area is a hot cake for metagenomics and environmental scientists as well.

4.3 Methods and Tools

The steps involved in metagenomics analysis have been shown in the flowchart given in Fig. 4.2, and each part is explained in detail along with the tool used in particular methods. Figure 4.2 shows flowchart for experiment design, sampling, sample fractionation for obtaining DNA, that is further analyzed using different computational tools to find out solution to various research problems.

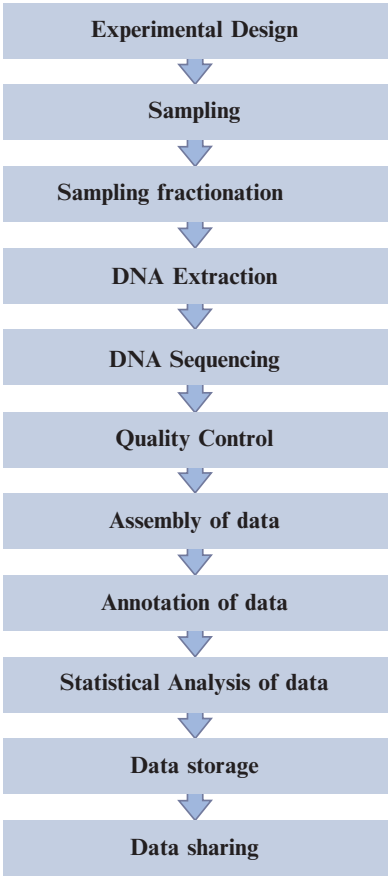

Fig. 4.2 Flowchart of experimental and computational methods that are used to retrieve the genomics information which is further analyzed by different bioinformatics tools. This analysis helps in screening and identification of uncultured microbes that are directly taken from environmental samples

4.3.1 Experimental Design

Experimental design plays a major role in getting accurate, reliable, and high-quality data. Researchers working in the field of metagenomics need to focus on number of replication of data, cost-effectiveness for the sequencing, and accuracy of methods that are used to perform the metagenomics data analysis. In order to obtain accurate and qualitative results in the field of metagenomics, there should be minimum standards during experimental design. While designing the experiment, one must consider the biological and technical replicates, budget should be fixed for sequencing, best protocols should be searched for high yield and good quality of DNA, and sequencing platform should also be discussed. The place should be clearly defined in the terms of certain parameters, from where the sample has to be taken (Cooke et al. 2017).

4.3.2 Sampling

After the experimental design, sample is collected from different sources, i.e., soil, air, water, biopsy, plants, etc. which is known as sampling. The quality of data we obtained from metagenomics depends on sampling (Thomas et al. 2012). While describing the biodiversity, the sample should represent whole population (Wooley et al. 2010) and it should also represent habitat. While collecting the samples, one should know about the time (i.e., day, date, and year of sample collection), number of samples, and volume of samples needed to describe the environmental conditions. Strategy of sampling method and variability of experimental methods should be clear. For collection of representative sample, it is very important to know the amplitude of variation in habitat environment, for example, soil communities with varying soil types like clay, silt and sand particles, plant matter in various stages of decomposition, and variety of invertebrates. So, while sampling one must consider the scale i.e. size of habitat, biological variation, experimental variability, reproducibility, repository and singletons.(The New Science of Metagenomics).

4.3.3 Sample Fractionation

Sample fractionation is a process of lysing the cell to extract the genomic DNA. It is done for obtaining the genomic DNA from abundant as well as rare representative of each taxonomic groups possessing different thickness of cell wall and cell membrane. During sample fractionation or cell lysis, genomic DNA is also exposed to different types of nucleases. So, it's very important to deactivate or inactivate the nucleases by adding strong denaturing agents to keep our genomic DNA safe (Virgin and Todd 2011; Claesson et al. 2012; Yatsunenko et al. 2012). Cell lysis can be performed by thermal, chemical, mechanical, and enzymatic methods (Felczykowska et al. 2015).

4.3.4 DNA Extraction

DNA extraction is a crucial step for analyzing the genome of unculturable microbe. So, it's very important to select a qualitative and quantitative DNA extraction method for getting high yield and good quality of DNA (Felczykowska et al. 2015). The sample contains DNA in various packages like virus particles, eukaryotic DNA, and prokaryotic DNA including free DNA. This may be suspended in liquid, bound to solid, or trapped in the biofilm or tissue. So, extraction methods are selected on the basis of medium present and interest of population. Basically, there are two methods for extraction of DNA, i.e., direct method and indirect method. In the first method, cells are lysed within the sample, and then DNA is extracted, e.g., viruses, and later one includes separation of sample from noncellular material before

lysis. The yield of DNA product is nearly 100 times lower in the indirect method of DNA extraction than direct, but the bacterial diversity of DNA recovered by indirect means was distinctly higher. (LaMontagne et al. 2002; Van et al. 1997; Ogram et al. 1987; Berry et al. 2003; Jacobsen and Rasmussen 1992).

4.3.5 DNA Sequencing

Generally, there are three types of sequencing methods, viz., amplicon sequencing, shotgun sequencing, and metagenomics sequencing. Amplicon sequencing is used for characterization of microbiota diversity and it is the most commonly used technique. It targets the small subunit of ribosomal RNA (16s) locus, which acts as marker which gives information about phylogeny and taxonomy (Pace et al. 1986; Hugenholtz and Pace 1996). This sequencing method is used to characterize a large range of microbial diversity in the human gut (Yatsunenko et al. 2012), *Arabidopsis thaliana* roots (Lundberg et al. 2012), ocean thermal vents (McCliment et al. 2006), hot springs (Bowen DeLeon et al. 2013), and Antarctic volcano mineral soils (Soo et al. 2009). Due to certain limitations of amplicon sequencing, shotgun sequencing came in the picture. Novel and highly diverged species were difficult to study using amplicon sequencing (Acinas et al. 2004).

Shotgun sequencing has capability to overcome the limitations of previous approach. This approach relies on extracting DNA from cells in community and fragmenting it into tiny parts (i.e., reads) that are used to align against the known genome and 16S rRNA. Hence, it provides opportunity to explore microbiota community with two aspects (Sharpton 2014). Shotgun sequencing has also limitation like large data handling, reads may not present in the whole genome, and sometimes two reads of the same gene don't overlap (Schloss 2008; Sharpton et al. 2011). Advancement in shotgun sequencing enables it to answer the above-raised questions and has been used for identification of new viruses (Yozwiak et al. 2012) as well as characterization of uncultured bacteria (Wrighton et al. 2012). This advanced metagenomics sequencing has been used to characterize the microbes associated with roots (Bulgarelli et al. 2013; Vorholt 2012) and also used for identification of taxa that are associated with the human gut (Morgan et al. 2012).

4.3.6 Quality Control

The sequencing data obtained from NGS technology is first subjected to quality control studies. It is the process of sorting out and screening low-quality reads, which affect the downstream analysis (Zhou et al. 2014). The accuracy of microbial biodiversity can be improved by quality filtering (Handelsman 2004). There are several tools available for quality control as shown in Table 4.1.

Table 4.1 List of online tools that are useful for assessing the overall quality of a sequencing run and are widely used in next-generation sequencing (NGS) data production environments as an initial quality control (QC) checkpoint

I.	FastQC (fast quality control)	Checks quality of data in terms of base quality, guanine and cytosine (GC) content, and sequencing length (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/)
II.	FastX ToolKit	Toolkit preprocessing of raw data is done, which includes read length trimming, identical read collapsing, adapter removing, and format conversion (http://hannonlab.cshl.edu/fastx_toolkit/)
III.	PRINSEQ (PReprocessing and INformation of SEQuences)	It is a web interface and provides more detail options for quality checking (Schmieder and Edwards et al. 2011)
IV.	NGS QC Toolkit (next-generation sequencing quality control)	This tool performs quality control and consults NGS data quality control using Roche 454 and illumine platform (Patel and Jain. 2012)

4.3.7 Assembly

Assembly means reconstruction of genome from smaller fragment of DNA, i.e., reads obtained through sequencing (Reich et al. 1984). Basically, there are two types of assemblies, i.e., de novo assembly in which the genome is constructed from reads data and the second is comparative assembly which is used to reconstruct the genome using a closely related organism (Medvedev et al. 2007). For the *de novo* assembly, three algorithm-based strategies are used named as greedy (Pop and Salzberg 2008), overlap layout consensus (Myers 1995), and De Bruijn graph (Zerbino and Velvet 2008; Pevzner et al. 2004). Improved *de novo* assemblies have been generated with the help of a known reference genome to form a comparative assembly like OSLay (optimal syntenic layout of unfinished assemblies) (Richter et al. 2007), Projector 2 (Van et al. 2005) and ABACAS (algorithm-based automatic contiguation of assembled sequences) (Assefa et al. 2009).

4.3.8 Annotation

Functional annotation of metagenomics data obtained after the assembling of reads involves predicting the gene, biological function, gene pathway annotation, and metabolic pathway annotation. The tools used for different functional annotations are shown in Table 4.2.

Table 4.2 List of tools and servers useful in metagenomics analysis. Some of them are freely available and compatible with Windows/Linux for functional annotation of metagenomics data and few are paid. Function of tool is shown in the first column and corresponding name is shown in the second column.

Annotation	Tools
Gene prediction	Metagenome annotator (Noguchi et al. 2008)
	Frag Gene Scan (Rho et al. 2010)
	Gene Mark(Zhu et al. 2010)
	Orphelia (Hoff et al. 2009)
	Gliommer MG (Kelley et al. 2012)
Functional annotation	IGM/M: The integrated microbial genomes and metagenomes (Markowitz et al. 2012)
	CAMERA: A community resource for metagenomics (Seshadri et al. 2007)
	MG-RAST: Metagenomics and rapid annotation using subsystems technology (Glass et al. 2010)
	METAREP: Metagenomics reports (Goll et al. 2010)
	RMMCAP: Rapid analysis of multiple metagenomes with a clustering and annotation pipeline (Li 2009)
	Smash community(Arumugam et al. 2010)
	MEGAN4: MEtaGenome ANalyzer (Huson et al. 2011)
	Comet (Linger et al. 2011)
	Web MGA: Metagenomic analysis (Wu et al. 2011)
	Amphora net (Kerpesi et al. 2014)

4.4 Metagenomics Databases and Online Resources

There are many databases and online tools for analyzing and retrieving metagenomics data. Table 4.3 shows the name along with link of such databases/servers. The European Bioinformatics Institute (EBI) Metagenomics enables us to submit, analyze, visualize, and compare our data (Mitchell et al. 2015). MG-RAST is a metagenomics analysis server for annotation of sequence fragments, their phylogenetic classification, functional classification of samples, and comparison between multiple metagenomes. It also computes an initial metabolic reconstruction for the metagenome and allows comparison of metabolic reconstructions of metagenomes and genomes (Wilke et al. 2016). MEGAN (Huson et al. 2011) is a comprehensive toolbox for analyzing microbiome data. One can perform the different analytics using this tool like taxonomic analysis, functional analysis, etc. QIIME (Quantitative Insights Into Microbial Ecology) is a freely available bioinformatics tool for performing microbiome analysis from raw DNA sequencing data. One can perform demultiplexing and quality filtering, OTU (operational taxonomic unit) picking, taxonomic assignment, phylogenetic reconstruction, and diversity analyses and visualizations (Caporaso et al. 2010). Mothur is an open-source, expandable software to fill the bioinformatics needs of the microbial ecology community (Schloss et al. 2009). RDP (ribosomal database) provides quality-controlled, aligned, and

Table 4.3 List of different tools and servers that are used for metagenomics data analysis. Some tools are freely available that can be downloaded and compatible with Windows and Linux, while servers are freely available online

Name of Tool	Link	Reference
EBI metagenomics	https://www.ebi.ac.uk/metagenomics/	Mitchell et al. (2015)
MG-RAST	http://metagenomics.anl.gov/	Wilke et al. (2016)
MEGAN	http://ab.inf.uni-tuebingen.de/software/megan	Huson et al. (2011)
QIIME	http://qiime.org	Caporaso et al. (2010)
Mothur	http://www.mothur.org	Schloss et al. (2009)
RDP 16S database	http://rdp.cme.msu.edu	Cole et al. (2009)
SILVA rRNA database	http://www.arb-silva.de	Quast et al. (2013)
Greengenes 16S database	http://greengenes.lbl.gov	DeSantis et al. (2006)
EzTaxon-e	http://eztaxon-e.ezbiocloud.net	Kim et al. (2012)
UNITE ITS database	http://unite.ut.ee	Abarenkov et al. (2010)
Real-time metagenomics	http://edwards.sdsu.edu/RTMg/	Edwards et al. (2012)
IGM/M	https://img.jgi.doe.gov/cgi-bin/m/main.cgi	Victor et al. (2005)
Metabenchmark	http://www.ucbioinformatics.org/metabenchmark.html	Lindgreen (2016)

annotated bacterial and archaeal 16S rRNA sequences, fungal 28S rRNA sequences, and a suite of analysis tools to the scientific community.

RDP is an online tool which is used to study the new fungal 28S rRNA sequence collection. RDP tools are now freely available in packages for users to incorporate in their local workflow (Cole et al. 2009). SILVA (from Latin *silva*) is an online freely accessible tool to check the quality of reads and aligned (16S/18S, small subunit ribosomal RNA) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) sequence data of bacteria, archaea, and eukarya (Quast et al. 2013). Real Time Metagenomics is an online freely available tool which performs annotation of metagenomes by relating the individual sequence reads with a database of known sequences and assigning a unique function to each read. They generated a novel approach to annotate metagenomes using unique k-mer oligopeptide sequences from 7 to 12 amino acids long (Edwards et al. 2012).

4.5 Bioinformatics-Based Data Analysis

Bioinformatics-based data analysis can be done using short reads and assembled contigs present in the short read archive (SRA) format (Fig. 4.3). The metagenomics SRA data is firstly treated to sort out high-quality reads or sequences. The pretreatment includes:

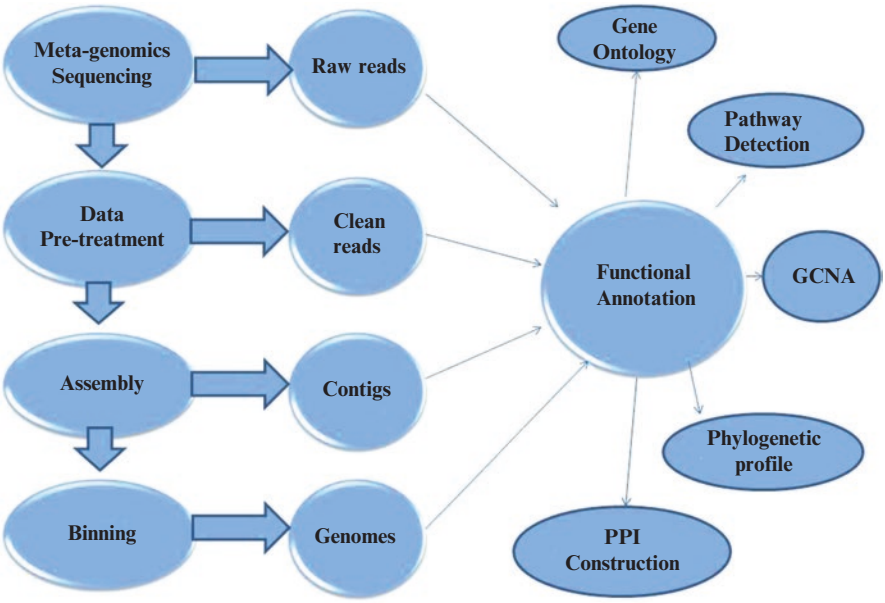

Fig. 4.3 Flowchart for analysis of data generated by different metagenomics experiments. The procedure involves use of several computational biology tools for retrieving functional information in terms of pathway, interaction network, and gene ontology hidden in the metagenomics data. GCNA (gene co-expression network analysis) and PPI (protein-protein interaction network) studies are useful for identification of interactors

(a) Removal of adapters and linkers
(b) Removal of duplicate sequences (dereplication)
(c) Quality assessment

Before pretreatment of data, quality of data is checked by checking base quality, GC content, sequence dereplication levels, and adapter content using FastQC. Quality control of metagenomics data is done by RSeQC (quality control of RNA-seq experiments) followed by RNA-SeQC (Wang et al. 2012; De Luca et al. 2012). Once data become clean, then it can be used for functional annotation. After pretreatment of data, assembling of reads is done for getting the functional contigs. Data size generated after sequencing can be reduced by metagenome assembly by using integrated computational approach (Howe et al. 2014).

Reference-based and de novo-based methods are used for assembling the reads. The previous one is used to align the short reads against the related genome, while the latter one is used to find out the novelty in genes against the similar reference genome. It requires a large memory and high computational methods. Once assembling is done, binning is performed. It is a computational process of clustering or assigning the contigs that may represent individual genome/taxon or closely related microbes. Homology-based tools are used to perform the binning, i.e., MetaPhlAn2, MetaPhyler, and CARMA (Segata et al. 2012; Liu et al. 2010a, b; Gerlach and Stoye 2011). Day by day, technology is improving which leads to reduction in sequencing cost; hence researchers can access the environmental

metagenome, and bioinformatics tools can be integrated with metagenome data to produce useful results and findings (Albertsen et al. 2013).

Structural and functional annotation of microbial community can be done by using assembled reads and unassembled reads too. It is well proven that unassembled short reads contain original information that can explain about functional genes, metabolic profile, and quantitative composition of microbial taxa (Davit Bzhalava and Joakim Dillner 2013).

4.6 Conclusion

Metagenomics is a continuously increasing and developing field. Modern tools and techniques like bioinformatics, NGS technology, and data analysis methods are proving to be facilitators of the trending research field. Biological data is continuously increasing its size; hence researchers have golden opportunity to solve or retrieve the hidden information present in assembled or unassembled reads using modern analytical tools more efficiently. Direct DNA sequencing of environmental samples has given opportunity to gather information about the microorganisms that were unexplored so far. Screening of useful bacteria that survive in extreme environmental conditions, heavily polluted soil, disease-affected tissues or cells, oil-contaminated water bodies, heavy metal-contaminated fields, etc. can be done easily by combining environmental and metagenomics approaches. The data obtained from environmental sample sequencing may be of great use in discovery of new drugs and antibiotics, new bacterial species, plant growth promoters, bioremediation, as well as many other industrial applications. This article presents a detailed account of applications of metagenomics especially in the field of environmental biotechnology with special focus on methods and tools useful in sample collection, sequencing, and analyzing the metagenomics data.

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Chapter 5
Biodegradation of Textile Azo Dyes

Veena Sreedharan and Kokati Venkata Bhaskara Rao

Contents

Abstract Azo dyes are known as industrially synthesized organic compounds, and these azo dyes are identified by their azo bonds (N=N). Mixtures of these synthetic dyes which are unbound to the fiber get released into the environment and that will ultimately lead to bioaccumulation. Bioaccumulation of these dyes constitutes a serious environmental hazard. Several physicochemical methods have been applied to the treatment of textile wastewater, but these methods have many limitations due to high cost, low efficiency, and secondary pollution problems. As an alternative to physicochemical methods, biological methods comprise bacteria, fungi, yeast, algae, and plants and their enzymes which received increasing interest due to their cost-effectiveness and eco-friendly nature.

Decolorization of toxic azo dyes by biological processes may take place either by biodegradation or biosorption. A variety of oxidative and reductive microbial enzymes may also be involved in the degradation of dyes. Azoreductase, peroxidase, laccase, and other important enzymes synthesized by these microbes have

V. Sreedharan · K. V. Bhaskara Rao (✉)
Molecular and Microbiology Research Laboratory, Department of Biomedical Sciences, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_5

shown 80–90% efficacy in decolorizing the textile dyes. Green synthesis of nanoparticles and their mediated azo dye degradation are the latest and effective methods used for treatment of hazards effluent samples. Toxicity evaluation of pure dyes and degraded dye product using phytotoxicity and biotoxicity study is given a clear chart of the most effective methods This review provides an overview of decolorization and degradation of azo dyes by biological processes and establishes the fact that these Microbes and enzymes are significantly effective biological weapons against the toxic azo dyes.

5.1 An Introduction on History and Discovery of Dyes

The history of dye begins in 2600 BC, according to the earliest written record, with the use of dye stuffs in China. During that time these dyes were originally obtained from animal and vegetable sources. Also, Egyptian mummies were found to be wrapped with red-dyed clothes made of madder plants. The Egyptians commonly dye clothes using plant dyes and natural earth dyes. They also had very good knowledge of attaching the dye to the fabrics. Anthraquinone dye requires a metallic salt to impart color to the fabrics, and it is believed that to accomplish this, Egyptians used the salt alum (Nicholson and Shaw 2000). The majority of dyes that are used in the present world are chemically synthesized, and origin of these dyes can be traced back to organic chemistry. W.H. Perkin (1856) who is known as the "father of dye industry" accidentally discovered mauveine dye while trying to synthesize quinine, an antimalarial drug (Tyagi and Yadav 2001). Synthesis of dye is a very complex process. Distillate aromatic molecules should undergo reduction, oxidation, condensation, and nitration. Bismarck brown dye was the first commercial azo dye obtained by diazotization, and most of the dye used today is obtained through the same procedure. Based on the different chemical structures, dyes are divided into different classes. Azo dyes are the largest class of dye compounds since among the 100,000 existing dyes, more than 2000 dyes belong to azo dye group (Stolz 2001; Vijaykumar et al. 2007). Azo dyes are the most commercially important and extensively studied ones; few of those are shown in Figs. 5.1 and 5.2. This is because of the superior properties that are found in these dye classes when compared to other dyes. The chemical structure that is found in azo dyes and the bond that is responsible for the nondegradable property of these dyes are R-N=N-R. Azo dyes can be synthesized easily and can attach well to the fabrics and will not fade easily (Jeong 2008). Based on the number of N=N, azo dyes are classified as mono azo dyes, diazo dyes, triazo dyes, and polyazo dyes. Degradation of azo dyes is a very difficult process due to the presence of N=N. A total number of azo bonds, functional groups, and their arrangements greatly influence its degradation capacity (Rani et al. 2009; Grekova et al. 2012).

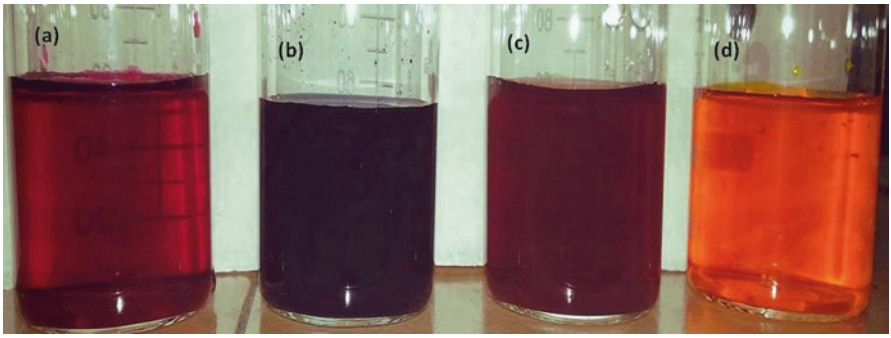

Fig. 5.1 Four different azo dyes which are used in almost all textile industries of India: (**a**) Reactive Red 195A, (**b**) Reactive Blue 198, (**c**) Reactive Brown F3B, (**d**) Reactive Yellow 145

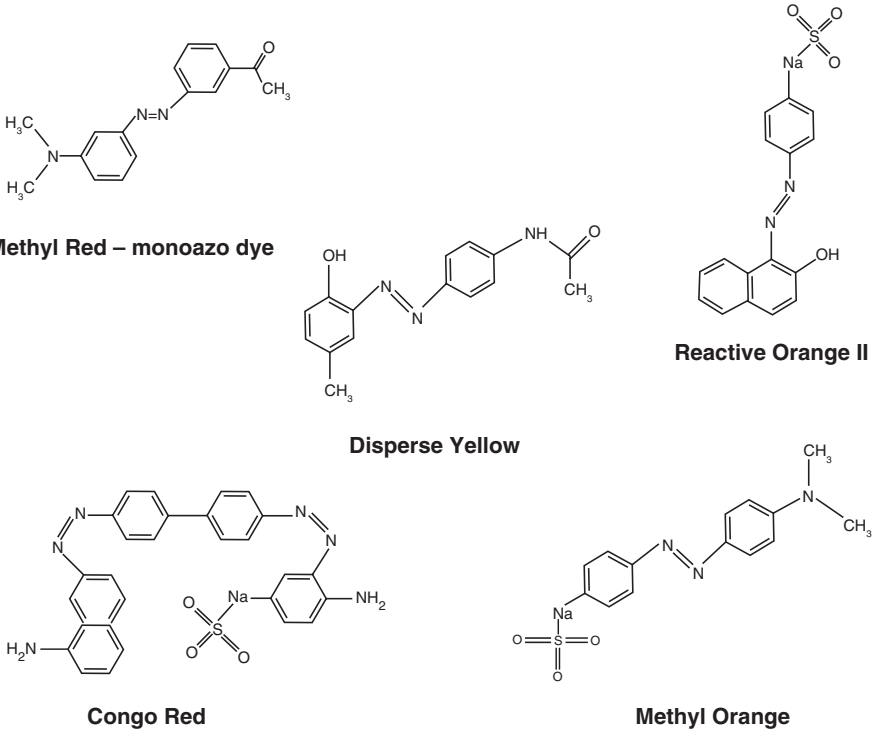

Fig. 5.2 Structure of toxic azo dyes Methyl Red, Disperse Yellow, Reactive Orange II, Congo Red, and Methyl Orange. (Sudha et al. 2014)

5.1.1 Impact of Azo Dyes

Increasing urbanization, globalization, and industrialization have caused different types of environmental pollution. Among various industries, the textile industries discharge large volume of wastewater after dyeing process. As azo dyes have poor exhaustion properties, the remaining unbounded dye particles to the fiber get released into the environment and lead to bioaccumulation (Zolinger 1987). Among all available synthetic dyes, azo dyes are the largest class of dyes with the wide range of colors and structures, and it represents a major portion of the total dyes used in textile industries (Lang et al. 2013). In every textile industry, to dye 1 kg of fabric, 40–60 g of dyestuff is required, and after the dyeing process, approximately 15–20% of dye remains in the effluent (Baban et al. 2003; Babu et al. 2007). This effluent then becomes a highly toxic solution with toxic chemicals like reactive dyes and azo dyes (N=N). Toxic effluents after discharge into the environment cause adverse effects on the fertility of soil, plants, animals, aquatic organisms, and human beings (Mester and Tien 2000; Puvaneswari et al. 2006; Solıs et al. 2012; Saratale et al. 2013). Phytoplanktons present in the environment show abnormal coloration and reduction in the photosynthesis ability due to the absorbance of light by these dyes that enters the water ecosystem (Duran and Esposito 2000; Mester and Tien 2000). This also affects the pH, biochemical oxygen demand, and chemical oxygen demands and provided intense coloration in water and hence decreases the quality of water. The presence of these toxic and unnatural colors in water is aesthetically unpleasant and shows the presence of contamination in water. These dyes and their contamination will remain in the environment for longer period of time if not treated adequately (Olukanni et al. 2006). So far many physicochemical and biological methods were adapted for the removal of these toxic dyes. Some of those methods are found to be effective but also showed many negative side effects, and few are very expensive. Chemical methods used for the degradation of azo dye are found to increase the toxicity in the environment since most of the organic compounds are normally toxic. The advantages and disadvantages of the methods used for the removal of dyes from the textile effluents are summarized in Table 5.1 (Andrea et al. 2005). Azo dyes are mainly used in textile industries, but its applications are also seen in food, pharmaceutical, paper, cosmetics, and leather industries (Saratale et al. 2011). The more we use these toxic dyes, the more our environment will get polluted. Industrial effluent samples consisting of these azo dyes lead to bioaccumulation that causes severe toxic effects on the environment since these N=N make azo dyes highly toxic. Most of the dyes are soluble in water and can be absorbed by skin contact and also inhalation which can lead to allergy, risk of cancer, and skin and eye irritation and cause high toxicity if inhaled or consumed (Nikulina et al. 1995). Para-phenylenediamine is an aromatic amine which is present in almost all dyes and causes skin irritation, chemosis, permanent blindness, and lacrimation. Entry of para-phenylenediamine inside the body causes edema on the neck, tongue, and face and also respiratory distress. These dyes also cause disease like acute tubular necrosis, vomiting gastritis, hypertension, vertigo, urinary bladder cancer, splenic sarcomas, nuclear anomalies, and chromosomal aberrations (Table 5.2). Degradation of

Table 5.1 Advantages and disadvantages of the current physical and chemical methods that are used for the removal of toxic azo dye from industrial effluents (Andrea et al. 2005)

Physical/chemical methods	Advantages	Disadvantages
Fenton's reagent	Effective for both soluble and insoluble dyes	Sludge generation
Ozonation	No alteration of volume since applied in gaseous form	Very short half-life (10 min)
Photochemical	Sludge not produced	Generation of toxic by-products
NaOCl	N=N cleavage	Releases aromatic amine
Cucurbituril	Good for degradation of many dyes	Highly expensive
Electrochemical destruction	Breaks down compounds into non-hazardous products	Requires high cost for electricity generation
Activated carbon	Removes wide variety of dyes	Highly expensive
Wood chips	Good for acid dyes	Requires very long retention time
Membrane filtration	Removes almost all types of dyes	Sludge production is very high in a very concentrated form
Electrokinetic coagulation	Economically feasible	Very high sludge production
Irradiation	Effective oxidation reaction in lab	Required a lot of dissolved oxygen
Ion exchange	No adsorbent loss	Not effective for all azo dyes
Silica gel	Effective only for the removal of basic dye	Commercially can't be used due to side reactions
Peat	Good adsorbent effect due to cellular structure	The specific surface is available for adsorption and is very less than activated carbon

Table 5.2 Different azo dyes and their severe effects reported on humans and animals

Name of the dye	Effects	References
Reactive brilliant red	Function of human serum albumin is inhibited	Li et al. (2010)
Acid Violet 7	Acetylcholinesterase in mice, lipid peroxidation, chromosomal aberration	Ben Mansour et al. (2010)
Disperse Red-1	Affects human lymphocytes – increases the frequency of micronuclei	Chequer et al. (2009)
Direct Black 38	Cancer of the urinary bladder	Cerniglia et al. (1982)
Direct Blue 15	Mutagenic	Reid et al. (1984)
Disperse Blue 291	DNA fragmentation in hepatoma cells; mutagenic, cytotoxic, and genotypic effects	Tsuboy et al. (2007)
Reactive Black 5	Decreases urease activity and ammonification of arginine rate in terrestrial ecosystem	Topac et al. (2009)

azo dyes is a bioremediation process which will remove toxicity from the environment. Therefore there is an urgent need for their removal and to reduce its toxicity before discharge of the waste effluent into the environment (Ayed et al. 2011). Research has been initiated in the field of biodegradation of azo dyes, i.e., azo dye degradation using microorganisms. Microbial degradation of azo dyes will also depend on the microbes such as bacteria, fungi, actinobacteria, bacterial consortium, and yeast and also on the culture condition provided. Biodegradation of azo dye is an easy, effective, and eco-friendly approach for the degradation and removal of toxic azo compounds from the environment. This review summarizes the recent achievements and methods that are used for the degradation of toxic azo dyes and also discusses the toxicity of degraded compounds and future perspective on the degradation of textile azo dyes.

5.2 Biodegradation of Azo Dye

Physical and chemical methods available for the removal of azo dyes include coagulation, precipitation, adsorption, flotation, flocculation, mineralization, and electrochemical destruction (Gogate and Pandit 2004). Mentioned techniques have many disadvantages such as high cost, release of the residue, time, and also inability to reduce the toxicity of degraded compounds (Copper 1993; Maier et al. 2004). Moreover these techniques will only minimize the toxicity level and not be able to completely remove the toxicity of the dyes (Copper 1993; Maier et al. 2004). To replace these techniques, microbial degradation methods can be used which show complete degradation of azo dyes and also detoxify the toxic compounds (Pandey et al. 2007). Biological treatment of textile effluents is an eco-friendly approach, and it is also gaining much importance in today's scenario. Microorganisms are very active in reducing azo dyes by secreting different enzymes like azoreductase, laccases, peroxidase, and hydrogenase. These reduced compounds are then broken down into smaller compounds which are then utilized as their energy source (Stozl 2001). The location of these reactions may be either intracellular or extracellular sites (Fig. 5.3). According to the available literature, microbes are more active under

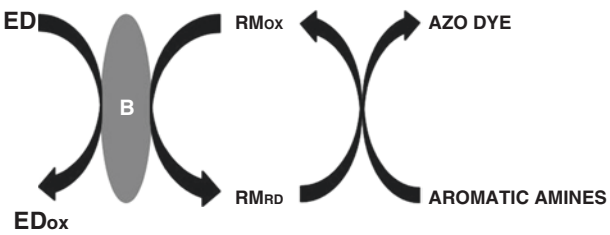

Fig. 5.3 Biological method mechanism behind degradation of reactive azo dyes using bacteria (*RM* redox mediator, *ED* electron donor, *B* bacteria)

combined effect of aerobic and anaerobic conditions (Waleed and Muhammad 2014). Almost all microorganisms are capable of degrading azo dyes including bacteria, yeast, actinomycetes, fungi, algae, and consortium of these microbes (Table 5.3). All these microorganisms have developed special enzyme systems for the discoloration and degradation of azo dyes under certain environmental conditions (Anjali et al. 2007).

5.2.1 Degradation Using Bacteria

Degradation of azo dyes using bacteria is normally nonspecific and faster (Sudha et al. 2014). Many aerobic and anaerobic bacteria such as *Staphylococcus* sp., *Enterococcus* sp., *Bacillus subtilis*, *Rhabdobacter* sp., *Xenophilus* sp., *Clostridium* sp., *Klebsiella* sp., *Acinetobacter* sp., and *Pseudomonas* sp. have been reported in many studies for the degradation of toxic azo dyes (Olukanni et al. 2006; Vijaykumar et al. 2007; Lin and Leu 2008). Most of the bacteria produces azoreductase enzyme for the degradation of N=N, and few bacteria show their activity in the presence of specific carbon and nitrogen sources (Caughlin et al. 2002). Bacterial degradation,

Table 5.3 Studies reported on degradation of azo dyes using different microbes such as bacteria, fungus, yeast, algae, and actinobacteria

Strain	Organisms	Dye	References
Bacteria	*Enterococcus faecalis*	Reactive Orange II	Subramani et al. (2007)
	Enterobacter sp.	Reactive Red 195	Kalyani et al. (2007)
	Bacillus subtilis	Acid Blue 113	Gurulakshmi et al. (2008)
	Brevibacillus laterosporus	Navy Blue 3G	Jirasripongpun et al. (2007)
	Enterobacter agglomerans	Methyl Red	Keharia and Madamwar (2003)
	Bacillus fusiformis	Acid Orange	Kolekar et al. (2008)
Fungus	*Geotrichum* sp.	Reactive Black 5, Reactive Yellow 27	Kuhad et al. (2004)
	Aspergillus ochraceus	Reactive Blue 25	Parshetti et al. (2006)
	Shewanella sp.	Acid Red 89	Chen et al. (2003)
	Phanerochaete chrysosporium	Reactive Orange II	Sharma et al. (2009)
Yeast	*Saccharomyces cerevisiae*	Methyl Red	Jadhav and Govindwar (2007)
	Kluyveromyces marxianus	Remazol Black B	Meehan et al. (2000)
Algae	*Cosmarium* sp.	Malachite Green	Daneshvar et al. (2007)
	Spirogyra rhizopus	Acid Red 247	Ozer et al. (2006)
Actinomycetes	*Streptomyces ipomoeae*	Reactive Orange II	Molina et al. (2009)

Table 5.4 Percentage decolorization of different toxic azo dyes using bacterial strains isolated from industrial effluent

Dye	Decolorization (%)	References
Golden Yellow HER, Orange 2R, Orange M2M	89	Kolekar et al. (2013)
Evans Blue and Brilliant Green	87	Zolgharnein et al. (2014)
Mixture of dyes	80	Saratale et al. (2013)
Amaranth, Acid Orange 52, Direct Blue 72	100	Liu et al. (2013)
Remazol Red, Direct Red 2B, Malachite Green	100	Kabra et al. (2013)
Acid Orange 7 and Acid Red 88	47	Vasconcelos et al. (2012)
Mixture of dyes	87	Naik and Singh (2012)
Reactive Orange P3R, Yellow P3R, Reactive Black V3R and Reactive Brown P5R	64.89	Jayan et al. (2011)
Rubine GFL, Reactive Brown 3REL	72.88	Kurade et al. (2011)
Mixture of Navy Blue RX, Golden Yellow HER, Direct Blue GLL, Reactive Red HE8B	61.4	Tamboli et al. (2010)
Eight textile dyes	89	Joshi et al. (2010)

when done individually under the aerobic or anaerobic condition, will show only the degradation, but there will not be any mineralization. Many researchers have experimentally proved that the combined effect or aerobic and anaerobic treatment methods can be an effective method (Feigel and Knackmuss 1993; Chen et al. 2003). Many reports are available on degradation of mixture of azo dyes using bacteria (Table 5.4). Kolekar and Kisan (2013) reported degradation of mixture of textile dyes using *Shewanella* sp. strain KMK6 isolated from soil sample contaminated with dyes. This study using bacteria showed a decrease in the color of the mixture of dye and chemical oxygen demand. Also the toxic mixture got converted into nontoxic degraded product. In another study, 87% of degradation in the mixture of dyes was observed using novel bacterial strain *Lysinibacillus* sp. RGS with a reduction within 48 h (Saratale et al. 2013). In a recent report, two bacterial isolates *Bacillus* sp. and *Aeromonas hydrophila* isolated from textile mill effluent showed more than 90% of Reactive Green and provisional pink dye within 5 days with a dye concentration of 50 mg/L (Parimala and Suruthi 2016). Few other strains of bacteria like *Pseudomonas fluorescens* and *Shewanella* have also been reported for degradation of azo dyes (Liu et al. 2013; Godlewska et al. 2014). In another study plant and bacterial synergistic systems were used for treatment of textile effluents, and their consortium was used for the degradation of mixture of dyes. This treatment method showed 100% degradation for the mixture of dyes (Kabra

et al. 2013). Anoxic culture of *Aeromonas hydrophila* was isolated and selected as dye degrading bacteria at a pH of 5.5–10 and at an optimum temperature of 20–30 °C (Naik and Singh 2012). Degradation parameter of textile effluent showed color and chemical oxygen demand removal when treated with culture of *Bacillus subtilis* (Jayan et al. 2011). Mixture of seven different dyes with different chemical structures showed 87% of degradation using *B. laterosporus* within 24 h when provided an optimum temperature of 40 °C. This study also came up with a very less toxic end product (Kurade et al. 2011). Biodegradation of selected dyes Reactive Black 5, Reactive Orange 16, Disperse Red 78, and Direct Red 81 was reported using bacterial isolates *Providencia rettgeri* and *Pseudomonas* sp. In this study, both isolates showed 97–99% degradation of all dyes within 30 h at a concentration of 100 mg/L (Harshad et al. 2014). It has been reported that mixed culture of bacteria can give better results when compared with the results shown by individual isolates.

5.2.1.1 Degradation Using Bacterial Consortium

Many bacterial isolates showed good azo dye degradation when applied together as a consortium rather than individually. Many reports are available on the dye degrading assays using bacterial consortium. Nigam et al. (1996) reported for the first time the combined ability of *M. luteus*, *Micrococcus* sp., and *P. polymyxa* in the degradation of azo dyes but individually showed no degradation. Similar work was carried by Moosi et al. (2007) using the same three isolates isolated from contaminated sites. A consortium of four bacterial isolates such as *P. putida*, *P. fluorescens*, *B. cereus*, and *S. acidaminiphila* showed degradation of Acid Red 88 within 24 h, but when inoculated individually, each isolate took more than 72 h for degradation (Khehra et al. 2005). In an another study, the effect of isolates *Klebsiella* sp., *Bacillus* sp., and *Clostridium* sp. showed good degradation ability under aerobic condition, while the same consortium showed no change under anaerobic condition (Cui et al. 2012). The fungal and bacterial consortium also plays a very good role and shows the high effect in azo dye degradation. *Aspergillus* sp. and *Pseudomonas* sp. together detoxified Rubine dye within 30 h (Lade et al. 2012). The same consortium showed very promising results by degrading 98% of textile effluents which consist of reactive dyes, disperse azo dyes, and sulfate within 35 h (Lade et al. 2012).

5.2.2 Degradation Using Fungi

A wide variety of fungal organisms are capable of decolorizing a wide range of textile azo dyes. Many of these fungi are employed either in living or inactive forms. Degradation of azo dyes using fungi has an advantage as it is cost-effective and

production of sludge is very less and environmentally friendly. Fungi possess a strong ability to degrade complex organic molecules by producing extracellular enzymes such as laccase and lignin peroxidase; hence researchers are paying more attention toward fungi-mediated dye degradation (Sudip et al. 2016). The mechanism of fungal degradation involves adsorption and enzymatic degradation or combination of both. Recently Wang et al. (2017) reported decolorization and degradation of Congo Red using *Ceriporia lacerata* a newly isolated white rot fungus isolated from decayed mulberry branches. This study showed 90% degradation of Congo Red dye with 48 h when 3 g of mycelia was inoculated in 20 mL of 0.1 mg/mL concentration of Congo Red solution. In another study two endophytic fungi, *Phlebia* sp. and *Paecilomyces formosus*, showed decolorization of Reactive Blue 19 and Reactive Black 5. Both isolates showed degradation activity with 0.1 g/ml of dye solution after 30 days (Ligia et al. 2017). Anand et al. (2017) reported biodegradation of Malachite Green using *Aspergillus flavus*. *Aspergillus flavus* showed complete degradation of 150 mg/L of dye solution within 8 days in Kirk's medium under static condition in the presence of sucrose and sodium nitrate as effective carbon and nitrogen sources, respectively. Table 5.5 depicts some of the different dye mixtures decolorized by fungal degradation.

Table 5.5 Percentage decolorization of different toxic azo dyes using fungal strains isolated from industrial effluent

Dye	Decolorization (%)	References
Azo anthraquinone dye mixture	74.93	Taha et al. (2014)
Brilliant Green and Diazo dye	80	Przystas et al. (2013)
Yellow FG, Red 3BS, Orange 3R, Blue RSP, Remazol Turquoise Blue	82	Idris et al. (2014)
Reactive Red dyes (Red, Black, and Orange)	88	Ambrosio et al. (2012)
Remazol Red, Golden Yellow HER, Rubine GFR, Scarlet RR	88	Waghmode et al. (2011)
Direct Red 80 and Mordant Blue 9	77–97	Pakshirajan and Singh (2010)
Reactive Blue 21, Reactive Black 5, Reactive Orange 13	60–66	Nordstrom et al. (2008)
Remazol Brilliant Orange, Procion Yellow, Cibacron Black 55, Drimaren Brilliant red 67	80–90	Machado et al. (2006)
Mixture of four reactive textile dyes, Azo and Anthraquinone dye	90	Harazono and Nakamura (2005)
Orange, Reactive Black, Reactive Red	88	Ambrosio and Takaki (2004)
Procion Orange MX2R, Remazol Red 3B, Remazol Black GF	97	Amaral et al. (2004)

5.2.3 *Degradation Using Yeast*

The growth rate of filamentous fungi is normally slow when compared with yeast; hence yeasts have an advantage over fungi from a biotechnological view for degradation of azo dyes. Yeast is a resilient microbe and is able to resist different environmental conditions like pH, organic wastewater, and high salt concentration. According to our literature survey, the first study presenting degradation of azo dyes by breaking N=N was published by Mecke and Schmahl (1957). However, this subject was actually brought into action after several years (Olteanu et al. 2008). Many reports available on yeast-mediated degradation are using *Candida curvata* and *Geotrichum candidum* with 90% and above degradation effect. *Kluyveromyces marxianus* showed the removal of diazo dye Remazol Black with 89% of degradation (Ertugrul et al. 2009). Similarly *Candida catenulata* and *Candida kefyr* degraded 90% of amaranth dye using biosorption techniques (Zeroual et al. 2007). *S. cerevisiae* and *C. tropicalis* are very active yeast isolates with the capacity of degrading more than one azo dye including Remazol Blue, Reactive Black, and Reactive Red. The action of these strains changes according to the dye concentration and exposure time (Aksu 2013; Donmez 2012). In a recent study, 12 out of the 44 isolated yeast colonies showed degradation; Reactive brilliant red K2 and those isolates were identified as *S. cerevisiae*, *Torulopsis candida*, and *Saccharomycopsis lipolytica.* Hence this feasible and metabolically versatile yeast should be considered for bioremediation process since a majority of yeast species have never been studied for azo dye degradation process.

5.3 Enzyme Involved in the Degradation of Azo Dyes

A number of microorganisms have been reported for the degradation of reactive azo dyes which include bacteria, yeast, fungi, and consortium of microorganisms and plants (Wesenberg et al. 2003; Olukanni et al. 2006). All these microbes have developed special enzyme systems for the degradation and discoloration of toxic azo dyes under suitable environmental conditions (Anjali et al. 2006). Although azo dyes have highly complex structural variations, they are degraded by a selected number of enzymes. Dye degrading enzymes are redox-active molecules which require a specific substrate for their action (Duran and Esposito 2000; Mester and Tien 2000). Microbes can either excrete the active enzymes into the used medium or the dye molecules move inside the microbial cell. Active enzymes are also potential in reducing or removing the toxicity from the dyes and effluents. The degrading capacity of microbes gets decreased by an increase in the concentration of dyes due to the microbial growth inhibition caused by the target molecules. To overcome this problem, we can extract the dye degrading enzymes from active microbes in bulk, and those enzymes can be used directly (Joshin and Chacko 2011). There are many reports on biologically synthesized dye degrading enzymes.

Peroxidase, azoreductase, and laccases are the major and most promising enzymes involved in azo dye degradation (Abadulla et al. 2000). Azoreductase is a major and the most important group of enzyme synthesized from bacteria and fungi. The mechanism of these enzymes is reductive cleavage of azo bonds and converting them into colorless aromatic amines (Pandey et al. 2007). Figure 5.4 shows the proposed mechanism for the degradation of azo dyes using azoreductase under anaerobic condition. In intracellular and extracellular sites of the bacterial cell wall, the reducing molecules such as NADH, NADPH, and $FADH_2$ help in the breaking of N=N (Zimmermann et al. 1982 and Zimmermann et al. 1984), while azoreductase plays a major role in the degradation process of bacteria, viz., *Escherichia coli*,

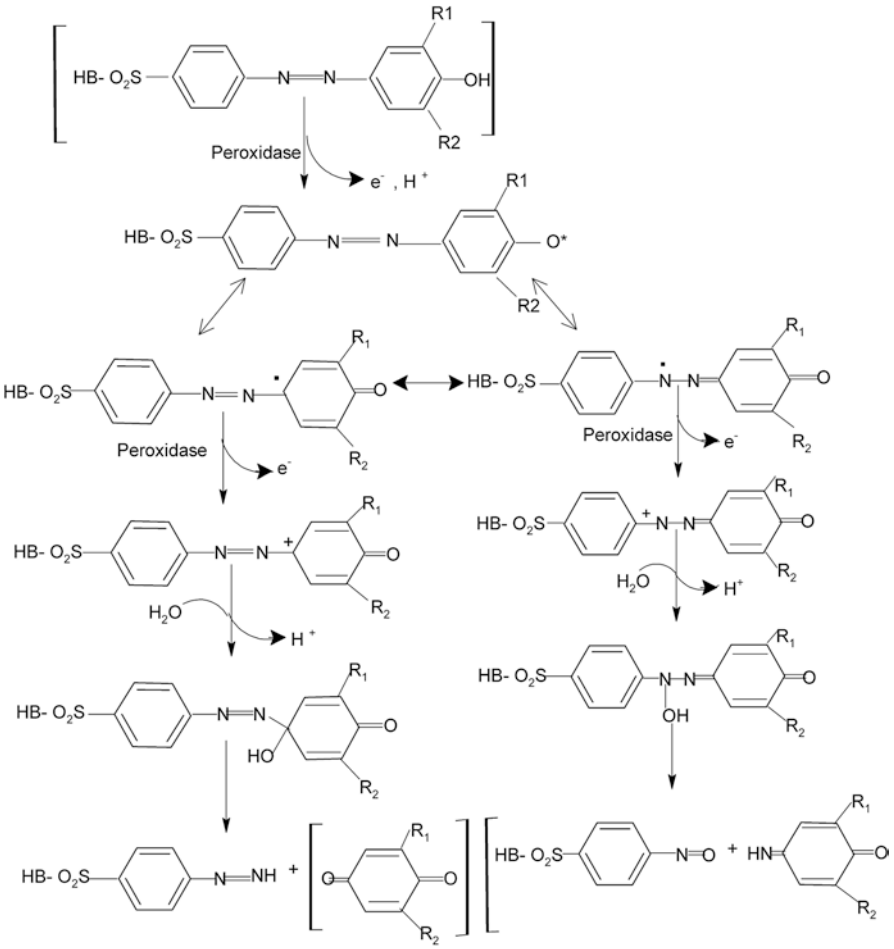

Fig. 5.4 Proposed mechanism for alternative asymmetrical and symmetrical cleavage of sulfonated azo dye by peroxidase enzymes generated from fungi. (Courtesy: McMullan et al. 2001)

Staphylococcus aureus, *Rhodobacter sphaeroides*, *Enterococcus faecalis*, and *Bacillus* sp. (Blumel and Stolz 2003, Yan et al. 2004 and Chen et al. 2005). Azoreductase enzyme extracted from *E. faecalis* YZ66 was able to degrade sulfonated azo dye Direct Red 18 and also detoxified their toxic effect (Sahasrabudhe et al. 2014). On the other hand, degradation of azo dyes using fungi generates two types of enzymes that are peroxidases and phenol oxidase (Ramya et al. 2010). Peroxidase enzymes are catalyzed in the presence of hydrogen peroxide (Fig. 5.5). These heme peroxidases are divided into different groups based on the organisms produced, substrate, and primary structure (Gumiero et al. 2010). The oxidative process of H_2O_2 which is catalyzed by chloroperoxidase was used for the degradation of azo dyes such as Orange G and S Yellow (Zhang et al. 2012). Lignin peroxidase enzyme isolated from *Tagetes patula* for the degradation of Reactive Blue 160 was reported by Patil and Jadha (2013). Another major enzyme that helps in the degradation of azo dyes is laccase enzyme (Fig. 5.6). These enzymes are also known as multicopper oxidase enzymes (MCO) as it belongs to the family of copper-containing polyphenol oxidases (Birhanli and Yesilada 2006; Arora and Sharma 2010; Giardina et al. 2010). Bertrand (1985) discovered laccase from the sap of a tree, *Rhus vernicifera*. Husain (2006) reported for the first time the importance of laccase enzyme in the degradation of textile color effluent. The major property of this enzyme that makes it a best azo dye degrading agent is its nonspecific oxidation capacity, a non-requirement of cofactors, and they do not require oxygen as an

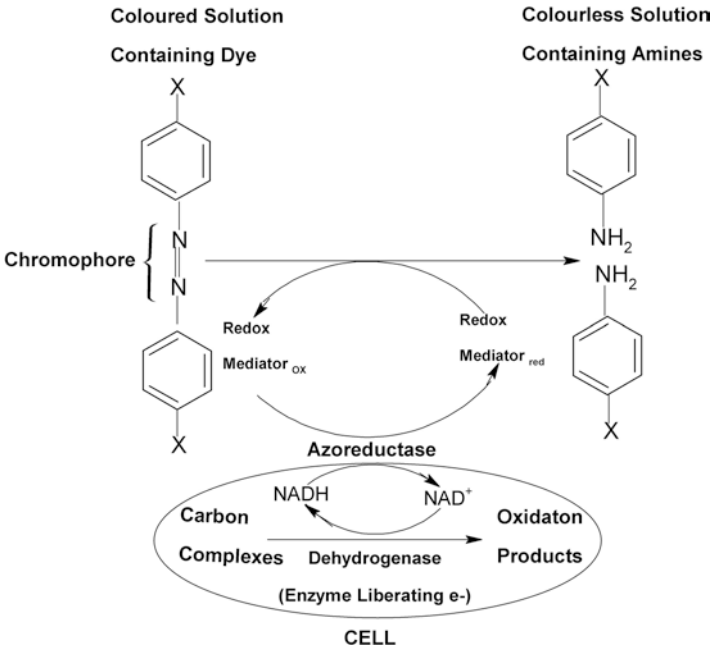

Fig. 5.5 Proposed mechanism for the degradation of azo dyes by azoreductase by converting toxic chromophore group N=N into nontoxic NH_2. (Courtesy: Keck et al. 1997)

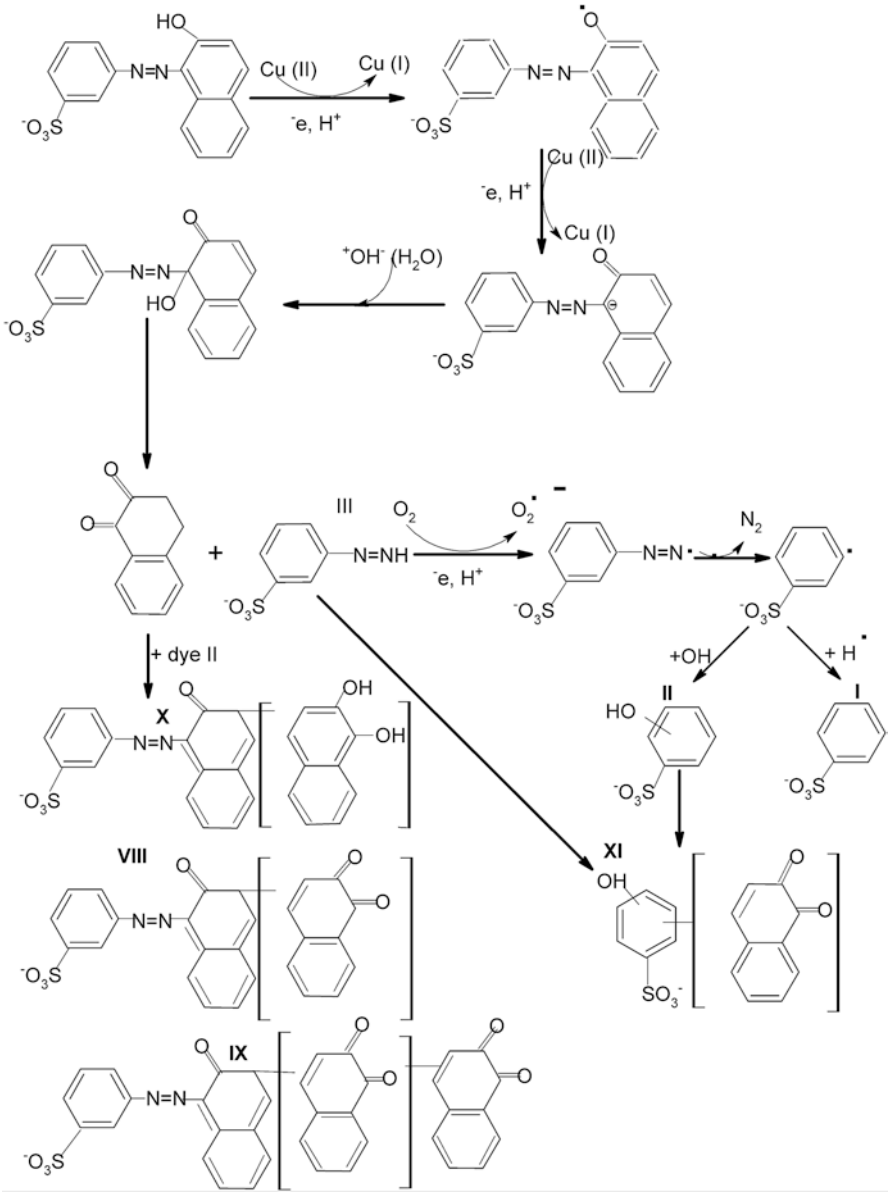

Fig. 5.6 Proposed mechanism of degradation using laccase enzyme, another major enzyme that helps in the degradation of azo dyes. (Courtesy: Andrea et al. 2005)

electron acceptor (Kalyani et al. 2012). In a recent study, laccase enzyme synthesized from white rot fungus, *Ganoderma lucidum* BCR 36123, showed 90% and above degradation azo dye Acid Orange AO7 (Chin et al. 2017). Purified laccases from mushroom *Hypsizygus ulmarius* showed degradation of azo dye Methyl Orange without using any redox mediator (Ravikumar et al. 2013). Enzymatic degradation of azo dyes also has significant potential to solve this problem due to their eco-friendly, inexpensive nature and also due to less production of sludges. Enzymatic processes are very promising for the degradation of toxic azo dyes; hence these enzymes can be considered as a molecular weapon for bioremediation of these dyes.

5.4 Nanoparticle-Mediated Photocatalytic Degradation

Photocatalytic degradation involves acceleration of a photoreaction in the presence of a catalyst. Light energy is absorbed by a provided semiconducting material which helps in the degradation of dyes. The concept of photocatalytic degradation using TiO_2 as a substrate for water decomposition was brought by Akira and Honda (1972), which is known as *Honda-Fujishima effect*. One of the advanced methods that are used for degradation of azo dyes is photocatalytic degradation (Zhao and Zhang 2008). The concept of nanoparticle-mediated degradation is very simple: the semiconducting materials absorb light of equal or more energy that will lead to the generation of electrons. These electrons can then further generate free radicals for the oxidation of substrates (organic matters). In many previous studies, this method has been broadly and highly explored (Yizhong 2000; Loannis and Triantafyllos 2004). Andrea et al. (2014) reported UV-induced degradation of Methyl Red and Methyl Orange azo dyes in the presence of TiO_2 nanoparticles which was immobilized at the bottom of the effluent passing channel. The concept of azo dye degradation using nanoparticles and photocatalysis is related to each other. Thermally active zinc oxide was used in a study for photocatalytic degradation of Congo Red, where the system showed 96% of degradation (Tapas and Naba 2014). In a recent report, ferric oxide Gallic nanostructures were used for the degradation of azo dyes. The nanostructure synthesized by two different ways was subjected to photocatalysis, and degradation percentage was compared (Minoo and Ali 2016). All these studies used chemically synthesized nanoparticles for azo dye degradation. Although these methods reduce the toxicity by a certain extent by breaking the azo bond, they can sustain moderate toxicity which may be due to the presence of nanoparticles. However, the toxic effect is less when compared with the chemical methods for effluent treatment. To overcome the abovementioned problem, researchers started green synthesis of nanoparticles and used them in azo dye degradation. Priyaragini et al. (2014) showed 84% and 85% degradation of Acid Red 79 and Acid Red 80, respectively, using marine actinobacterial-mediated TiO_2 nanoparticles. Photocatalytic degradation of Rhodamine Blue in the presence of actinobacterial-mediated TiO_2 nanoparticles showed 95% and above degradation

effect (Veena et al. 2016). Similarly in another study, UV and solar photocatalytic degradation of azo dyes and dye effluents in different time intervals was tested using the crude extract of coconut-mediated silver nanoparticles (Mariselvam et al. 2016). Photocatalytic degradation of azo dye can be considered as an easy approach, however it is time consuming and effective method when compared to all other techniques. Photocatalytic activity was used first for self-cleaning property by Akira Fujishima (1972), and now it is used in the process of cleaning our environment and protects it from becoming toxic for the coming generations. When we talk about nanoparticle-mediated azo dye degradation, we should focus on biologically synthesized nanoparticles rather than going with chemically synthesized nanoparticles, since our purpose is to remove toxicity completely without leaving a single trace of toxic compounds. Also, the green synthesis of nanoparticles will make azo dye degrading product cheaper when compared with chemically synthesized nanoparticles. Hence, it can be concluded that nanoparticle-mediated photocatalytic degradation of azo dye is an easy, economical, fast, and eco-friendly technique.

5.5 Degraded Compounds and Their Toxicity

Degradation and decolorization of azo dyes only are not enough; emphasis should also be given to verify the detoxification of azo dyes as well. The degraded dye should break down into nontoxic compounds. After degradation, analysis researchers should focus whether the highly toxic azo dyes got converted to nontoxic compounds or not, and also the reduction in the toxicity levels can be checked. In this field the first attempt was done by removing the toxicity and mutagenicity of direct red in the presence of *B. velezensis* strain (Bafana et al. 2008). Toxic Remazol Black B was converted into nontoxic derivatives by using *Zinnia angustifolia* and *Exiguobacterium aestuarii*, a plant and bacterial remediation, respectively (Khandare et al. 2012). In another study, toxicity evaluation was done using *Daphnia magna* under microaerophilic process (Harshad et al. 2015). Here complete detoxification of all the selected textile azo dyes was observed. Few reports are available on toxicity removal by a combined effect of ozonation and biofilm reactor. Toxicity of azo dye solution decreased within 2 min when subjected to ozonation, but toxicity increased when kept for longer time. Along with the removal of toxicity, evaluation of toxicity is also a necessary process. Biotoxicity and phytotoxicity assays are two majorly used assays to evaluate the toxicity of degraded compounds. In many reported works, biotoxicity assay was done using brine shrimp eggs, and the test is known as brine shrimp hatchability test. This test is used widely since it uses convenient organisms for the evaluation of toxicity and is a simple and inexpensive method. In a study less toxic nature of degraded dye was evaluated by observing the survival of 50% of brine shrimp eggs at a much higher concentration than that of the azo dyes

(Arun and Bhaskara Rao 2012). In a recent report, toxicity evaluation of degraded azo dye Direct Yellow 4 was reported using phytotoxicity assay. Here phytotoxicity assay showed a considerable decrease in the toxicity of degraded dyes when compared with the pure dye (Shazia et al. 2017). Mutagenicity, cytotoxicity, and phytotoxicity of biodegraded textile effluent using fungal ligninolytic enzyme have been evaluated in a recent report (Muhammad et al. 2016). The cytotoxicity (*Allium cepa*, *Daphnia magna*, and brine shrimp), phytotoxicity (*Triticum aestivum*), and mutagenicity study using Ames test revealed that biodegradation of textile effluent using fungal-mediated enzymes detoxifies the toxic compounds present.

5.6 Future Perspective

Wastewater discharge by textile industries has become a great environmental concern for scientists because of the prevailing hazards in our ecosystem. Accumulation of industrial dyestuffs and dye wastewater not only creates environmental pollution, but it can also lead to medical problems and problems in the exquisiteness of our environment. There should be technically possible and cost-effective treatment methods for the removal of these toxic dyestuffs from the environment since in the present world environment regulations are becoming even stricter. Dye degradation using microbes bears a significant potential in solving these problems since microbes and their products are eco-friendly, inexpensive, and easily available. This review clearly stated the importance of microbial dye degradation, nanoparticle-based degradation, photocatalytic degradation, enzymatic degradation, and toxicity of degraded compounds. As an emerging technique, using microbes and their mediated nanoparticles is an eco-friendly, less expensive, and easy way to degrade toxic dyes and to remove toxicity from our environment. Lab-scale work will be entirely different when it reaches to the industrial level. Microbial degradation of azo dyes should be focused using small-scale effluent treatment fermenter designing which later can be applied to different textile industries to treat these toxic dye-filled effluents. Similarly, all techniques should be studied with a design so that they can be applied at the industrial level. Industries should get involved with universities or research institutes to carry out these lab works to the next level. The enzyme responsible for degradation of these toxic dyes should be produced in a large amount with the help of industries and should be brought in action as soon as possible at least for small-scale textile industries or dyeing units. Also, there's a need to formulate the effective product that can be delivered to remote places. These dyes get concentrated at the end of the food chain and lead to severe medical problems such as tumor, cancer, asthma, nervous disorder, and even death. So to avoid these entire problems and to protect our environment, textile effluents have to be free from toxic azo dyes and its toxicity before it reaches the environment.

5.7 Conclusion

Azo dyes constitute the largest and most versatile class of synthetic dyes used in a variety of industries including textile, pharmaceutical, food and cosmetics industries and represent major components in wastewater from these industrial dyeing processes. The presence of dyes imparts an intense color to effluents which leads to environmental as well as aesthetic problems. Many researchers are working on degradation of azo dyes; however, there is still a need to generate relative performance data on industrial effluents. Hence this review concludes that azo dye degradation is an extremely serious topic to be focused on, and it can be done using microbes, nanoparticles, and photocatalytic methods. As we are aware of the effect of water scarcity in our country, wastewater treatment is an issue that should be taken into consideration. Also, it's our duty to keep our environment clean and to protect our natural resources from all toxic compounds. Azo dye also comes in the list of toxic compounds or environmental pollution-causing products; hence removal of these toxic dyes using all motioned techniques will help in the process of keeping our environment clean and healthy.

Acknowledgment Authors are very thankful to the management of VIT University and CSIR Delhi, India, for supporting this study.

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Chapter 6
Epoxide Hydrolase for the Synthesis of Chiral Drugs

Priya Saini and Dipti Sareen

Contents

P. Saini · D. Sareen (✉)
Department of Biochemistry, Panjab University, Chandigarh, India
e-mail: diptsare@pu.ac.in

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_6

Abstract Since the racemic enantiomers have different physiologic effects, there are strong recommendations by US FDA for the production of chiral drugs, and since then the chiral drug industry has been growing with 15% growth rate projected for the period 2010–2022. For the synthesis of chiral drugs, enantiopure epoxides and diols serve as important precursors. Though several chemo-catalytic strategies have been employed for their production, nowadays due to a rising environmental concern, there is an upsurge in the development of greener technologies for the production of chiral drugs. Thus, biocatalysis appears as a green alternative.

Here, we have reviewed several biocatalysts for the synthesis of enantiopure epoxides and diols. Among them, epoxide hydrolases from microbes have emerged as one of the key catalysts as they are ubiquitously present, do not require any additional nucleophile or cofactors, are stable and have broad substrate spectra. To identify novel epoxide hydrolases, several screening strategies like enrichment and metagenome screening, 16SrRNA sequencing and genome mining have been adopted. With the expansion of publically available genome database, genome mining provides a quicker, cheaper and easier method for epoxide hydrolases identification.

We have also reviewed the assays available, in detail here, to establish the functional state of epoxide hydrolases. There are spectrophotometric methods like 4-(*p*-nitrobenzyl) pyridine assay, adrenaline assay, sodium-metaperiodate assay, *p*-nitrostyrene oxide assay and fluorophotometric assay along with the chromatographic techniques like gas chromatography and high-performance liquid chromatography. The spectrophotometric methods have some limitations as 4-(*p*-nitrobenzyl) pyridine assay is not very accurate at low epoxide conversion ratio; only aromatic epoxide can be detected in sodium-metaperiodate, while fluorogenic assay requires additional screening step with industrially important epox-

ides. The chromatographic method like gas chromatography involves extra step of derivatization of diols before analysis; compounds should be volatile and must not degrade when heated at high temperature, whereas in high-performance liquid chromatography, detectors are non-destructive, and samples do not require any further treatment before analysis. All the studies reviewed here establish epoxide hydrolases as vital green biocatalysts, for the production of chiral pharmaceutical drug intermediates.

6.1 Introduction

Chiral drug production is an expanding subject of the time. Chirality is a property of a molecule in which it cannot be superimposed on its mirror image, for example, left and right hands. The two mirror images of chiral molecules which are non-superimposable are called enantiomers, and enantioselectivity is a property of a reaction where one enantiomer is expressed either exclusively or predominantly over the other (Nguyen et al. 2006).

Chirality has significant effects on the physiological activity of biomolecules, as is evidenced by various examples of the different activities shown by (*R*)- and (*S*)-enantiomers of the racemic drug in a biological system. One enantiomer produces therapeutic effect and the other may have undesirable or even toxic effects or no effect at all in our body. One well known example of a racemic drug which caused birth abnormalities as its side effects is Thalidomide™ (von Moos et al. 2003). Its (*R*)-enantiomer had the desired tranquillizing effect on pregnant women with morning sickness, while its (*S*)-enantiomer had a teratogenic activity leading to foetal abnormalities. Due to the different physiological effects of enantiomers, the importance of a single enantiomer utility is appreciated, and several methods are being adopted to resolve the racemic compounds into optically pure entity which always has advantage over the racemate due to which several chiral drugs are being presented as a single enantiomer for approval. The US FDA has made strong regulations on racemic drugs. Therefore, the pharmaceutical companies are being directed to develop single enantiomeric drugs. Chiral drugs have shown a continuous growth worldwide, and thus, majority of the topmost selling drugs are chiral. In the market, approximately 50% drugs are chiral, and out of these about 50% are still mixtures of the two enantiomers rather than single enantiomer (Hutt 2002). Numerous synthetic chiral intermediates have been widely produced nowadays to meet the increasing demands for chiral pharmaceuticals by various chemo-catalytic and biocatalytic methods (Breuer et al. 2004).

Chiral epoxides and diols are gaining importance as they are being used as intermediates for the preparation of enantiomeric drugs (Peeliwal et al. 2010). The manufacturers are ardent to make available the optically pure drugs to evade the undesirable side effects of the distomer. As a result, it is perceived that chiral enantiomers have pronounced benefit in chiral drug development and they lead to

develop a molecule of low dose and metabolic load and higher efficiency to produce the desired response. Although the decision to use a single enantiomer against a racemic mixture of enantiomers of a specific drug is challenging, it should be made intelligently on the basis of the data from clinical trials and clinical experiences. Therefore, the use of chiral drugs can potentially lead to more simpler and selective pharmacologic profiles, improved therapeutic indices, simpler pharmacokinetics due to different rates of metabolism of enantiomers and decreased drug interactions (Peeliwal et al. 2010).

This chapter provides a comprehensive overview about the industrially important epoxide hydrolase enzymes as green catalysts for the production of enantiopure epoxides and diols. Initially, we have discussed about the various types of chemo-catalytic methods and then focused on the biocatalytic methods especially epoxide hydrolases for the production of enantiopure epoxide and diol. Different methodologies for the mining of epoxide hydrolases were detailed along with their structure, catalytic and resolution mechanisms besides their nomenclature, epoxide hydrolase activity and enantioselectivity detection methods and universal presence of epoxide hydrolases in mammals, plants, insect and microbes.

6.2 Chemo-catalytic Methods for Chiral Epoxide and Diol Synthesis

Epoxides and diols are versatile building blocks for the enantiopure pharmaceuticals owing to their high reactivity with other nucleophilic groups such as halides, nitrogen, oxygen and sulphur. There are many methods available, both chemical and biocatalytic, for the asymmetric synthesis of enantioenriched epoxides and diols. The two major chemical approaches for the synthesis of chiral epoxides are the direct stereospecific epoxidation of alkenes and the hydrolytic kinetic resolution (HKR) of racemic epoxides (Kumar et al. 2007), both of which have been successfully devised by synthetic chemists. HKR is a means of differentiating two enantiomers in a racemic mixture. Two enantiomers react with different reaction rates in a chemical reaction with a chiral catalyst or a reagent where water is used as a nucleophilic agent for ring opening of epoxides which results in enantioenriched sample of the slow-reacting enantiomer. For the synthesis of enantiomerically pure 1,2-diols, numerous chemical methods have been reported (Milo and Neumann 2010). One of the methods is the reduction of α-oxoaldehydes which is the only chemical method, which uses α-and β-oxoaldehydes as preliminary materials for the production of 1,2-diols using $TiCl_3$ (4 equiv)/NH_3 as a catalyst for the reduction to produce racemic phenyl-1,2-ethanediol in 80% yield (Clerici et al. 2002). Asymmetric epoxidation of prochiral allylic alcohols and dihydroxylation (Katsuki and Sharpless 1980; Sharpless 2002), asymmetric epoxidation of unfunctionalized *cis*-substituted alkenes and hydrolytic kinetic resolution by Katsuki–Jacobsen (Irie et al. 1990; Zhang et al. 1990; Jacobsen et al. 1991), epoxidation of *trans*-substituted alkenes by

Shi's method (Tu et al. 1996) and Darzen's enantioselective reaction are the main asymmetric and excellent classical chemical approaches for the synthesis of chiral epoxides and diols (Botes and Mitra 2006; Lin et al. 2011a). Terminal epoxides in enantiomerically pure form are less accessible as more secondary interactions are required between catalyst and substrate in an asymmetric catalysis. They were synthesized later on by Jacobsen's group by HKR using the technology based on (salen) Co(III)(OAc) as catalysts (Tokunaga et al. 1997; Kumar et al. 2007). Using different enantiomers of the (salen) Co(III)(OAc) complex, either enantiomer of the corresponding chiral epoxides with >99% enantiomeric excess (ee) can be obtained through this process (Schaus et al. 2002).

In Sharpless epoxidation, primary allylic alcohols were enantioselectively epoxidized and catalysed by l-(+)/d-(−)-diisopropyl tartrate and titanium tetraisopropoxide, using *tert*-butyl hydroperoxide as the oxidant (Riera and Moreno 2010). Katsuki–Jacobsen asymmetric epoxidation of *cis*-alkenes can be achieved using the chiral Mn (III)-salen catalyst introduced with NaOCl/PhIO as an oxidant in CH_2Cl_2 at room temperature, giving good yields and selectivity. But these direct epoxidations usually suffer from insufficient enantioselectivities (Hamada et al. 1996). These drawbacks were partially overcome by using the Jacobsen's HKR method which has many salient features like the high accessibility and applicability to racemic terminal epoxides, most of which are quite inexpensive, production of enantioenriched products with close to theoretical yields, use of commercial enzymes with low load (0.2–2 mol %) and recyclability at low cost. Here, water is used as the nucleophile for epoxide ring opening and is used as the only reagent without the need for any other solvent; the protocol is practical and easy as products can be easily separated from unreacted epoxide due to large boiling point and polarity differences (Kumar et al. 2007). The maximum theoretical yield reached by using HKR method is limited to 50%, while maximum ee can be reached with diverse terminal oxides (Lin et al. 2011a). However, the asymmetric reactions using the chemo-catalysts result in less enantioselectivity and also suffer from the need for complex catalysts, use highly toxic reagents and have limited substrate scope. These may be suited for the benchtop reactions, but on an industrial scale, they are harmful to the environment (Clouthier and Pelletier 2012).

Therefore, biocatalytic methods remain particularly attractive for the kinetic resolution of racemic epoxides using various enzymes and recombinant cells as a biodegradable catalyst, like in direct epoxidation of olefin substrates by monooxygenases or peroxidases, dehalogenation of halohydrins by halohydrin dehalogenases, enantioselective hydrolysis of epoxides by epoxide hydrolases, etc. isolated from microorganisms, plants and animals. They catalyse reaction in a regio-, diastereo-, and enantioselective manner under mild reaction conditions in economic and environmental-friendly manner giving high enantiopurity, yield, specificity, catalytic efficiency and activity (Breuer et al. 2004; Wohlgemuth 2010; Lin et al. 2011a). Enzymes are available in so much diversity that they exhibit broad substrate spectra for the generation of enantiopure epoxides at ambient temperature and cause much less pollution as compared to chemo-catalytic processes (Lin et al. 2011b).

Still, the availability of commercial enzymes is limited, and therefore, there is a need for the development of novel biocatalysts (Choi 2009). The biocatalytic scope and potential of the enzymes have been expanded by the advancement of new techniques for the screening and selection of enzymes and also by exploiting new microbial sources, as the number of sequenced genomes is increasing day by day. Several enzymes have now been commercialized like chloroperoxidase from *Caldariomyces fumago* as a suspension in a phosphate buffer and the epoxide hydrolases from *Aspergillus niger* and *Rhodococcus rhodochrous* as a lyophilized powder, both from Sigma-Aldrich (Lin et al. 2011a). With the increasing industrial applications of substrates, greener routes for the enantioselective preparation of fine chemicals are being explored (de Vries and Janssen 2003). Some of the biocatalysts for the synthesis of epoxides and diols which are used as intermediates in synthesis of pharmaceutically important drugs are described ahead.

6.3 Biocatalysts for Enantiopure Epoxide and Diol Production

6.3.1 Monooxygenases (EC 1.14.14.1)

Monooxygenases are involved in varied biological processes like drug detoxification, biodegradation of aromatic compounds, biosynthesis of antibiotics and siderophores, etc. (Eisendle et al. 2004; Lombó et al. 2006; Cashman 2008). In the reactions catalysed by monooxygenases, one atom of dioxygen gets introduced into the substrate, and the other atom gets reduced to H_2O molecule in the presence of NAD(P)H as the reducing agent. Electrons from NAD(P)H gets delivered to the enzyme-substrate complex via a redox system (Fig. 6.1) (Harayama and Kok 1992). Monooxygenases require a variety of cofactors for catalytic activity like FAD, tetrahydropteridine or copper ion (Torres Pazmiño et al. 2010). Out of the wide variety of reactions carried out by monooxygenases, they can also catalyse transformation of alkenes to the corresponding oxides with good enantioselectivities such as styrene monooxygenase (SMO), xylene monooxygenase (XMO) and alkene/alkane

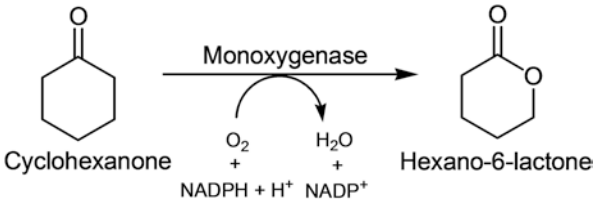


Fig. 6.1 Monooxygenase-catalysed reaction**.** Monooxygenases oxidize a substrate by transferring one atom of oxygen from the dioxygen and reduce the other oxygen atom to H_2O molecule, in the presence of NAD(P)H as the reducing agent

monooxygenase (Nolan and O'Connor 2008). SMO can enantioselectively epoxidize styrene to *(S)*-styrene oxide with >99% ee. SMO shows good regio- and enantioselectivities and reacts under mild reaction conditions with the use of oxygen as an inexpensive non-toxic oxidant (Lin et al. 2011a, c). SMO from *Pseudomonas fluorescens* ST and *P. fluorescens* VLB120 was used to study the bioconversion of α- and β-methyl substituted styrenes to 2-amino and 1- and 2-phenyl ethanols giving good yield and high enantiopurity (Schmid et al. 2001; Sello et al. 2006). Another SMO from *Pseudomonas* sp. LQ26 transformed α- and β-substituted conjugated styrene derivatives to chiral epoxides with excellent >99% ee (Lin et al. 2011b). Recently, a review by Liu et al. focused on the improvement of SMO from *Pseudomonas* sp. LQ26 (Liu et al. 2016). Recombinant whole cells harbouring SMO can react with various derivatives of styrene as well as with nonconjugated alkenes. It is also involved in a cascade reaction along with a ketoreductase and produced epoxy ketones and allylic epoxy alcohols with >99% ee and is also a subject of protein engineering studies which are underway (Liu et al. 2016). Another whole cell suspension of *Methylosinus trichosporium* IMV 3011 containing methane monooxygenase biosynthesize epoxyethane (6.6 n mol) from ethylene which is a useful and important intermediate for industrial chemical production (Xin et al. 2017). A recombinant engineered toluene o-xylene monooxygenase from *Burkholderia cepacia* G4 carried out industrially significant oxidation of ethylene to ethylene oxide by >5500-fold relative to the native enzyme (Carlin et al. 2015).

6.3.2 Chloroperoxidases (EC 1.11.1.10)

Chloroperoxidase primarily catalyses the halogenation of organic compounds in the presence of halide ions and peroxides such as H_2O_2 (Libby et al. 1982) (Fig. 6.2). It can also catalyse the halide-independent reactions such as asymmetric epoxidation, allylic hydroxylation, sulfoxidation, oxidative halogenation, oxidation of alcohols, aldehydes and amines. Epoxidation reaction catalysed by chloroperoxidase is cofactor-independent, making the procedure much easier (Valery 2003). Chloroperoxidase can catalyse the asymmetric epoxidation of *cis*-disubstituted alkenes bearing alkyl groups such as *cis*-1-methyl substituted alkenes giving up to 97% ee of the corresponding (*R, S*)-oxides (Hager et al. 1998). Chloroperoxidase

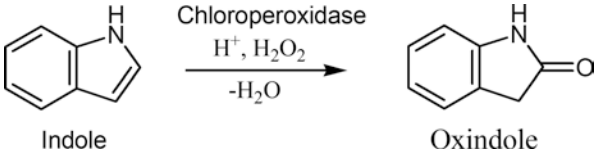


Fig. 6.2 Chloroperoxidase-catalysed reaction. Chloroperoxidase is a haloperoxidase that catalyses the halogenation of organic compounds in the presence of halide ions (chloride, bromide and iodide) and peroxides (H_2O_2) and results in the formation of carbon-halogen bond

can also give excellent enantioselectivities (91–97% ee) by asymmetric epoxidation of functionalized *cis*-2-alkenes, such as unsaturated carboxylic ester, and alkenes with a terminal bromine atom (Hu and Hager 1999) and form *(R)*-epoxides with 89–95% ee in presence of 1,1-disubstituted terminal alkenes with one substituent as methyl using H_2O_2 as the terminal oxidant (Hager et al. 1998). Due to the poor activity and stability of chloroperoxidase in solvents with low water content, alternative solvents like ionic liquids have been explored for the first time by Wu et al. (2010). Asymmetric epoxidation of 3-chloropropene was catalysed by chloroperoxidase from fungus *Caldariomyces fumago* with 97.1% ee and 88.8% yield of (*R*)-epichlorohydrin in homogenous phosphate buffer/ionic liquid mixtures (Wu et al. 2010). Chiral epichlorohydrin is widely used in organic synthesis and medicinal chemistry. However, to overcome the lack of long-term stability of chloroperoxidase, various enzyme immobilization studies were carried out (Munoz-Guerrero et al. 2015; Dong et al. 2017). Immobilization on mesoporous structure and in the presence of co-solvent increased the operational stability of chloroperoxidase 246% in presence of 3-nitrostyrene compared to free enzyme (Munoz-Guerrero et al. 2015). Chloroperoxidase from *Caldariomyces fumago* has already been commercialized by Sigma-Aldrich.

6.3.3 Haloalcohol Dehalogenases (EC 3.8.1.5)

Haloalcohol dehalogenases (also known as halohydrin dehalogenases, hydrogen-halide lyases, halohydrin epoxidases) convert vicinal haloalcohols to an epoxide and halide by reversible dehalogenation (Fig. 6.3) as well as the enantioselective ring opening of epoxides in the presence of a nucleophile such as N_3^-, CN^- or NO_2^- and play a role in the biodegradation of xenobiotic halogenated compounds. Haloalcohol dehalogenases along with azide catalyse the ring opening of *p*-substituted styrene oxides (Spelberg et al. 2001) producing the *(R)*-epoxide and resulting in the remaining *(S)*-epoxide and the formed *(R)*-2-azido-1-phenylethanol with E > 200 (de Vries and Janssen 2003). Azidolysis of aromatic epoxides by haloalcohol dehalogenase from *Arthrobacter* sp. AD2 produced enantiomerically pure (*S*)-β-azido alcohols and (*R*)-α-azido alcohols (ee>99%) (Hrenar et al. 2016). Amino alcohols are vital molecules and can be prepared from the azido alcohol through reduction. Novel haloalcohol dehalogenases identified from *Agrobacterium*

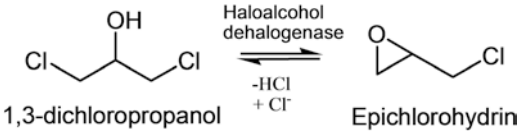


Fig. 6.3 Haloalcohol dehalogenase-catalysed reaction. Haloalcohol dehalogenases are bacterial enzymes that catalyse cofactor-independent dehalogenation of vicinal haloalcohols, thereby producing the corresponding epoxide

radiobacter AD1 (Jin et al. 2012a), *Agrobacterium tumefaciens* (Liu et al. 2014) and *Tistrella mobilis* ZJB1405 (Xue et al. 2015b) were majorly applied in the production of chirally important epichlorohydrin. In order to achieve high ee and improve the efficiency, haloalcohol-mediated reactions were performed in a specially designed equipment by immobilized cells (Jin et al. 2013a), improved mutants of haloalcohol dehalogenase were created by site directed mutagenesis (Liu et al. 2014), or synthesis of enantiopure epoxide using one pot chemo-enzymatic approach (Wu et al. 2016).

Above are some of the examples of biocatalysts which can produce enantiopure epoxides or diols. In these enzyme-catalysed reactions, they also require certain nucleophiles or other cofactors for the completion of the reactions which is not the case with epoxide hydrolases, and hence they are discussed in detail herein.

6.3.4 Epoxide Hydrolases (EC 3.3.2.x)

Epoxides are industrially important substrates and are widely used as chiral intermediates for the production of pharmaceutically important drugs (de Vries and Janssen 2003). Epoxides are present commonly in both simple and complex biologically active molecules and can easily undergo stereoselective ring-opening reactions with a varied range of nucleophiles (Lee and Shuler 2007). In biocatalytic reactions, epoxides are kinetically resolved by epoxide hydrolases producing enantioenriched diols and remaining epoxides from racemic substrates (de Vries and Janssen 2003). Enantiopure epoxides and diols are valuable intermediates in organic synthesis for the production of optically active pharmaceuticals (Lee and Shuler 2007). Out of the above-mentioned enzymes, enantioselective and enantioconvergent epoxide hydrolases have several advantages as they are ubiquitously present in the environment, i.e. from prokaryotes to eukaryotes to arthropods, and can be easily cloned from various microorganisms and produced in abundant amount as recombinant proteins; they are stable proteins and do not require any cofactors or added nucleophiles for their activities and have broad substrate specificity. Due to these characteristics, epoxide hydrolases are most commonly used commercially important biocatalysts (Choi and Choi 2005). Everyday scientists are researching for new strategies to make the application of epoxide hydrolases user-friendly and to increase their stability, robustness and reusability. These studies have been analysed critically in our recent review (Saini and Sareen 2017) using approaches like reaction media optimization, use of greener ionic solvents, immobilization approaches and engineering of epoxide hydrolases.

Epoxide hydrolases are present mostly in all types of organisms like mammals, invertebrates, plants, fungi and bacteria which provide hidden treasures of epoxide hydrolases and hence can be exploited to discover novel and potential epoxide hydrolases to be used industrially for the production of enantiopure drug intermediates (Choi and Choi 2005). Novel epoxide hydrolases can be discovered using different methodologies as described ahead.

6.4 Mining of Epoxide Hydrolases

Various approaches have been followed to identify regio- and stereo-selective epoxide hydrolases from microbes:

6.4.1 Enrichment Screening

The classical approach which has long been used to identify epoxide hydrolases is enrichment screening. In this technique, desired microorganisms capable of metabolizing a specific compound are isolated by providing their specific substrate (epoxide) as a sole carbon source (Dagher et al. 1997). Epoxides being toxic molecules are present in petroleum contaminated soil or other polluted sites from where microbial isolates were screened for the presence of enantioselective epoxide hydrolases (Choi et al. 2008; Zhang et al. 2010; Bala et al. 2011; Woo et al. 2015).

6.4.2 Metagenome Screening

Another approach to find novel epoxide hydrolases is by metagenome screening. In metagenomics, the genome of the uncultured microbial species from the environment is cloned into vectors using recombinant techniques to form metagenomic libraries which are then screened for the identification of novel epoxide hydrolase biocatalysts. There are two types of screening methods to identify epoxide hydrolases or other biocatalysts: first is the function-based screening in which the clones are expressed and desired clones are selected from the libraries which shows epoxide hydrolase catalytic activity and second is the sequence-based screening method which is based on the conserved DNA sequence of the target gene. In this screening method, DNA probes or primers derived from the conserved regions of the already known genes are used to PCR amplify the cloned genes in metagenomic libraries, and the amplified recombinant clones are selected (Yun and Ryu 2005). Epoxide hydrolases from *Streptomyces* species has been identified by metagenomic screening of forest soil of high Andean forest (Montaña et al. 2012). Recently, epoxide hydrolases along with haloalkane dehalogenases, xylanase and amylase were also identified from mangrove soil metagenomes in oil-contaminated sites (Jimenez et al. 2015; Ronzella et al. 2017). A thermostable limonene epoxide hydrolase was discovered from Russia and China's hot terrestrial environment metagenome (Ferrandi et al. 2015b) and used in the pilot-scale industrial biotransformations (Ferrandi et al. 2015a).

6.4.3 16S rRNA Sequencing Method

Epoxide hydrolases are also identified by 16S rRNA gene sequencing method (Woo et al. 2015; Woo and Lee 2013; Xue et al. 2014) in which genomic DNA is isolated from the bacterial isolates showing epoxide hydrolase activity which are grown in harsh conditions and then amplified by PCR using degenerate primers and sequenced. These sequenced 16S rRNA genes are compared to 16S rRNA sequences of the strains present in public databases using bioinformatic alignment tools, and phylogenetic tree is constructed which leads to identification of related or new microbial species (da Cruz et al. 2010). 16S rRNA gene sequencing of genera *Bacillus* from copper mine drainages of Brazil has been identified as a source of epoxide hydrolases (Zucoloto et al. 2016).

However, the above-described conventional screening techniques are still labour-intensive and time-consuming. Consequently, with the advancement in bioinformatics and recombinant techniques, significant progress has been made in the identification of novel biocatalysts from the public genome databases quickly and in an economical way as discussed ahead.

6.4.4 Genome Database Mining

There are 101,542 prokaryotic genomes (as of 22 June, 2017) which have been sequenced and present in public databases. Several epoxide hydrolases have been identified by genome mining approach (van Loo et al. 2006; Saini et al. 2014, 2017; Hu et al. 2015; Wu et al. 2015a). In this approach, template of already reported epoxide hydrolases containing all the conserved epoxide hydrolase residues is chosen, and BLAST search is performed, which yields homologous protein sequences. Sequence with highest percentage identity with the template sequence is selected and then analysed by multiple sequence alignment tools (CLUSTALW or MUSCLE, etc.) for the conserved key residues and subjected to molecular cloning, overexpression and epoxide hydrolase activity analysis (Luo et al. 2012). With the advancement in technology and evolution of new methods for the exploration of industrially competent epoxide hydrolases, they can be identified with much ease and less expenditure of time and resources.

Earlier, epoxide hydrolases were studied for their role in the detoxification in mammals as they catalyse the degradation of epoxides to more polar diols, which can be converted to water-soluble products and are easily eliminated (Archelas and Furstoss 1998). With the discovery of epoxide hydrolases in microbial sources, they were exploited for the production of chiral epoxides and diols using kinetic resolution and enantioconvergent processes, as microbial epoxide hydrolases can be produced in large amounts with the help of recombinant DNA technology (Lee et al. 2004; Kim et al. 2006). Epoxide hydrolases can be used in the form of whole cells from both wild-type and recombinant strains or in the lyophilized and immobilized

forms for the preparation of enantiopure epoxides and diols by the hydrolysis of epoxides (Lee and Shuler 2007). Epoxide hydrolases catalyse synthesis of enantiomerically pure epoxide and diol intermediates which can be used for the synthesis of drugs, e.g. 1-phenyl-1,2-ethane diols used for the synthesis of fluoxetine which is an anti-depressant (Mahajabeen and Chadha 2011) along with Tembamide and Aegeline drugs which have hypoglycaemic activity (Sadyandy et al. 2005), *(S)-p*-isobutyl-α-methyl styrene oxide used as an intermediate for *(S)*-Ibuprofen (Cleij et al. 1999), etc. Some of the epoxides which are not preferred or reported by the Jacobsen HKR can also be hydrolysed by epoxide hydrolases such as pyridyloxirane, 2,2-disubstituted, 2,3-disubstituted epoxides and trisubstituted racemic epoxides (Osprian et al. 1997; Weijers 1997; Genzel et al. 2001). Most of the bacterial epoxide hydrolases showed preference to hydrolyse *(S)*-epoxides, thus producing *(R)*-epoxides with >99% ee (Orru et al. 1998). However, epoxide hydrolases with preference for *(R)*-epoxides are also available (Monfort et al. 2004). Despite with good ee, the maximum theoretical yield that could be reached is only 50% (Jin et al. 2004). However, the low theoretical yield has been overcome by using epoxide hydrolase from *Solanum tuberosum* which catalyses the enantioconvergent hydrolysis of *meta*-chloro styrene oxide and producing *(R)*-diol with 97% ee and 88% yield (Monterde et al. 2004). In another example, bacterial and marine fish epoxide hydrolases combination gave the enantiopure *(R)*-diol with 90% ee and 94% yield (Kim et al. 2008; Mahajabeen and Chadha 2011). Therefore, new epoxide hydrolases are consistently being explored with improved ee and yield.

6.5 Structure of Epoxide Hydrolase

Epoxide hydrolases belong to α-/β-hydrolase fold enzyme superfamily, which is one of the largest groups of structurally related enzymes (Ollis et al. 1992). The members of this versatile enzyme family have wide applications, which range from the kinetic resolution of precursors of pharmaceutical compounds (Patel 2004; Bornscheuer and Kazlauskas 2006), degradation of pollutants (Pavlova et al. 2009) and bulk applications such as lipid modification and laundry detergents (Bornscheuer 2000). The members of the α-/β-hydrolase fold enzyme superfamily showed divergent evolution as they have evolved from a common ancestor and have some conserved catalytic residues (Ollis et al. 1992). X-ray crystal structure of epoxide hydrolases has been elucidated from microorganisms *Agrobacterium radiobacter* AD1 (Nardini et al. 1999), *Aspergillus niger* (Zou et al. 2000), *Mycobacterium tuberculosis* H37Rv (Biswal et al. 2008) and *Bacillus megaterium* ECU1001 (Kong et al. 2014). Epoxide hydrolases generally exist as homodimers with the exception of plant epoxide hydrolases (Mowbray et al. 2006). α-/β-Hydrolase family is characterized by the α-/β-hydrolase fold which has a conserved topology with a two-domain structure. One is the main domain that consists of a central β-sheet having eight β-strands (1–8) with seven parallel and second is antiparallel β-strand. β-sheet is alternated by seven α-helices which covers both sides. Second is the lid or cap

domain located at the top of the substrate binding site above the main domain between β-strands 6 and 7 composed of five α-helices as shown in Fig. 6.4 (Rink et al. 1997; Nardini et al. 1999; van Loo et al. 2006). The catalytic triad of epoxide hydrolases consists of a nucleophile, an acid and a histidine which always occur in the same order in the primary sequence. The sequence around the nucleophile is Sm-X-Nu-X-Sm-Sm (Sm, small residue; X, any residue; Nu, nucleophile) which occurs between β-strand 5 and α-helix C forming a catalytic elbow (Fig. 6.4). Nucleophile is mainly Asp. Acid of the triad which can be Asp or Glu is on a loop following β-strand (Fig. 6.4). The highly conserved catalytic histidine is present at the end of a β-strand 8 (Fig. 6.4). The catalytic triad residues are located on loops above the β-sheet which forms a scaffold for them and brings them together to form an active site (Jochens et al. 2011). Two backbone nitrogen atoms form the oxyanion hole, where one nitrogen atom is contributed by Phe present immediately after the nucleophile and the second comes from HGXP motif (H, His; G, Gly; X, usually aromatic amino acid; and P, Pro) and is present in a turn between β-strand 3 and α-helix A which stabilizes a tetrahedral intermediate (Fig. 6.4) (Ollis et al. 1992; Rink et al. 1997; Nardini et al. 1999). GXS_mXS/T motif is located between the HGXP motif and the catalytic nucleophile, and its function is not known yet (van Loo et al. 2006). HGXP and GXS_mXS/T motifs are located on loops which emerge from the β-sheet in proximity of the cap domain (Ollis et al. 1992; Rink et al. 1997) (Fig. 6.4). Epoxide hydrolases possess two tyrosines which protrude from the cap domain into the binding pocket located between the α-/β-hydrolase fold domain and the cap domain positioned close to the catalytic nucleophile and catalyse the oxirane ring opening (Arand et al. 1996; Yamada et al. 2000). N-terminal region is highly variable among various types of epoxide hydrolases and possess phosphatase

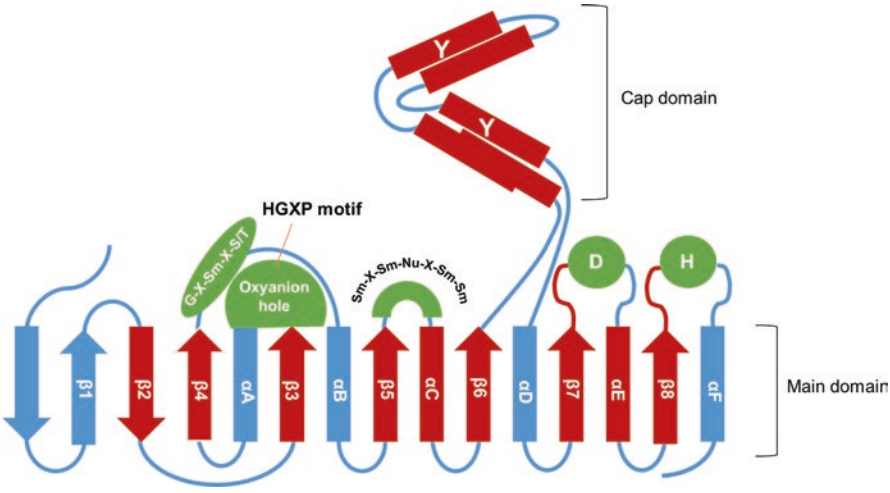


Fig. 6.4 Structure of epoxide hydrolase. Epoxide hydrolase structure depicting the cap domain consisting of tyrosine residues and main domain consisting of a catalytic triad (Asp-His-Asp), HGXP (oxyanion hole) and GXS_mXS/T motifs

activity (Homburg et al. 2002). In bacteria, microsomal epoxide hydrolases lack N-terminal region, while mammalian and insect microsomal epoxide hydrolases possess it (Barth et al. 2004). N- and C-terminal domains of epoxide hydrolase are bound by a linker segment which is ~18 residues long, and C-terminal possesses the epoxide hydrolase activity, while the function of the N-terminal domain is still unknown (Archelas and Furstoss 1998). In addition to the role of established active site triad (Asp-His-Asp) and the two lid residues (tyrosines) in epoxide hydrolase catalytic mechanism, important role of two new residues E35 and His104 has been uncovered recently in potato soluble epoxide hydrolase, which form an ion pair and are highly conserved among epoxide hydrolases and related hydrolytic enzymes (Amrein et al. 2015).

On the basis of α-/β-hydrolase Fold Enzyme Family 3DM database (ABHDB), a structure-based multiple sequence alignment tool, α-/β-hydrolase fold enzymes are divided into subfamilies based on the conserved core residues of the families for which structures are available (Kuipers et al. 2010). Family I is a superfamily which contains most of the known α-/β-hydrolase fold enzymes, and the residues of the GXSXG motif (catalytic elbow with the nucleophile) are highly conserved throughout the whole superfamily where G is Gly, S Ser and X variable amino acid residues. 3DM analysis showed that the residues X are highly conserved in the individual enzyme families of the α-/β-hydrolase superfamily which are divided into five families based on the composition of the catalytic elbow: **II** GHSXGG, **III** GESAGA, **IV** GDSAGG, **V** GNSMGG and **VI** GESYAG. His in the nucleophile-His-acid catalytic triad is highly conserved with acid, and nucleophile loops accommodate more than one type of amino acid among different families of α-/β-hydrolase fold enzymes (Ollis et al. 1992). Oxyanion hole composition also differs between different enzyme families. The following motifs are present in the oxyanion hole in the five different families **II** HGX, **III/IV** HGGG (A) X, **V** HGSG and **VI** NGGP (Kourist et al. 2010). Family **II** consists of epoxide hydrolases along with other members having highly diverse functions including haloalkane dehalogenases, haloacid dehalogenases, haloperoxidases and GX-esterases. Other families in α-/β-hydrolase fold superfamily database includes family III which consists of esterases and lipases, family IV includes hormone sensitive lipases, family V comprises hydroxynitrile lyases and family VI contains carboxypeptidases (Kourist et al. 2010).

Enzymes are always considered to be specific for their substrates and the reactions they catalyse, but sometimes there is a divergence from this rule leading to promiscuity. A promiscuous enzyme along with catalysing the reaction for which it is evolved also catalyses the side reaction for which it has not functionally evolved (Hult and Berglund 2007). Many α-/β-hydrolase fold enzymes show catalytic promiscuity, i.e. they are able to catalyse more than one type of chemical transformation (Henke Erik and Bornsheuer 2003; Fujii et al. 2005; Hult and Berglund 2007; Li et al. 2008). Epoxide hydrolases have the tendency to show promiscuous behaviour mainly to haloalkane dehalogenases, haloacid dehalogenases, haloperoxidases and esterases as they fall in the same family **II** and have similar structure and catalytic mechanism as of epoxide hydrolases, but they contain conserved key residues that determine the chemoselectivity of the enzymes. Jochens et al. converted an

esterase from *Pseudomonas fluorescens* into an epoxide hydrolase by substituting some amino acid residues and a loop which suggests that interconversion of enzyme activities is possible within the α-/β-hydrolase fold family (Jochens et al. 2009). In recent times, another enzyme from α-/β-hydrolase fold family *Candida antarctica* lipase B was explored to be used as a scaffold for designing new epoxide hydrolases in which further studies need to be carried out in the future (Bordes et al. 2015). Thus, enzyme catalytic promiscuity can be explored to develop new efficient and sustainable biocatalysts for industrial use (Humble and Berglund 2011).

The similarity among the sequences of the epoxide hydrolase family members is low (<50%) and confined to some specific areas such as the N-terminal region where two motifs are located, i.e. the HGXP and the GXS_mXS/T, and active site residues of the catalytic triad and in secondary structures in the inner core of the enzyme, mainly in the central β-sheet. The residues corresponding to the peripheral regions and cap domain show no sequence similarity among epoxide hydrolases and are involved in determining the substrate specificity (Rink et al. 1997).

6.6 Catalytic Mechanism of Epoxide Hydrolase

Epoxide hydrolase reaction mechanism was elucidated on the basis of reaction mechanism of haloalkane dehalogenase as the sequence of epoxide hydrolase is quite similar to haloalkane dehalogenase and they share the same α-/β-hydrolase fold (Arand et al. 1994; Sato et al. 2007). Ring opening of epoxides can be carried out nucleophilically under acidic, basic and neutral conditions (Smith 1984). In acidic conditions, ring opening of epoxides is carried out by following SN_1 mechanism with protonation of the epoxide, and ring opening occurs before the nucleophile attacks at the more substituted end of the epoxide. Acid-catalysed reaction mechanism has been less studied as compared to the base-catalysed reaction mechanism. In basic or neutral conditions, epoxides are hydrolysed through an anti-Markovnikov-type (commonly referred to as *trans*) nucleophilic attack of the oxirane ring preferably at the least sterically hindered carbon atom following SN_2 mechanism (opening and attack concurrently) accomplished by the back side attack of the epoxide ring (inversion of configuration) (Hammock et al. 1980). Epoxide hydrolase-catalysed reaction occurs through a two-step process: in the first step, carboxylic acid of Asp performs a nucleophilic back side attack on the most reactive, less hindered primary carbon atom of epoxide (Jacobs et al. 1991; Arand et al. 1996; Rink et al. 1997) and facilitates the epoxide ring opening assisted by two acidic tyrosines present in the active site (cap domain) by hydrogen bonding and protonation of the epoxide oxygen (general acid catalysis) and forms a covalently bound ester intermediate by forming an ester bond between the carboxylic acid of an enzyme and an alcohol of a diol (Fig. 6.5a) (Rink et al. 1999; Argiriadi et al. 2000; Yamada et al. 2000). In the second step, water molecule directly attacks the covalently bound alkyl-enzyme intermediate activated by the His of the catalytic triad which acts as a proton acceptor (general base catalysis) assisted by the acidic

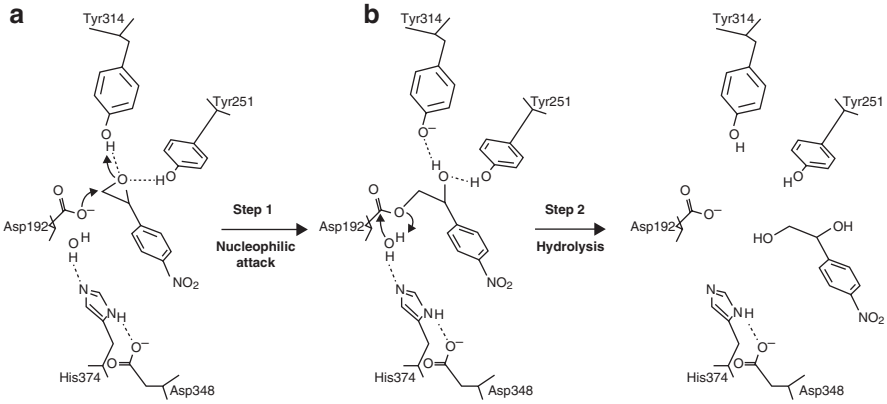


Fig. 6.5 Catalytic mechanism of epoxide hydrolase. (**a**) Ring opening of the epoxide is catalysed by the nucleophile Asp assisted by tyrosines. (**b**) Alkyl-enzyme intermediate is hydrolysed by activated water molecule. (Adapted from Zou et al. 2000)

Asp/Glu residues and releases the product diol where one oxygen atom being incorporated from the epoxide and the other originates from the water regenerating the active site of the epoxide hydrolase (Lee and Shuler 2007) (Fig. 6.5b). This is the rate limiting step of the reaction (Spelberg et al. 2002). During hydrolysis of the alkyl-enzyme intermediate, negative charge gets developed on the carbonyl oxygen of the nucleophilic Asp which is stabilized by the oxyanion binding site (Nardini and Dijkstra 1999). Reaction mechanism of all epoxide hydrolases is similar except limonene and cholesterol epoxide hydrolases and is discussed in their respective sections.

6.7 Resolution Mechanisms of Epoxide Hydrolases

In industries, there is a need for a pure enantiomer for the production of chiral drugs which are without the other unwanted enantiomer. One process by which this limitation can be overcome is kinetic resolution in which enantiomers are hydrolysed at different rates and enantiopure product and unreacted substrate each can be obtained in 50% theoretical yield such as the precursors for the synthesis of *(R)*-Eliprodil which is used a neuroprotective agent and is produced with a product yield of 93% and ee of 96% (Manoj et al. 2001). Merck has patented the process for the synthesis of intermediate (1*S*, 2*R*-indene oxide) with 100% ee and 14% yield using epoxide hydrolase from *Diplodia gossipina* ATCC 16391 which inhibits the HIV protease (Jinyou et al. 1995; Chartrain et al. 1998). The limitation of 50% yield can be overcome by using three approaches which are discussed below.

6.7.1 Asymmetric Synthesis of Prochiral Substrates

In this process, chiral product is synthesized stereo-selectively from a non-chiral (prochiral) or *meso*-compound (M) via resolution of the racemate using epoxide hydrolase as a biocatalyst and produces chiral products compared to prochiral substrate molecules in unequal amounts (Fig. 6.6) (Schober and Faber 2013). Asymmetric hydrolysis of 2,2-disubstituted epoxide using lyophilized cells of *Rhodococcus* sp. NCIMB 11216 leads to (*S*)-1,2-diols and *(R)*-epoxides with E > 100 (Wandel et al. 1995), *para*-nitrostyrene oxide hydrolysis using epoxide hydrolase from *A. niger* produces 97% ee of *(S)*-isomer with a conversion of 47% (Morisseau et al. 1997) and asymmetric hydrolysis of 1,2-epoxyoctane using whole cells of *Chryseomonas luteola* gives 98% ee of remaining *(S)*-epoxide and 86% ee of formed *(R)*-diol (Botes et al. 1998). These are the few examples of asymmetric hydrolysis. After asymmetric synthesis, another approach for enantiopure synthesis of products, i.e. dynamic kinetic resolution, has evolved.

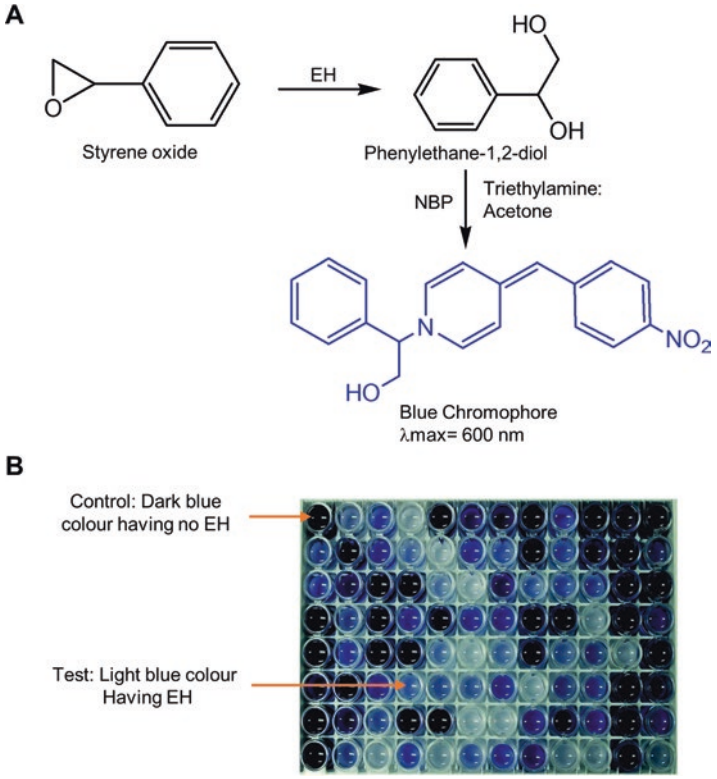


Fig. 6.6 Asymmetric hydrolysis of prochiral substrates. Asymmetric resolution of a prochiral or meso-compound M producing B as the predominant enantiomer where M is prochiral or *meso*-substrate and B, *ent*-B are product enantiomers. (Adapted from Schober and Faber 2013).

6.7.2 *Dynamic Kinetic Resolution (DKR)*

DKR is like kinetic resolution (KR) but is an improvement of KR. In KR, two enantiomers of a racemate are transformed into product at quite different rates, i.e. one enantiomer is converted to the desired product, while the other remains unchanged (Fig. 6.7a). This procedure has limitation as the maximum theoretical yield cannot exceed 50% as described above. This problem is overcome by DKR. In DKR, also one enantiomer reacts slowly compared to the other in the given reaction conditions in the presence of a biocatalyst and gives quantitative yield of one of the enantiomers, and the other slow-reacting enantiomer gets converted by in situ equilibration or racemization to the chirally labile substrate which gets converted to the single product isomer leading to 100% yield (Fig. 6.7b) (Pellissier 2003). Racemic-2-methylglycidyl benzyl ether was hydrolysed by using *Rhodococcus* sp. CBS 717.73 and provides the (*R*)-epoxide and the (*R*)-diol with >97% ee and E > 200. Further, the acid-catalysed hydrolysis of (*R*)-epoxide gives (*R*)-1-benzyl-oxy-2-methylpropane-2,3-diol with >97% ee and 78% yield (Simeó and Faber 2006). Epoxide hydrolase from *Corynebacterium* C12 catalysed the resolution of (±)-1-methyl-1,2-epoxycyclohexane to (*1S,2S*)-1-methylcyclohexane-l,2-diol and gives 80% yield with an ee of >95% (Archer et al. 1996). In 2003, Chang et al. catalysed the hydrolysis of a *meso*-epoxide (N-benzyloxy-carbonyl-3,4-epoxy-pyrrolidine) and cyclohexene oxide using an epoxide hydrolase from bacteria

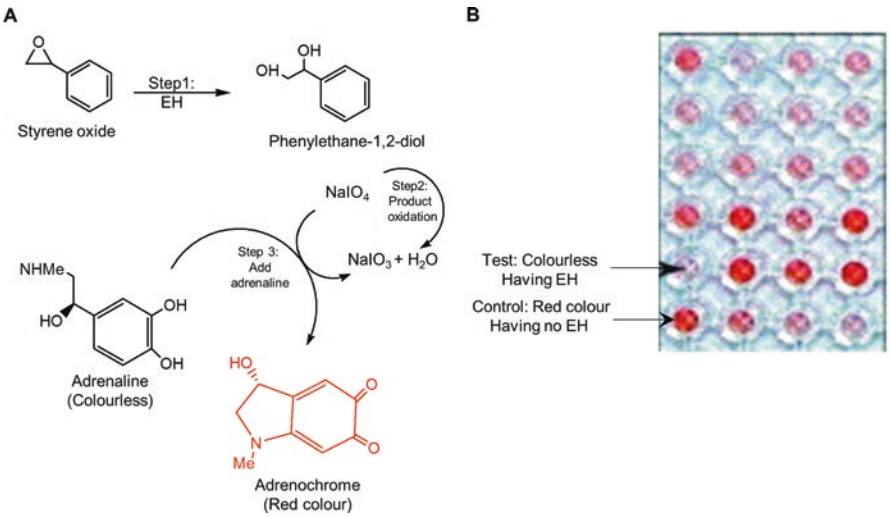


Fig. 6.7 Kinetic and dynamic kinetic resolution of racemic epoxides. (**a**) Kinetic resolution of a pair of enantiomers A and *ent*-A producing B as the predominant enantiomer and *ent*-A as the unreacted enantiomer, where A, *ent*-A are substrate enantiomers and B, *ent*-B are product enantiomers. (**b**) Dynamic kinetic resolution of a pair of enantiomers A and *ent*-A producing B as the sole product where A, *ent*-A are substrate enantiomers and B, *ent*-B are product enantiomers. (Adapted from Schober and Faber 2013)

Sphingomonas sp. HXN-200, producing corresponding vicinal *trans*-diols with 96% ee and 100% yield (Chang et al. 2003).

6.7.3 Enantioconvergent Hydrolysis

One hundred percent yield can also be achieved by enantioconvergent hydrolysis where single enantiomeric product can be synthesized from a racemic mixture of substrate via independent pathways by retention or inversion of configuration (Fig. 6.8). Enantioconvergent epoxide hydrolases were identified from various organisms which prevent 50% loss of the substrate and allow an expensive substrate to be used (Monterde et al. 2004; Cao et al. 2006). Recently, epoxide hydrolase from *Vigna radiata* catalyses enantioconvergent hydrolysis of racemic *p*-nitrostyrene oxide producing *(R)-p*-nitrophenyl glycol with 99% ee and 71.5% yield (Wu et al. 2015a, b), another enantiocomplementary epoxide hydrolases from *Aspergillus tubingenesis* TF1 produced *(R)*-(−)-1-phenyl-1, 2-ethane diol from racemic styrene oxide with 97% ee and >99% yield (Duarah et al. 2013). Hwang and co-workers also identified epoxide hydrolase from *Caulobacter crescentus* which hydrolyses racemic *p*-chlorostyrene oxide forming corresponding (*R*)-diol with ee of 95% and 72% yield, preparatively (Hwang et al. 2008). In our lab, an epoxide hydrolase was identified from *Cupriavidus metallidurans* CH34 which catalysed hydrolysis of both (*R*)- and (*S*)-enantiomers of styrene oxide and produced both (*R*)- and (*S*)-diol products with regioselective coefficients for α(*R*)/β(*R*) as 66/34 and α(*S*)/β(*S*) as 79/21. This makes it a potential candidate for mutational studies as it already has a preference for α-carbon of (*S*)-styrene oxide, and by altering its regioselectivity, it will attack at β-carbon of (*R*)-styrene oxide making it an enantioconvergent epoxide hydrolase so that it produces enantiopure (*R*)-1-phenyl-1,2-ethanediol (PED) (Kumar et al. 2011). Enantioconvergent epoxide hydrolases give close to the 100% theoretical yield and ee, thus accelerating the industrial potential of epoxide hydrolases for the production of enantiopure drugs.

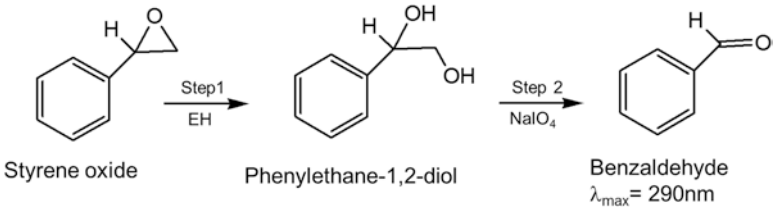


Fig. 6.8 Enantioconvergent hydrolysis of racemic epoxides. Enantioconvergent hydrolysis of A and *ent*-A enantiomers through retention and inversion of configuration producing B as the sole product where A, *ent*-A are substrate enantiomers and B is product enantiomer. (Adapted from Schober and Faber 2013)

6.8 Types of Epoxides

6.8.1 Monosubstituted Epoxides (Type I)

Type I epoxides contain phenyl group such as in styrene oxide and its derivatives like *p*-chlorostyrene oxide, *p*-nitrostyrene oxide, trifluoro methyl styrene oxide, glycidyl aryl ether derivatives, etc. (Fig. 6.9a). Monosubstituted epoxides are flexible and slim molecules due to which they are not easily recognized by epoxide hydrolases from various sources especially fungal and bacterial enzymes (Steinreiber and Faber 2001), while red yeasts (*Rhodotorula, Rhodosporidium and Trichosporon*) are capable of resolving them enantioselectively (Botes et al. 1999). There are few exceptions to fungal and bacterial epoxide hydrolases like fungus *Aspergillus niger* which gave E value of 40 with terminal aryl monosubstituted epoxide (*p*-nitrostyrene oxide) having 97% regioselectivity (Morisseau et al. 1999). Asymmetric hydrolysis of terminal aliphatic monosubstituted epoxide was catalysed by bacterial epoxide hydrolase *Nocardia* which yielded 54% ee_s and 54% ee_p of octane diol but with low selectivity (Cagnon et al. 1999). The enzymes for these substrates show preference for *(R)*-enantiomer of oxirane with only a few exceptions (Orru and Faber 1999; Woo et al. 2007).

6.8.2 Styrene Oxide-Type Epoxides (Type II)

Type II epoxides are like styrene oxide substrate, and they possess a benzylic carbon atom which enable the formation of carbocation which is stabilized by the aromatic moiety (Fig. 6.9b). As the attack at these types of substrates can occur at both the oxirane carbon atoms, hence the enantioselectivity values have to be reported correctly where regioselectivity is involved (Steinreiber and Faber 2001). Fungal enzymes are the catalysts of choice for the styrene oxide-type substrates. *Beauveria densa* CMC 3240 fungal epoxide hydrolase resolves a series of *p*-substituted styrene oxides (Grogan et al. 1997), yeast strains like *Rhodosporidium toruloides* UOFS Y-0471 and *Rhodotorula glutinis* UOFS Y-0653 can also catalyse the biocatalysis of nitro substituted styrene epoxides (Yeates et al. 2003), *Chryseomonas*

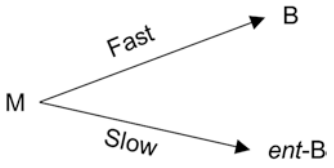


Fig. 6.9 Structure of various types of epoxides. (**a**) Monosubstituted epoxide (Type I), (**b**) styrene oxide-type epoxide (Type II), (**c**) 2,2-disubstituted epoxide (Type III) and (**d**) 2,3-disubstituted and trisubstituted epoxide (Type IV)

luteola bacterial epoxide hydrolase produced high selectivity of the remaining *(S)*-epoxide and formed *(R)*-diol with >98% and 86% ee, respectively, from 1,2-epoxyoctane (Botes et al. 1998), *Sphingomonas* sp. HXN-200 catalyses biohydrolysis of substituted styrene oxides with 98-99% ee of *(S)*-epoxides (Wu et al. 2013), and α-methyl styrene oxide derivatives produced *(S)*-ibuprofen, a non-steroidal anti-inflammatory drug using epoxide hydrolase from *A. niger* (Steinreiber and Faber 2001).

6.8.3 Disubstituted Epoxides (Type III)

Bacterial epoxide hydrolases especially from actinomycetes show best selectivities with 2,2-disubstituted (Fig. 6.9c) and 2,3-disubstituted epoxides (Type IV) (Fig. 6.9d) (Osprian et al. 2000; Steinreiber et al. 2000; Hellström et al. 2001; Steinreiber and Faber 2001). *Rhodococcus equi* IFO 3730 and *Mycobacterium paraffinicum* NCIMB 10420 hydrolyse 1,1-disubstituted epoxides with good enantioselectivity E>200 (Faber et al. 1996). Actinobacteria *Rhodococcus* and *Nocardia* spp. showed preference for *(S)*-configuration of 2,2-disubstituted epoxides with high enantioselectivity, while methylotrophic bacteria hydrolysed *(R)*-2,2-disubstituted epoxides with somewhat reduced enantioselectivity (Krenn et al. 1999). In addition to bacterial epoxide hydrolases, fungal epoxide hydrolases isolated by Botes and his co-workers can also hydrolyse 2,2-disubstituted epoxides (Botes et al. 2005). 2,3-Disubstituted epoxides were also hydrolysed by bacterial strain of *Nocardia* EH1 giving 91% ee and 79% yield (Kroutil et al. 1997). *cis*-2,3-Disubstituted haloalkyl epoxides were hydrolysed by bacterial epoxide hydrolase like *Rhodococcus ruber* DSM 44541 to produce single enantiomeric product with 92% ee and 79% yield (Steinreiber et al. 2001a).

6.8.4 Trisubstituted Epoxides (Type IV)

There is a limited data available on the enzymatic hydrolysis of trisubstituted epoxides (Archer et al. 1996; van der Werf et al. 1999; Weijers and de Bont 1999) (Fig. 6.9d) and that too not in an enantioconvergent manner due to the bulkiness of the trisubstituted epoxides which makes their enantioconvergent hydrolysis a difficult task as they require attack at the sterically crowded carbon atom (Steinreiber and Faber 2001). In 2001, Steinreiber et al. successfully hydrolysed trisubstituted epoxides enantioconvergently using whole cells of *Rhodococcus* and *Streptomyces* spp. (Steinreiber et al. 2001b).

6.9 Nomenclature of Epoxides

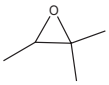
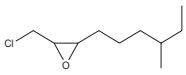
Epoxides are cyclic ethers having the oxygen atom in a three-membered ring. Epoxides are also called as oxiranes. They have a C-O-C bond angle of 60 ° which is a considerable deviation from the tetrahedral bond angle of 109.5 °. Thus, epoxides have an angle strain making them more reactive than other ethers. One epoxide can be named by various ways, so it is important to understand the nomenclature of epoxides to name them correctly.

Naming of epoxides can be done in three different ways as described below (www.chem.ucalgary.ca).

6.9.1 Epoxyalkanes

This method uses IUPAC nomenclature. If the epoxide is part of another ring system, it is named by the prefix epoxy. The root name is based on the longest chain with the ring to which the oxygen atom is attached and numbered so that the epoxide unit gets the lowest possible locant. Two numbers are used to designate the location of the atoms to which the oxygen is bonded. Then, the prefix epoxy is added before the root name along with both the locants (Table 6.1). For more substituted epoxides, this method should be used.

Table 6.1 Different methods for nomenclature of epoxides. Epoxides can be named using either of the below mentioned methods

Structure of the epoxide	IUPAC name		Common name
	Epoxy method	Oxirane method	Alkene oxide method
	1,2-Epoxy ethane	Oxirane	Ethylene oxide
	1,2-Epoxypropane	Methyl oxirane	Propene oxide
	2,3-Epoxy-2-methylbutane	2,2,3-Trimethyl oxirane	2-Methyl-2-butene oxide
	*cis*-2,3-Epoxy butane	*cis*-2,3-Dimethyl oxirane	*cis*-2-Butene oxide
	1,2-Epoxy cyclohexane	–	Cyclohexene oxide
	1-Chloro-2,3-epoxy-7-methyl nonane	–	1-Chloro-7-methyl-2-nonene oxide

6.9.2 Oxiranes

This method also uses IUPAC nomenclature. Oxiranes are simplest epoxides having two carbon atoms and one oxygen atom in a ring. Epoxides can be named as derivatives of oxiranes. Oxirane ring is numbered such that the oxygen atom is at first position and the first substituent is at second position (Table 6.1). In monosubstituted oxiranes, there is no need to number substituents. When there is a larger molecule with an epoxide or epoxide has many substituents, it is better to name it with an epoxyalkane method.

6.9.3 Alkene Oxides

Common names of epoxides are named as alkene oxides as they are prepared by adding an O atom to an alkene. The root name is for the corresponding alkene (by imaginarily removing the oxygen and adding C=C at that location), and then, the term oxide is added to it (Table 6.1).

Some examples of naming the epoxides are shown in Table 6.1.

6.10 Enzymatic Assays to Detect Epoxide Hydrolase Activity

There are different kinds of enzymatic methods to determine epoxide hydrolase activity as well as enantioselectivity, and they have their own pros and cons as discussed.

6.10.1 Spectrophotometric Assays

There are several spectrophotometric assays which are used to determine the epoxide hydrolase activity as well as enantioselectivity and are also used to screen the large mutant enzyme libraries obtained through various approaches of directed evolution by measuring the increase or decrease in absorption (Zocher et al. 1999). The epoxides/diols have usually small extinction coefficients compared to substances present in the crude cell extract, so epoxides or diols are measured by forming coloured complexes with substances which have high extinction coefficients in various assays.

6.10.1.1 4-(*p*-Nitrobenzyl) Pyridine (NBP) Assay/Blue Assay

In order to determine the epoxide hydrolase activity, NBP is the most frequently used assay, and the activity is determined by decrease of the epoxide concentration. This assay is based on the formation of dark blue colour between the epoxide and NBP in alkaline (provided by triethylamine) or acidic media. The unpaired electrons from the pyridine ring of NBP acts as a nucleophilic agent, which attacks the oxirane ring of epoxides to yield a blue chromophore. In the presence of epoxide hydrolase, epoxide gets consumed and lesser will be the concentration of epoxide, thus, lesser the intensity of blue dye that will in turn show lesser absorbance as compared to a control reaction. Thus, enzymatic activity is determined by measuring the absorption at 600 nm. The reading is taken rapidly as the coloured adduct is not stable in alkaline medium (Fig. 6.10). Zocher et al. had developed a modified NBP assay to determine the epoxide hydrolase activity in whole cells or with purified epoxide hydrolase in microtiter plates by using filter paper-based assay for rapid and accurate screening of epoxide hydrolases (Zocher et al. 1999). The one disadvantage of NBP assay is that all epoxides do not react with NBP to form a blue colour. Therefore, NBP assay with little modification has been developed by Doderer et al., to determine the epoxide hydrolase activity in bacterial cell extracts. In this method, the diol formed by epoxide hydrolase-catalysed reaction reacts with

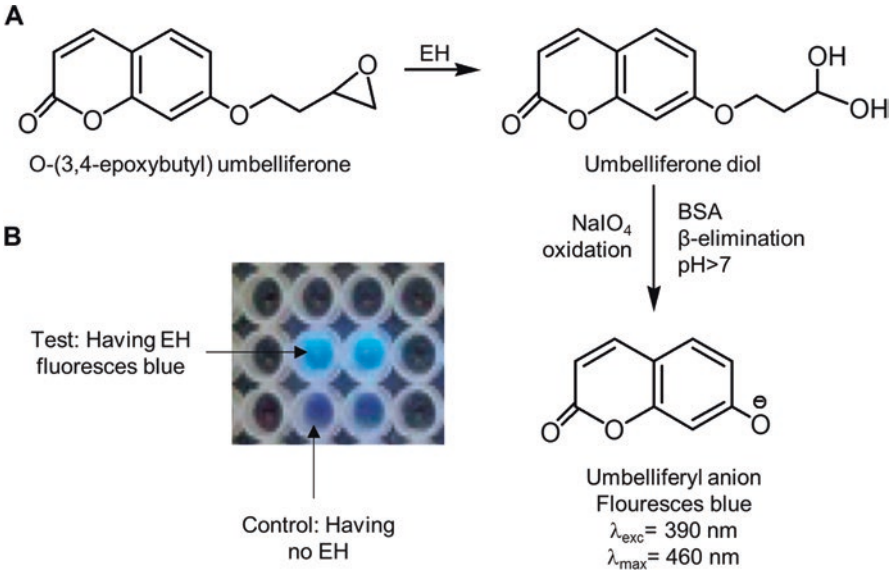


Fig. 6.10 4-(p-nitrobenzyl) pyridine assay. (**a**) Epoxide hydrolase catalyses the ring opening of styrene oxide to phenylethane-1, 2-diol. The unreacted styrene oxide then reacts with 4-(p-nitrobenzyl) pyridine and forms blue chromophore. (Adapted and modified by the permission from Alcalde et al. 2004). (**b**) A microtiter plate showing activity screening of the library of mutated epoxide hydrolases. (Adapted from Xue et al. 2015b and modified by the permission from Yu-Guo Zheng) (*NBP* 4-(p-nitrobenzyl) pyridine, *EH* epoxide hydrolase)

periodate and generates aldehydes and/or ketones which then react with Schiff's reagent. These are then colorimetrically detected by the resulting magenta dye monitored spectrophotometrically at 560 nm. This method can be used to detect any epoxide indirectly having at least one hydrogen substituent (Doderer et al. 2003). Despite these limitations, NBP is one of the most widely used method for epoxide hydrolase activity analysis due to its rapid and accurate results (Duarah et al. 2013; Xue et al. 2014, 2015a; Saini et al. 2014, 2017).

6.10.1.2 Adrenaline Assay/Red assay

Another assay to determine the epoxide hydrolase activity is adrenaline assay, also called as red assay, due to the formation of red chromophore. In adrenaline assay, the diol formed by epoxide hydrolase hydrolysis of aliphatic or aromatic epoxide is oxidized by sodium-metaperiodate ($NaIO_4$), and the remaining unreacted $NaIO_4$ reacts with adrenaline (or epinephrine) and forms red-coloured adrenochrome which is measured spectrophotometrically at 490 nm (Fig. 6.11a) (Wahler and Reymond 2002). Adrenaline assay is based on the quantification of product diol unlike the NBP assay which is based on the measurement of the unreacted epoxide. Adrenaline assay is an indirect process in which, the more the diol formed after epoxide hydrolase reaction, the more $NaIO_4$ will be consumed, so less $NaIO_4$ will be available to react with adrenaline, and less red colour will be formed (Fig. 6.11b). In addition to the epoxides, this assay is also sensitive to tributyrin, triglycerides and phytic acid which are converted from $NaIO_4$-resistant substrates to $NaIO_4$-sensitive reaction products by esterases, lipases and phytases, respectively. One limitation of this assay is that $NaIO_4$ also reacts with any other diol present in the reaction medium like glycerol which is used in preservation of the enzymes, so care must be taken while performing this assay and it should be free from any other diol (Goddard and Reymond 2004a). In 2008, Kahakeaw and Reetz had successfully applied adrenaline test for high-throughput screening of whole cells of *Aspergillus niger* mutants (Kahakeaw and Reetz 2008). Recently, Oliveira had discovered epoxide hydrolase from *Trichoderma reesei* strain QM9414 and performed its

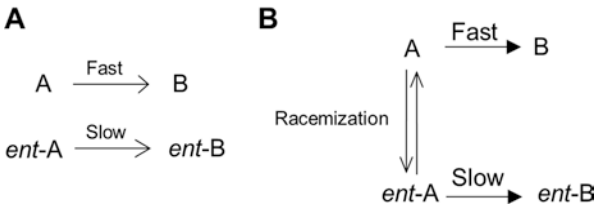


Fig. 6.11 Adrenaline assay. (**a**) Phenylethane-1, 2-diol produced after epoxide hydrolase reaction of styrene oxide (Step 1) gets oxidized by $NaIO_4$ (Step 2), and the remaining unreacted $NaIO_4$ reacts with adrenaline to form red-coloured adrenochrome (Step 3). (**b**) A microtiter plate showing activity screening of the library of mutated epoxide hydrolases. (Adapted and modified from Goddard and Reymond 2004a, b) (*EH* epoxide hydrolase)

enantioselectivity studies by Red assay and was found to preferentially hydrolysing (*S*)-styrene oxide. Cedrone et al. had strikingly compared the blue and red assays in terms of epoxide hydrolase activity and enantioselectivity. In the comparison, red assay outstands the blue assay, as the red assay is more sensitive and reproducible and the reaction rates can be measured at different substrate concentrations, and therefore, the kinetic parameters can be determined by red assay. Blue assay is somewhat cumbersome and not very accurate at low conversion ratio as it measures the decrease in epoxide concentration (Cedrone et al. 2005).

6.10.1.3 Sodium-Metaperiodate Assay

Sodium-metaperiodate assay is like adrenaline assay with minor difference as described ahead. Adrenaline assay is for both aliphatic and aromatic epoxides, but in sodium-metaperiodate assay, epoxide hydrolase activity of only aromatic epoxides can be measured. The diol formed by the enzymatic hydrolysis of aromatic epoxide is then oxidized by $NaIO_4$ into benzaldehyde which has a strong UV absorbance at 290 nm (Fig. 6.12) (Mateo et al. 2003) unlike the adrenaline assay in which aliphatic epoxides which do not give absorbance directly have to be visualized by formation of adrenochrome with adrenaline. On the contrary, sodium-metaperiodate assay is a direct process in which more the diol formed, more it will react with $NaIO_4$ and higher will be the absorbance due to abundant amount of benzaldehyde formed. This assay is rapid, sensitive and accurate and can be used for the kinetic studies of the enzyme. However, this method cannot be used with the aromatic epoxides, like *ortho*-chlorostyrene oxide and 2-pyridyloxirane as the aldehydes of these epoxides are not stable in aqueous solutions (Mateo et al. 2003).

6.10.1.4 *Para*-nitrostyrene Oxide (*p*-NSO) Assay

p-NSO assay described by Bhatnagar et al. can also be used to measure the epoxide hydrolase activity and kinetic parameters of the enzyme. In this assay, after epoxide hydrolase reaction with *p*-NSO, *p*-nitrostyrene diol (*p*-NSD) gets formed. Both *p*-NSO and *p*-NSD give strong UV absorbance at 280 nm (Bhatnagar et al. 2001).

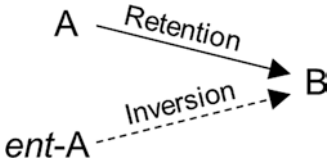


Fig. 6.12 Sodium-metaperiodate assay. After epoxide hydrolase reaction of the epoxide and formation of phenylethane-1, 2-diol (Step 1), it is oxidized by $NaIO_4$ to form benzaldehyde which is measured at 290 nm. (Adapted from Mateo et al. 2003 and modified by the permission from Roland Furstoss) (*EH* epoxide hydrolase)

After the enzymatic reaction, the formed *p*-NSD was extracted with chloroform and quantified spectrophotometrically at 280 nm. This assay is based on the direct measurement of the diol produced rather than decrease in the concentration of the epoxide. The limitation of this assay is that it can be used to measure the epoxide hydrolase activity with only those substrates which have strong UV absorbance or restricted to epoxide hydrolases which have significant activity on *p*-NSO, therefore limiting its applicability. Another limitation is that it also needs an additional extraction step which is not present in other spectrophotometric assays.

6.10.1.5 Fluorometric Assay

In fluorometric assays, signal intensity is higher, and interferences due to cell debris do not affect the epoxide hydrolase activity measurements significantly as in the case of other optical methods (Doderer and Schmid 2004). For the first time, Badalassi et al. described the fluorogenic assay for the screening of epoxide hydrolases (Badalassi et al. 2000). In fluorogenic assay, fluorogenic substrates themselves are nonfluorescent but release a fluorescent product upon reaction. After epoxide hydrolase reaction with the fluorogenic umbelliferone epoxide, the fluorescent product umbelliferone diol gets oxidized in the presence of $NaIO_4$ and is unstable in the basic medium and undergoes β-elimination catalysed by bovine serum albumin (BSA) and releases the umbelliferyl anion which produces a fluorescent signal at 460 nm after excitation at 390 nm (Badalassi et al. 2000) (Fig. 6.13a). On the basis of fluorescence-based screening with whole cells, epoxide hydrolases have been identified from *Agrobacterium tumefaciens*, *Pichia stipitis* and *Trichosporon cutaneum* (Bicalho et al. 2004). The umbelliferon-based fluorogenic assays are specific for only one substrate (i.e. umbelliferone epoxide and cannot be used with range of epoxides in the initial reaction to detect epoxide hydrolase activity). Therefore, an additional screening step with industrially important substrate or epoxides needs to be performed. Recently, Beloti et al. characterized an epoxide hydrolase from *Aspergillus brasiliensis* using the fluorogenic probe O-(3,4-epoxybutyl) umbelliferone, and enantioselectivity studies were performed using styrene oxide (Beloti et al. 2013). To determine the many reaction products, a high-throughput fluorometric assay based on the sensitivity of carboxyfluorescein towards periodate was developed. After epoxide hydrolase reaction of any substrate, the formed diols were cleaved oxidatively by periodate, and periodate is reduced to iodate. The remaining periodate then reacts with carboxyfluorescein and reduces its fluorescence. After excitation at 480 nm, the remaining fluorescence of carboxyfluorescein was measured fluorometrically at 515 nm by spectrofluorimeter (Doderer and Schmid 2004). Using chiral fluorogenic probes enantioselectivity of the epoxide, hydrolases were assessed by fluorometric assay (Mantovani et al. 2008). Substrate and enantioselectivity of dinoflagellate *Karenia brevis* were assessed using the sensitive fluorescent assay while that of the substrates having no fluorescent reporter group were evaluated by GC-MS analysis (Sun et al. 2016).

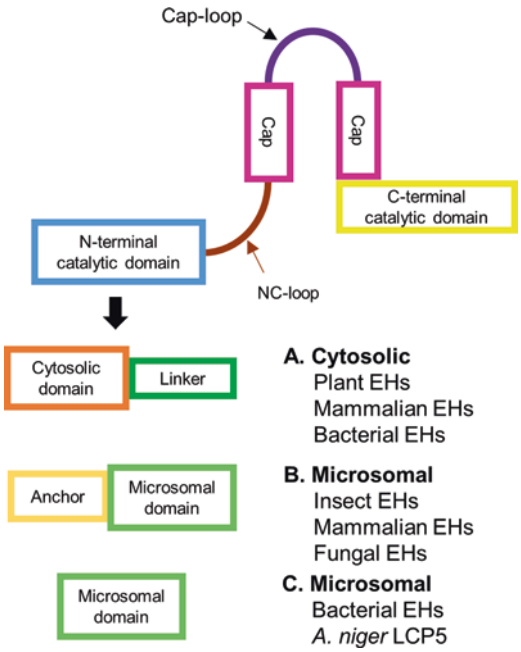


Fig. 6.13 Fluorometric assay. (**a**) Epoxide hydrolase reaction of fluorogenic epoxide produced diol which after $NaIO_4$ oxidation and β-elimination by bovine serum albumin produced umbelliferyl anion which produced fluorescent signal at 460 nm after excitation at 390 nm. (Adapted from Beloti et al. 2013 and modified by the permission from A.P. Souza). (**b**) Fluorogenic assay on microtiter plate showing blue fluorescence of umbelliferone under UV illumination in presence of epoxide hydrolase and no fluorescence in absence of epoxide hydrolase. (Adapted and modified from Reymond 2001) (*EH* epoxide hydrolase, *BSA* bovine serum Albumin)

These spectrophotometric assays can also be used for enantioselectivity analysis of the epoxide hydrolases by using enantiomerically pure epoxides (Bhatnagar et al. 2001; Cedrone et al. 2005), but the results obtained are not as reliable as achieved by chromatographic methods. In order to check the epoxide hydrolase activity with substrates such as chromophore-releasing epoxy carbonates (2*S*,3*S*)-nitrophenyl-2,3-epoxy-3-phenylpropyl-carbonate, (3*R*,3*R*)-nitrophenyl-2,3-epoxy-3-phenylpropyl-carbonate and 1-naphthyl-*trans*-2,3-epoxy-phenyl-propyl-carbonate by spectrophotometric methods, only purified epoxide hydrolases have to be used as whole cells containing esterases, or lipases can cleave the carbonate (Dietze et al. 1994).

6.10.2 Chromatographic Methods

Although the above-described spectrophotometric assays have been successfully used for the epoxide hydrolase activity analysis over the years especially for the screening of the large mutant libraries as they provide the efficient high-throughput system with ease in the epoxide hydrolase activity analysis, there are only few

reports where these methods have been used for the enantioselectivity analysis (Bhatnagar et al. 2001; Mateo et al. 2003; Beloti et al. 2013) as the degree of conversion and ee of the epoxide and diol cannot be determined precisely by these assays. Moreover, they can also result in false positives or negatives during the screening of epoxide hydrolases due to high background in cell-based assays or presence of protein in supernatant (Kahakeaw and Reetz 2008). So, there is a need for more accurate and proficient assays. Chromatographic methods like gas chromatography (GC) and high-performance liquid chromatography (HPLC) are being used in the epoxide hydrolase activity and enantioselectivity analysis since long time (Giuliano et al. 1980; Westkaemper and Hanzlik 1980; Van den Wijngaard et al. 1989). Though both HPLC and GC are time-consuming, expensive techniques and involve separation of both substrate and product by extraction before analysis (Doderer et al. 2003), they offer many advantages compared to other techniques in epoxide hydrolase activity analysis as they are highly sensitive and provide extraordinary resolution with good accuracy and precision in results, with no false positives and highly reproducible results. These techniques are also fast with less time involved in analysis, automatic and can detect very less amount of sample in microliters (Scott 2003). In both GC and HPLC, after the enzymatic reaction, the sample is extracted with organic solvents and dried over anhydrous sodium sulphate and analysed by the respective techniques. GC involves an extra step of derivatization of diols before analysis. Earlier, these methods were not used for screening as a large number of samples are involved which cannot be detected as fast as with spectrophotometric methods, but with the advancement in technology, these techniques are made user-friendly which can be handled by a single person and can be used to screen large libraries of epoxide hydrolases (Kotik et al. 2011).

6.10.2.1 Gas Chromatography

In GC, sample is vapourized and injected into the chromatographic column and carried along the system by an inert gas such as helium which constitutes the mobile phase. Even though the hydrogen gas produces better separation and efficiency, due to its flammable nature, its use is mostly prohibited. The column contains liquid stationary phase which is adsorbed onto the surface of an inert solid (Scott 2003). The column can be achiral or chiral according to the requirements of analysis. In 1989, Van den Wijngaard et al. and Pedragosa-Moreau et al. had reported epoxide hydrolase activity in three undesignated bacterial cultures using GC and identified enantiocomplementary epoxide hydrolases in *Aspergillus niger* (LCP 521) and *Beauveria sulfurescens* (ATCC 7159) using chiral GC (Van den Wijngaard et al. 1989; Pedragosa-Moreau et al. 1993). Since then, it has been used widely for both screening (Zocher et al. 2000; Choi et al. 2008; Kotika et al. 2010; Kotik et al. 2011; Woo et al. 2015) and enantioselectivity analysis (Jin et al. 2012b, 2013b; Beloti et al. 2013) of epoxide hydrolases due to shorter time involved in analysis and highly reproducible results. The limitations with this method are that only volatile compounds can be analysed and the sample must not get degraded when heated at

high temperature as the sample has to be vapourized before analysis. If the compound to be analysed is not volatile, it has to be derivatized to make it suitable for analysis. Most common detectors used in GC are destructive, i.e. they destroy the sample after analysis like flame ionization detector which is extremely sensitive detector (Wilson and Walker 2010). Sometimes, epoxide hydrolase activity and kinetic parameters are measured by using achiral columns and enantioselectivity, and regioselectivity of the epoxide hydrolase is measured by using chiral columns in GC (Kotika et al. 2010).

6.10.2.2 High-Performance Liquid Chromatography

In HPLC, mobile phase is liquid, and stationary phase is solid, and it separates the epoxides and diols based on their polarity and interaction with the stationary phase in the presence of the mobile phase, due to which they travel at different speeds and separate out. It can be applied to any compound which is both volatile or non-volatile, thermally labile and soluble in liquid phase unlike GC. The detectors in HPLC are non-destructive, and the analytes can be collected for further analysis (Wilson and Walker 2010). In comparison to GC, HPLC takes more time for result analysis and sometimes shows peak or band broadening which results in lower resolution (Cziczo 2004). In spite of these drawbacks, HPLC is used widely and it is more cost-effective than GC. For the direct separation and analysis of enantiomers, chiral HPLC is one of the most widely used method. Chiral HPLC can be performed by two methods: one is the indirect method where samples were derivatized with a chiral reagent (which is a chiral auxiliary used to convert enantiomers into diastereomers, i.e. from isomers having same physical properties to isomers with different physical properties to analyse the quantities of each enantiomer) which can be separated by either normal phase or reverse phase HPLC (Nguyen et al. 2006) and the other method is the direct HPLC, where a chiral selector is used either in stationary phase called chiral stationary phases (CSPs) or in mobile phase called chiral mobile phase additives (CMPA). CMPA is rarely used for enantiomeric analysis of epoxide or diols because of its high cost and low efficiency (Nguyen et al. 2006). Chiral separations using CSPs are more commonly used nowadays for epoxide and diol enantiomeric analysis. Earlier in 1980s, reverse phase HPLC was used to detect microsomal and cytosolic epoxide hydrolase activity in rat liver (Giuliano et al. 1980; Westkaemper and Hanzlik 1980). With the passage of time, both normal phase and reverse phase HPLC have been used widely for characterization (Nakamura et al. 1994; Manoj et al. 2001; Yu et al. 2013; Wu et al. 2014, 2015a; Hu et al. 2015; Saini et al. 2017) and screening of epoxide hydrolases (Archer 1997; Rui et al. 2005; Bala et al. 2010; Kotik et al. 2013; Kong et al. 2014). Thus, HPLC method has been established as a good alternative in long run and gives consistent results with high accuracy without any need for further treatment of samples as compared to GC.

6.11 Diversity in Epoxide Hydrolases

In nature, there are various types of epoxide hydrolases present in different organisms performing their functions which vary from organism to organism. They basically exhibit three main functions: detoxification, catabolism and regulation of signalling molecules (Morisseau and Hammock 2005). Epoxide hydrolases have been classified into microsomal epoxide hydrolases and soluble epoxide hydrolases, based on their cellular location (Fretland and Omiecinski 2000) which are further categorized into HYL1 and HYL2 (where HYL stands for hydrolase) separate families, respectively, of the superfamily of α-/β-hydrolase fold, based on the amino acid comparison (Archelas and Furstoss 1998). All epoxide hydrolases exhibit identical catalytic mechanism as described above in Sect. 6.7 except for limonene and cholesterol epoxide hydrolases. They are briefly discussed here with special emphasis on microbial epoxide hydrolases. The schematic diagram showing domains of various types of epoxide hydrolases is shown in Fig. 6.14. Both cytosolic and linker domains are present in epoxide hydrolases of plants, mammals and bacteria (Fig. 6.14a). Microsomal epoxide hydrolases of insects, mammals and fungi contain N-terminal anchor and microsomal domain (Fig. 6.14b), while microsomal epoxide hydrolases of bacteria and *A. niger* LCP521 (exception) contain only microsomal domain with no N-terminal anchor (Fig. 6.14c). N-terminal region is extremely variable among different families of epoxide hydrolases. Epoxide hydrolases of all families composed of a variable NC-loop of 16–57 amino acids which connects the conserved N-terminal catalytic domain to conserved cap domain having variable cap loop of 5–59 amino acids. The cap domain is followed by the conserved catalytic C-terminal domain. Epoxide hydrolases present in various organisms has been discussed below.

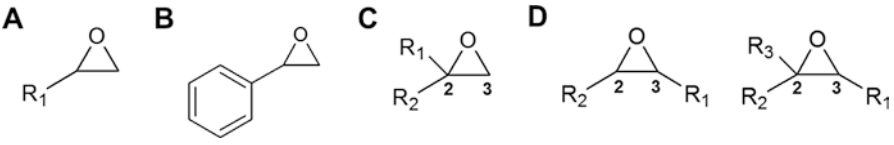


Fig. 6.14 Diagrammatic representation of domains of different homologous epoxide hydrolase families. (**A**) Plant, mammalian and bacterial soluble epoxide hydrolases consist of cytosolic domain and linker. (**B**) Insect, mammalian and fungal microsomal epoxide hydrolases consist of N-terminal membrane anchor and microsomal domain, while (**C**) bacterial and *A. niger* LCP521 microsomal epoxide hydrolases consist of only microsomal domain. (A), (**B**) and (**C**) structures described are present in all families of epoxide hydrolases: N-terminal catalytic domain, NC-loop, Cap-loop of variable length and C-terminal catalytic domain. (Adapted and modified from Barth et al. 2004) (*EHs* epoxide hydrolases)

6.11.1 Mammalian Epoxide Hydrolases

Mammalian epoxide hydrolases have been studied since long time as epoxide hydrolases have role in detoxification of epoxides to more polar metabolites or diols which can be converted to water soluble products and thus eliminated from the body (Shou et al. 1996). In mammals, five types of epoxide hydrolases are present, but soluble epoxide hydrolase and microsomal epoxide hydrolase are the two major mammalian epoxide hydrolases present in the liver named after their fractionation at 100,000g in supernatant and microsomal pellet, respectively. Microsomes are not naturally present in living cells; they are vesicle-like artefacts reformed from the pieces of the endoplasmic reticulum when eukaryotic cells are broken up in the laboratory (Voet and Voet 2004). Microsomal epoxide hydrolases and soluble epoxide hydrolases are related as they show structural characteristics signifying origin from a common ancestral gene but still have different physical properties. Both the enzymes have broad and partially overlapping substrate specificity, but their individual substrate preferences are quite distinct. Generally, mono- and *cis*-disubstituted epoxides bearing a lipophilic substituent are good substrates for microsomal epoxide hydrolase and tri- and tetra-substituted epoxides, and in particular, several *trans*-disubstituted epoxides are excellent substrates for soluble epoxide hydrolases (Fig. 3.9) (Newman et al. 2005).

6.11.1.1 Mammalian Microsomal Epoxide Hydrolase (EC 3.3.2.9)

Mammalian microsomal epoxide hydrolase was the first epoxide hydrolase characterized and isolated from mammalian liver (Newman et al. 2005). It is the best studied epoxide hydrolase which is abundantly expressed in the liver and other organs such as lungs, kidneys, intestine, brain, prostate, heart and testes (Coller et al. 2001). Human microsomal epoxide hydrolase has been named EPHX1 located on the long arm of chromosome 1 composed of eight introns and nine exons. Microsomal epoxide hydrolase is located in the endoplasmic reticulum, mostly attached to the smooth endoplasmic reticulum (Decker et al. 2009), but has also been reported in association with plasma membrane, and its molecular mass is 52 kDa (Arand et al. 1994) with a strongly hydrophobic transmembrane anchor of around 20 residues at the N-terminal (Friedberg et al. 1994; Zhu et al. 1999). The C-terminal domain containing catalytic residues is homologous to a haloalkane dehalogenase, like the soluble epoxide hydrolase (Arand et al. 1994; Beetham et al. 1995). Microsomal epoxide hydrolase generally contains glutamate as the acidic residue (Decker et al. 2009). Microsomal epoxide hydrolases are mainly involved in the detoxification processes by the conversion of lipophilic substances into more water-soluble, readily excretable compounds, and its physiological role is the hydrolysis of epoxides derived from xenobiotics, including polycyclic aromatic hydrocarbons. Generally good substrates for the microsomal epoxide hydrolase are lipophilic substituted epoxides of *cis*-configuration such as *cis*-stilbene oxide.

Typical substrates include toxic and procarcinogenic compounds, as well as commonly used anticonvulsant drugs (Decker et al. 2009). Microsomal epoxide hydrolase catalysed hydrolysis of several β-alkyl substituted styrene oxide derivatives, the *cis*-1, 2-disubstituted epoxides and *trans*-epoxides and resulted in the production of enantiopure diols with good enantioselectivity. Mammalian microsomal epoxide hydrolase showing high substrate and product enantioselectivities appeared to be a useful biocatalyst for the production of enantiopure epoxides and vicinal diols (Weijers and de Bont 1999). Like soluble epoxide hydrolase, microsomal epoxide hydrolase is also associated with polymorphisms with the onset of various cancers in humans (Baxter et al. 2002).

6.11.1.2 Mammalian Soluble Epoxide Hydrolase (EC 3.3.2.10)

Mammalian soluble epoxide hydrolase was discovered after mammalian microsomal epoxide hydrolase. It is also called as cytosolic epoxide hydrolase and is predominantly localized in the cytosol along with some amount in peroxisomal matrix of liver cells (Newman et al. 2005). The X-ray structure of murine (Argiriadi et al. 1999) and human (Gomez et al. 2004) soluble epoxide hydrolases has been solved. The human soluble epoxide hydrolase gene EPHX2 is located on the chromosome 8, ~54 kb in size, and consists of 19 exons and 18 introns, respectively (Sandberg and Meijer 1996). It is a homodimer of 62 kDa monomeric subunits and consists of two catalytic domains, a 25 kDa phosphatase domain and 35 kDa epoxide hydrolase domain at the N- and C-terminus, respectively, and separated by a proline rich linker (Decker et al. 2009; Harris and Hammock 2013). N-terminal domain plays a crucial role in the stabilization and dimerization of the enzyme (Argiriadi et al. 1999). It is largely distributed, existing in almost every organ like liver, lungs, kidney, heart, brain and ovary (Enayetallah et al. 2004; Sura et al. 2008) restricted mainly in cytosol, but sometimes it shows dual localization, cytosolic and peroxisomal due to a defective C-terminal peroxisomal targeting sequence (PTS-1) (Arand et al. 1991; Mullen et al. 1999). It can hydrolyse various epoxides derived from xenobiotic compounds, and their primary biological function is the metabolic transformation of epoxides produced from endogenous substrates, especially from fatty acids, i.e. lipid metabolism and regulation of blood pressure and inflammation (Lee and Shuler 2007). Through metabolism of lipid signalling molecules like epoxyeicosatrienoic acids (Imig et al. 2012), they are involved in several diseases like hypertension, cardiac hypertrophy, arteriosclerosis, cancer, pain, etc. (Morisseau and Hammock 2013) and are anticipated as a pharmacological target for which small molecular inhibitors are available (Shen and Hammock 2012). The two bifunctional domains of soluble epoxide hydrolases are result of gene fusion event (Beetham et al. 1995) as the C-terminal domain of soluble epoxide hydrolase is homologous to bacterial haloalkane dehalogenase as well as to single domain epoxide hydrolases of fungi, plants and invertebrates (Newman et al. 2005; Harris et al. 2008) and the N-terminal domain is homologous to bacterial haloacid dehalogenase (Beetham et al. 1995) (Fig. 6.14). Soluble epoxide hydrolases generally catalyse the

hydrolysis of *trans*-substituted epoxides as well as aliphatic epoxides. Soluble epoxide hydrolase activity has been found in many vertebrates like rat (*Rattus norvegicus*), domestic horse (*Eqqus caballus*), primates such as rhesus monkey (Macaca mulatta), baboon (*Papio* sp.), human (*Homo sapiens*), etc. (Newman et al. 2005). In soluble epoxide hydrolase, several single nucleotide polymorphism sequences have been identified which are associated with the onset of several diseases and cancers (Kiyohara et al. 2002). The substrate and product enantioselectivity of soluble epoxide hydrolase is lower than microsomal epoxide hydrolase. Therefore, mammalian soluble epoxide hydrolase soluble epoxide hydrolase has only a subsidiary role in biocatalysis compared to microsomal epoxide hydrolase (Lee and Shuler 2007)**.**

6.11.1.3 Hepoxilin A_3 Epoxide Hydrolase (EC 3.3.2.7)

It is a cytosolic or soluble epoxide hydrolase which metabolizes hepoxilins to trioxilins (Fretland and Omiecinski 2000). It is partly characterized, and its structural information is also not available (Decker et al. 2009). It is ubiquitously present as a monomeric enzyme in mammalian tissues, and its biological role is mainly studied in tissues of vascular or central nervous system origin (Pace-Asciak 1994). Its preferred substrate is hepoxilin A_3 (a hydroxy epoxide derivative of arachidonic acid) and shows negligible activity towards leukotriene or styrene oxide (Pace-Asciak and Lee 1989). Hepoxilins are lipid signalling molecules and derivatives of arachidonic acid formed by 12-lipooxygenase pathway. The molecular weight of hepoxilin A_3 epoxide hydrolase partly purified from rat liver cytosol is 53 kDa (Cronin et al. 2011). The coinciding substrate specificity and subcellular localization of hepoxilin A_3 epoxide hydrolase to mammalian soluble epoxide hydrolase suggest that they may have complimentary roles and are identical, and like the soluble epoxide hydrolases, they also play role in regulation of inflammation (Newman et al. 2005; Cronin et al. 2011). The catalytic mechanism of this epoxide hydrolase is not yet identified (Fretland and Omiecinski 2000). Structurally hepoxilins are classified into two groups: one is the γ-hydroxy epoxides (hepoxilin As) and the other is the α-hydroxy epoxides (hepoxilin Bs) (Pace-Asciak 1994).

6.11.1.4 Leukotriene A_4 Hydrolase (LTA_4H) (3.3.2.6)

LTA_4H is an atypical monomeric soluble epoxide hydrolase (Arand et al. 2005) of 69 kDa having three domains, N-terminal, C-terminal and a catalytic domain (Thunnissen et al. 2001), and catalyses hydration of leukotriene A_4 (5,6-epoxide) into leukotriene B_4 (5,12-diol) which is involved in respiratory and inflammatory disorders, particularly in erythrocytes (Mcgee and Fitzpatricks 1985). LTA_4H is a bifunctional enzyme as it possesses, in addition to epoxide hydrolase activity, a peptidase activity and the catalytic sites of both are overlapping but are not similar. Also, in the reaction mechanism of both (epoxide hydrolase and peptidase), different amino acid residues Asp^{375} in case of epoxide hydrolase and Glu^{296} in case of

peptidase are used for the activation of water molecule. The catalytic domain of LTA_4H contains zinc-binding motif which is structurally similar to that present in thermolysin with the signature HEXXH-X_{18}-E having three zinc binding ligands His-295, His-299 and Glu-318, and fourth ligand is always an activated water molecule and contains 1 mole of zinc per mole of protein. LTA_4H is classified as a member of M1 family of zinc metallopeptidases based on its zinc signature and aminopeptidase activity (Haeggström 2004). Zinc as a cofactor is required for both epoxide hydrolase and peptidase activity. Sequence alignment of LTA_4H is not related to other soluble epoxide hydrolases or microsomal epoxide hydrolases, and the reaction mechanism is also different which involves formation of a carbocation (Beetham et al. 1995; Andberg et al. 1999) which suggests that it does not belong to α-/β-hydrolase fold enzyme family and forms non-vicinal diol as a product (Decker et al. 2009). LTA_4H gene in humans is located on chromosome 12q22 and has been characterized (Mancini and Evans 1995), and its crystal structure has been elucidated (Thunnissen et al. 2001). Haeggstrom et al. has published reviews on LTA_4H detailing the mechanism, substrate specificity and catalytic important residues (Haeggström 2000; Haeggström et al. 2007).

6.11.1.5 Cholesterol Epoxide Hydrolase (3.3.2.11)

Cholesterol epoxide hydrolase is a microsomal epoxide hydrolase which catalyses the hydration of cholesterol 5α-, 6α- and 5β- and 6β-oxide to cholestane 3β-, 5α- and 6β-triol and has limited substrate specificity. It shows fivefold preference for α- over the β-diastereomer (Sevanian and Mcleod 1986). Its gene has not yet been cloned or characterized (Fretland and Omiecinski 2000), so there is not much data available on it. It has wide tissue distribution with highest enzyme activity in mammalian liver microsomes (Astrom et al. 1986). Recently, it was shown that cholesterol epoxide hydrolase is a hetero-oligomeric complex composed of anti-oestrogen-binding site (AEBS), which consists of two subunits: 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I, also known as the emopamil-binding protein (EBP)) and 3β-hydroxysterol-Δ^7-reductase (DHCR7). Both enzymes are required for post-lanosterol cholesterol biosynthesis (de Medina et al. 2010). Thus, identification of molecular identity of this enzyme opened new perceptions regarding the role of cholesterol epoxide hydrolase with cancer as defined by (Silvente-Poirot and Poirot 2012). Its reaction mechanism does not involve formation of covalent ester-enzyme intermediate with its substrate and involves a one-step general base mechanism with the formation of positively charged transition state (Nashed et al. 1986). This is the first mammalian epoxide hydrolase member having reaction mechanism similar to limonene epoxide hydrolase from *Rhodococcus erythropolis* bacteria (Decker et al. 2009) which is described in detail ahead. Further, its size is too small (Watabe et al. 1986), and it is not structurally related to microsomal epoxide hydrolases or soluble epoxide hydrolases (Muller et al. 1997); therefore, it does not fall in the category of typical α-/β-hydrolase fold enzymes. Cholesterol epoxides are reported to be weak direct-acting mutagen as when they

get accumulated in cells, they get converted to cholestane triols which are more toxic and potent inhibitor of DNA synthesis than epoxide but at concentrations more than 17.8 μM. Therefore, the production of cholesterol epoxide and cholestane triols favours mutagenicity and cytotoxicity, respectively (Sevanian and Peterson 1984). Hence, a detailed characterization of cholesterol epoxide hydrolase will decipher its physiological role completely (Decker et al. 2009).

6.11.2 Plant epoxide Hydrolases

They are soluble monomeric or dimeric ~35 kDa proteins (Summerer et al. 2002) homologous to C-terminal domain of mammalian soluble epoxide hydrolase (Beetham et al. 1995). Plant epoxide hydrolases have the highest number of soluble epoxide hydrolases expressed in *E. coli* among the eukaryotes (Koschorreck et al. 2005). The structure of plant soluble epoxide hydrolases is similar to mammalian soluble epoxide hydrolases with the exemption that there is an additional loop present in the lid domain which prevents their dimerization (Mowbray et al. 2006). Newman et al. described that plant soluble epoxide hydrolases have been known in various plants and have been isolated from fruits, roots, tuber, leaves, etc. (Newman et al. 2005). Recently, an enantioconvergent soluble epoxide hydrolase from *Vigna radiata* producing 99% ee and 71.5% yield of *(R)-p*-nitrophenyl glycol has been expressed in *E. coli* (Wu et al. 2015a, b). The favourable substrates for plant soluble epoxide hydrolases are *trans*-epoxides (Bellevik et al. 2002), and they prefer epoxide containing fatty acids as the endogenous substrates. They also produce ample range of epoxides containing lipids in biochemical pathways which are associated with plant defence responses (Blee 2002; Howe and Schilmiller 2002) and cutin polymer biosynthesis (Kolattukudy 2001; Lequeu et al. 2003). The first and the most studied and well characterized of the plant epoxide hydrolases are from soya bean (Blee and Schuber 1992). Cutin is a waxy coating covering the outer surface of a plant and provides physical barrier to pathogens while allowing the gaseous exchange (Heredia 2003). For protection against pathogens, plant epoxide hydrolases also secrete antifungal substances like linoleate-derived triols (Hamberg 1999). As epoxides are reactive molecules, hence their accumulation in plants during stress is toxic, and they are degraded by epoxide hydrolases (Murray et al. 1993). There are two major clades; EH1 and EH2 present in plant epoxide hydrolases with the exception of *Arabidopsis thaliana* which has only EH2 type genes (Wijekoon et al. 2008). The detailed characterization of EH1- and EH2-type epoxide hydrolases has been discussed by Huang and Schwab on epoxide hydrolases from *Nicotiana benthamiana* (Huang and Schwab 2013).

6.11.3 Insect/Juvenile Hormone Epoxide Hydrolase (EC 3.3.2.3)

Insect epoxide hydrolase is a microsomal enzyme which is involved in metabolism of toxic compounds and detoxification of allelochemicals (plant chemical defence substances) found in the diet and regulation of developmental chemical mediator, juvenile hormone. Juvenile hormone epoxide hydrolase is a significant enzyme in the regulation of the insect/juvenile hormone and hence greatly influences insect physiology (Newman et al. 2005). Juvenile hormone titres are strictly regulated in different stages of development through balance between biosynthetic and degradation pathways. In insects, seven types of juvenile hormones (JHs) (JH 0, JH I, JH II, JH III, 4-methyl JH I, JH III skipped bisepoxide and JH III bisepoxide) have been identified. Among them, JH III is the most abundant (Kotaki et al. 2009). Structurally, all juvenile hormones are sesquiterpenes composed of an epoxide at one end or near the end of the molecule and α, β-unsaturated methyl ester at the other end. In insects, juvenile hormone is metabolized by three enzymes: juvenile hormone esterase (EC 3.1.1.1), juvenile hormone epoxide hydrolase and juvenile hormone diol kinase. The methyl ester portion of juvenile hormone is degraded by juvenile hormone esterase which converts juvenile hormone to juvenile hormone acid and juvenile hormone epoxide hydrolase hydrolyses the epoxide part in juvenile hormone to juvenile hormone diol and also degrades juvenile hormone acid to juvenile hormone acid diol. Then, yet again juvenile hormone esterase degrades juvenile hormone diol. Finally, juvenile hormone diol kinase metabolizes juvenile hormone diol to juvenile hormone diol phosphate which gets excreted out of the body (Share and Roe 1988). Juvenile hormone esterase and juvenile hormone epoxide hydrolase are expressed in almost all tissues of the insects, while juvenile hormone diol kinase is observed mainly in the midgut with trace levels in the Malpighian tubules and haemocytes (Hua-jun et al. 2011). The comparative role of epoxide hydration and ester hydrolysis in juvenile hormone catabolism differs with species and insect life stage (Slade and Zibbitt 1972). Juvenile hormone epoxide hydrolase shows structural resemblance and are homologous to mammalian microsomal epoxide hydrolases; hence, the mechanism of juvenile hormone epoxide hydrolase hydrolysis is similar to them (Morisseau and Hammock 2005). Like mammalian microsomal epoxide hydrolase, juvenile hormone epoxide hydrolase also contains N-terminal membrane anchor and is a monomeric enzyme. Several juvenile hormone epoxide hydrolases from different insects have been characterized like *Manduca sexta* (tobacco hornworm) (Debernard et al. 1998), *Bombyx mori* (silkworm) (Zhang et al. 2005), *Tribolium castaneum* (red flour beetle) (Tsubota et al. 2010), *Heliothis virescens* (tobacco budworm) (Kamita et al. 2013b), *Homalodisca vitripennis* (glassy-winged sharpshooter)

(Kamita et al. 2013a), *Anopheles gambiae* (mosquito) (Xu et al. 2014), *Leptinotarsa decemlineata* (Colorado potato beetle) (Lü et al. 2015) and *Culex quinquefasciatus* (the southern house mosquito) (Xu et al. 2015). The study of juvenile hormone epoxide hydrolase from *Anopheles gambiae* suggests that mammalian soluble epoxide hydrolase homologs are also present in insects (Xu et al. 2014). The copy number of juvenile hormone epoxide hydrolase genes varies in various insects (Lü et al. 2015). Large-scale production of insect epoxide hydrolases is still challenging which limit their biocatalytic applications (Weijers and de Bont 1999).

6.11.4 Microbial Epoxide Hydrolases

Interest in microbial epoxide hydrolases has increased over the years as they provide great potential as a source of enantioselective epoxide hydrolases with unlimited production by the use of recombinant DNA techniques and green technology which lessens the chemical pollution in the environment (Archelas and Furstoss 2001). In microbes, epoxides are formed as intermediates in the degradation of alkenes or halohydrins which are used as carbon and energy source for growth by them. The advent of new methods for the screening and selection of enzymes from microbial sources has opened the unexplored treasures of epoxide converting biocatalysts (de Vries and Janssen 2003). Microbial epoxide hydrolases comprise of fungal and bacterial epoxide hydrolases, and they consist of both microsomal epoxide hydrolases and soluble epoxide hydrolases. Best substrates for fungal epoxide hydrolases include styrene oxide-type substrates and for red yeasts the monosubstituted oxiranes (Botes et al. 1999), while 2,2- and 2,3-disubstituted epoxides are the catalyst of choice for bacterial epoxide hydrolases (Kroutil et al. 1997; Osprian et al. 2000). The reaction mechanism of microbial epoxide hydrolases (as already explained) is different from limonene epoxide hydrolase reaction mechanism.

6.11.4.1 Fungal Epoxide Hydrolases

More and more epoxide hydrolases have now been explored from fungal sources which comprise of eukaryotic organisms: unicellular microorganisms like yeasts and moulds as well as multicellular microorganisms like mushrooms. Fungus *Aspergillus niger* and basidiomycetes red yeasts *Rhodotorula glutinis* and *Rhodosporidium toruloides* have been greatly explored for the enantioselective resolution of epoxides (Archelas and Furstoss 2001; Smit 2004). Recombinant *Aspergillus niger* is commercially available from Fluka (Monfort et al. 2002). Fungal epoxide hydrolases are mostly microsomal. *A. niger*, *R. glutinis* and *R. toruloides* microsomal epoxide hydrolases have been demonstrated to have great potential as biocatalysts as they are active against a wide range of substrates and are stable when present as lyophilized enzyme preparations (Smit 2004). Fungal epoxide hydrolases react particularly with aryl- and substituted alicyclic substrates but not

with aliphatic epoxides. However, yeast epoxide hydrolases can react even with unbranched terminal aliphatic-1,2-epoxides enantioselectively (Weijers and de Bont 1999). Fungal epoxide hydrolase from *Aspergillus niger* LCP521 is an exception, as according to the amino acid sequence alignment, it has been grouped with microsomal epoxide hydrolases though it is found in the soluble fraction and has 25% sequence similarity to microsomal epoxide hydrolases of mammals and insects than to other soluble epoxide hydrolases. Like mammalian microsomal epoxide hydrolases, *A. niger* LCP521 gene has nine exons and eight introns which are remarkably a high number for an *A. niger* gene. The only difference between the *A. niger* epoxide hydrolase and the microsomal epoxide hydrolases is the absence of the N-terminal membrane anchor in the *A. niger* epoxide hydrolase. It also shows high turnover number in comparison to other mammalian epoxide hydrolases. The fungal epoxide hydrolase has an aspartic acid as the acidic residue in the catalytic triad of the charge relay system instead of glutamic acid which is present in all microsomal epoxide hydrolases (Arand et al. 1999). Microsomal epoxide hydrolases from *Rhodotorula*, *Rhodosporidium* and *Xanthophyllomyces dendrorhous* are all membrane associated with N-terminal anchor. An enantioconvergent epoxide hydrolase from fungus *Aspergillus tubingensis* TF1 carried out the biotransformation of racemic styrene oxide to *(R)*-1-phenyl-1, 2-ethane diol with >99% yield and 97% ee (Duarah et al. 2013). A number of epoxide hydrolases of fungal and yeast origin have been discovered such as *Botryosphaeria dothidea* ZJUZQ007 (Sheng et al. 2011), *Rhodosporidium paludigenum* (Labuschagne et al. 2004), *Talaromyces flavus* (Wei et al. 2012) and *Xanthophyllomyces dendrorhous* (Visser et al. 1999) which can enantioselectively hydrolyse racemic epoxides. An epoxide hydrolase from fungus *Diplodia gossypina* commercialized by Merck resolves racemic indene oxide to (1*S*, 2*R*)-indene oxide and *(R)*-indane diol. Chiral (1*S*,2*R*)-indene oxide is used as a precursor for the synthesis of the side chain of the HIV protease inhibitor MK 639 (Jinyou et al. 1995). *Aspergillus* species is a store house of epoxide hydrolase genes as a lot of epoxide hydrolase have been sequenced and cloned from different *Aspergillus* species like *A. niger* M200 (Kotik et al. 2005), *A. niger* SQ-6 (Liu et al. 2007), *A. niger* ZJB-09173 (Jin et al. 2012b), *A. brasiliensis* CCT 1435 (Beloti et al. 2013), *A. niger* ZJUTZQ208 (Chen et al. 2013) and *A. usamii* E001 (Hu et al. 2015). Due to the stability of epoxide hydrolases in whole cells, they are also being rapidly employed for the hydrolytic reactions of styrene oxide (Duarah et al. 2013) or glycidyl ether derivatives (Martins et al. 2011).

6.11.4.2 Bacterial Epoxide Hydrolases

Bacterial epoxide hydrolases are both microsomal and cytosolic, but most microsomal bacterial epoxide hydrolases lack N-terminal anchor (Barth et al. 2004). Like fungi, many epoxide hydrolases have been known from bacteria which can catalyse the resolution of racemic epoxides for the production of industrially significant chiral diols. Different approaches like genome database mining (van Loo et al. 2006; Saini et al. 2014, 2017), metagenome screening (Montaña et al. 2012; Jimenez et al.

2015) and enrichment strategies (da Cruz et al. 2010) have been adopted for the screening of enantioselective epoxide hydrolases from bacterial strains. Epoxide hydrolysis for the first time was reported by the use of bacterial strain *Pseudomonas putida* (Allen and Jakoby 1969) for the industrial synthesis of *L*- and *meso*-tartaric acid. Initially, much focus was on the mammalian epoxide hydrolases but due to limitation in their large scale production, attention moved to fungal and bacterial epoxide hydrolases (Archelas and Furstoss 1998). Apart from soil, since oceans cover a large part of the earth, they also offer abundant resources for the discovery of new epoxide hydrolases for the production of pharmacologically important drugs (Woo et al. 2009, 2010a). Recently, marine bacteria *Rhodococcus* sp. YSM104 and YSNA32 and *Roseobacter* sp. TSBP12 have been isolated from oil-spilled offshore which showed high epoxide hydrolase activities for the production of various chiral chlorinated derivatives of styrene oxide (Woo et al. 2015). Glycidyl phenyl ether which is a useful intermediate in the synthesis of chiral amino alcohols and bioactive compounds like β-blockers can be produced from (*R*)-glycidyl phenyl ether having >99.9% enantiopurity using epoxide hydrolase from marine bacterium *Rhodobacterales* HTCC2654 (Woo et al. 2010b). *Erythrobacter litoralis* HTCC2594, a marine bacteria, possesses three epoxide hydrolases genes: *eeh1* which is similar to mammalian microsomal epoxide hydrolases but lacks N-terminal membrane and is soluble when overexpressed and shows enantioselectivity towards *(R)*-styrene oxide, while *eeh2* and *eeh3* are related to mammalian soluble epoxide hydrolases. *eeh2* could hydrolyse both *(R)*-styrene oxide and *(S)*-styrene oxide at an equal ratio, and *eeh3* showed enantiopreference towards *(S)*-styrene oxide (Woo et al. 2007). As enantiopure molecules are one of the important requirement for the production of chiral drugs, this was accomplished by various bacterial strains such as *Tsukamurella paurometabola* (Wu et al. 2015a, b), *Nocardia tartaricans* (Wang et al. 2013), *Bordetella* sp. BK-52 (Pan 2010), *Streptomyces* sp. (Zocher et al. 2000), *Streptomyces globisporus* (Lin et al. 2009), *Sphingomonas* sp. HXN-200 (Wu et al. 2013), *Acinetobacter baumannii* (Choi et al. 2008), and *Streptomyces carzinostaticus* (Lin et al. 2010). Commercialization of bacterial epoxide hydrolase from *Rhodococcus rhodochrous* by Sigma-Aldrich for the production of enantiopure epoxides or diols has increased the attention of the pharmaceutical industries to discover more novel microbial epoxide hydrolases having high enantioselectivity.

6.11.5 Limonene Epoxide Hydrolase (EC 3.3.2.8)

Limonene epoxide hydrolase has been cloned and characterized from *Rhodococcus erythropolis*. It does not share any similarity of conserved regions and mechanism with any α-/β-hydrolase fold enzyme, and its size is relatively small of 150 amino acids, and thus, it does not belong to α-/β-hydrolases. It is a monomeric cytoplasmic enzyme with MW of 17 kDa which converts limonene-1, 2-epoxide to limonene-1, 2-diol. It can grow on limonene as a sole source of carbon and energy (Barbirato et al. 1998; Werf et al. 1998). It has a narrow substrate spectrum and shows activity

with only limonene-1,2-epoxide, 1-methylcyclohexene oxide, cyclohexene oxide and indene oxide (Barbirato et al. 1998). In addition to bacteria *R. erythropolis* (Barbirato et al. 1998) and *Mycobacterium tuberculosis* (Johansson et al. 2005), limonene epoxide hydrolase has also been found in fungus *Fusarium oxysporum* (Molina et al. 2015) and *Grosmannia clavigera* (Wang et al. 2014) and was also identified by metagenomics approach (Ferrandi et al. 2015b). The catalytic reaction of limonene epoxide hydrolase proceeds through single step in contrast to α-/β-hydrolases. Structure of limonene epoxide hydrolase comprise of cone–shell-like single domain fold consisting of six β-strands and three α-helices. The catalytic triad in limonene epoxide hydrolase consists of Asp-Arg-Asp. The reaction mechanism follows S_N2 pathway and does not involve formation of covalent ester-enzyme intermediate. The reaction follows general acid/base catalysis. The substrate-binding site consists of two aspartate, Asp^{101} and Asp^{132}, present at the bottom which are actively involved in catalysis. Arg^{99} forms a network of hydrogen bonds with these aspartates and participates in catalysis by positioning the carboxylate group of these aspartates. Asp^{132} activates the catalytic water by proton abstraction supported by Tyr^{53} and Asn^{55}, which are lining the substrate binding cavity (base catalysis). These two also form hydrogen bonds with water and hold it in proper position for the nucleophilic attack on the substrate. Simultaneously, Asp^{101} donates its proton to the oxygen of the epoxide for its ring opening (acid catalysis). Lastly, after release of the hydrolysis product, the proton which shifted from Asp^{101} to Asp^{132} gets transferred back rapidly through Arg^{99} (Arand et al. 2003) (Fig. 6.15). In this pathway, enzyme limonene epoxide hydrolase can be regenerated very rapidly (Arand et al. 2005). Limonene epoxide hydrolase structure shows high resemblance to structure of cholesterol epoxide hydrolase found in *M. tuberculosis.* Cholesterol epoxide hydrolase is also present in mammals, but limonene epoxide hydrolase present in *R. erythropolis* and *M. tuberculosis* did not show any resemblance to mammalian cholesterol epoxide hydrolase (Johansson et al. 2005).

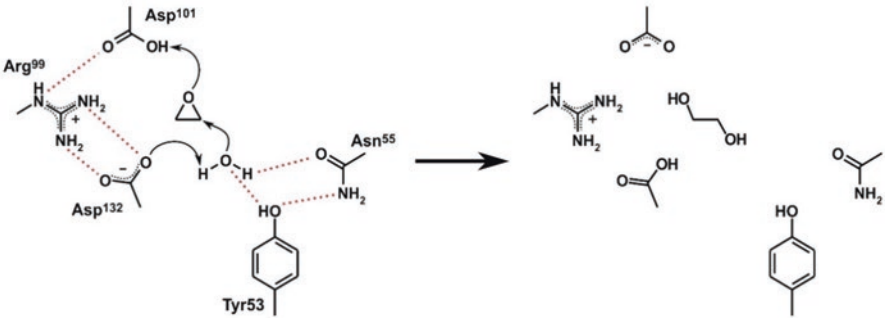


Fig. 6.15 Catalytic reaction mechanism of limonene epoxide hydrolase. Reaction mechanism of limonene epoxide hydrolase is different than other epoxide hydrolases and occurs in a single step having catalytic triad Asp-Arg-Asp instead of Asp-His-Asp and does not involve formation of a covalent ester-enzyme intermediate. (Adapted from Widersten et al. 2010)

As can be viewed from the above-discussed literature, there are abundant sources of epoxide hydrolases which can be seen as a potential source of industrially important enzymes. Abundantly available epoxide hydrolases can react with wide range of racemic epoxides in an enantioselective manner to produce epoxides and diols as chirally important drug intermediates.

6.12 Conclusion

This chapter enlightens about the need for greener biocatalytic methods, especially epoxide hydrolases, over the chemo-catalytic methods for the production of enantiopure chiral epoxides and diols. As the growth in chiral pharmaceutical industry has soared up, these chiral epoxides and diols serve as important drug intermediates and cause less toxic pollution, when synthesized biocatalytically. Microbial epoxide hydrolases provide great potential as a source of enantioselective epoxide hydrolases as they can be produced in larger amounts. Here, advantages and disadvantages of the various screening methodologies of epoxide hydrolases have been discussed in detail, along with the wide spectrum of epoxide hydrolases in various life forms and their broad substrate spectra. This study will give researchers a quick and detailed insight about these enantioselective epoxide hydrolases as promising industrial biocatalysts.

Acknowledgements This work was financially supported by the Department of Biotechnology (DBT) via Grant No. BT/PR/4694/PID/6/633/2012, Government of India, New Delhi. PS gratefully acknowledges DBT for the SRF. The financial assistance received from the Department of Science and Technology-Promotion of University Research and Scientific Excellence (DST-PURSE) and University Grants Commission-Special Assistance Programme (UGC-SAP) (DRS Phase-I) is duly acknowledged.

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Chapter 7
Nanoimmobilization of β-Galactosidase for Lactose-Free Product Development

Ethiraj Selvarajan, Anbazagan Nivetha, Chandrasekaran Subathra Devi, and Vaithilingam Mohanasrinivasan

Contents

E. Selvarajan
School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

Department of Genetic Engineering, School of Bioengineering, SRM Institute of Science & Technology, Kattankulathur, Chennai, Tamil Nadu, India

A. Nivetha · C. Subathra Devi · V. Mohanasrinivasan (✉)
School of Bio Sciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India
e-mail: v.mohan@vit.ac.in

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_7

Abstract It is estimated that over 70% of the world's adult population have problems in digesting lactose resulting from absent or reduced β-galactosidase activity in the small intestine. Estimates of the number of Americans affected by lactose intolerance (LI) range between 30 and 50 million, whereas approximately 75 million Americans are lactose maldigesters. Maldigestion is also a common occurrence in adults who have low-intestinal lactase activity. Lactose that is not digested transits to the lower small intestine and large intestine, thus creating the potential for symptoms. β-Galactosidase is one of the relatively few enzymes that have been used in large-scale processes to perform lactose hydrolysis and galacto-oligosaccharide production. Immobilization is the limitation of movement of biocatalysts according to chemical or physical treatment. Immobilized molecules technique using biomaterials and nano-biotechnology is a very interesting topic that is touching almost all aspects of our life. This review outlines information regarding lactose intolerance, overview of β-galactosidase and recent advances of nanoimmobilization on β-galactosidase to study lactose hydrolysis potential. The plausible advantages with their use include their (1) biocatalyst efficiency, (2) specific surface area, (3) mass transfer resistance and (4) effective enzyme loading. Enzyme immobilization is a usual requirement as a solution to obtain reusable biocatalysts and thus decrease the price of the expensive biocatalysts. Various immobilization methods have been developed, and in particular, specific attachment of enzymes on metal oxides such as ZnO has been an important focus of attention. The method of immobilization has an effect on the preservation of the enzyme structure and retention of the native biological function of the enzyme. Enzymes immobilized onto nanoparticles showed a broader working pH and temperature range and higher thermal stability than the native enzymes.

7.1 Introduction

7.1.1 Lactose Intolerance

Lactose is the major disaccharide found in milk and milk products. It is catabolized into glucose and galactose by the enzyme β-galactosidase. Normally, it gets absorbed into the intestine and travels through the bloodstream after getting broken down into these two forms of sugar. Lining the wall of the small intestine is the enzyme lactase that helps with the breakdown process, and in its complete absence or partial deficiency, the stomach can't digest the lactose (Roth 2006). Inside a lactose-deficient

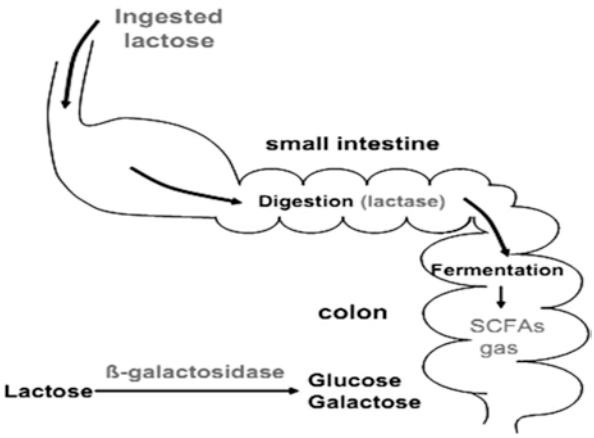

Fig. 7.1 Fate of lactose in the gastrointestinal tract (Venema 2012). To digest lactose, the body produces a digestive enzyme in the gut called lactase. If the individual does not produce enough of the lactase enzyme to completely digest the lactose, the undigested portion remains in the small intestines ultimately moving into the colon where it is left to ferment. This fermentation process is what produces the gases and symptoms associated with lactose intolerance

person's gastrointestinal system, lactose is left untouched until it reaches the large intestine (colon) where bacteria finally break them apart (Fig. 7.1). This flawed metabolism leads to production of lactic acids and other chemicals which cause distress. The signs and symptoms of lactose intolerance usually begin 30 min to 2 h after drinking or eating foods that contain lactose (Mayo Clinic Staff 2010). Lactose is not completely catabolized in lactose-intolerant individuals because of lactase deficiency, and this will lead to certain symptoms such as diarrhoea, nausea, abdominal cramps, bloating, flatulence, loss of appetite, etc. Feeling sick or presenting with these symptoms after consuming lactose-containing milk for one time doesn't make the person lactose intolerant; the symptoms have to repeat every time a product containing lactose is consumed. Lactose-free dairy products such as ice cream and aged cheese are sometimes tolerated by people who are lactose intolerant (Rosdahl and Kowalski 2008). Lactose concentration in milk is inversely related to the content of fat and protein. Fascinatingly, human milk contains the highest concentration [7%] of lactose in mammals. Besides this, lactose is a sugar with a high biological oxygen demand, low sweetness, low solubility and a strong tendency to adsorb flavours and odours when compared to glucose and galactose (Ladero et al. 2000, 2001).

Lactose intolerance appears to be hereditary, passed down from parents through genes. Specific population shows high levels of lactose intolerance, while others do not. In India, about 20% of the population is suffering from lactose intolerance, particularly infants or newborn babies and adults. Lactose intolerance occurs in 70% of the world's adult population, and Eastern Asia has the highest number of lactose malabsorbers with more than 90% of its population (Vrese et al. 2001). To avoid lactose intolerance problems, milk and milk-related products are enzymatically

hydrolysed using β-galactosidase. Lactose hydrolysis causes several changes with potential value for the manufacture and marketing of dairy products (Hettwer and Wang 1990).

Many people have low levels of lactase, but most don't experience signs and symptoms. Only people with both low lactase levels who also have associated signs and symptoms have, by definition, lactose intolerance. In certain populations, there are many individuals in whom the activity of lactase is insufficient to produce satisfactory lactose hydrolysis, provoking bloating, abdominal cramps, flatulence, nausea, diarrhoea and loss of appetite, known as lactose intolerance (Di Stefano and Veneto 2001).

7.2 Classification of Lactose Intolerance

Lactose intolerance can be classified into primary, secondary and congenital.

7.2.1 Primary Lactose Intolerance

The human body naturally produces large amounts of lactase during infancy and early childhood as it gets fed with mother's milk primarily. But as the baby's breastfeeding schedule is reduced and more varied nutrient sources are introduced, the production of lactase decreases. This type of decrease in production usually begins after about age 2 and may not present with any symptoms until the adulthood age is reached, and therefore, sometimes it is also known as adult hypolactasia (Moore 2003). It is believed to be genetically programmed from the beginning, however, and the differences in prevalence of adult hypolactasia among different ethnic groups are very noticeable (Heyman 2006). Nearly 75% of the world's population have primary lactase deficiency (Kretchmer 1972).

7.2.2 Secondary Lactose Intolerance

Secondary lactose intolerance though is more common during infancy; it can develop at any age because it is caused by illness in the intestinal walls of the digestive system. Such gastrointestinal diseases include celiac disease, gastroenteritis and an inflammatory bowel syndrome of Crohn's disease; they can involve injury or rupturing of the epithelium linings where lactase enzymes are located (Savaiano and Levitt 1987; Scrimshaw and Murray 1988; Srinivasan and Minocha 1998).

7.2.3 Congenital Lactose Intolerance

Congenital lactose intolerance is a rare form, in which babies are born with complete absence of lactase. It occurs due to mutation of gene responsible for lactase production and is inherited from parents. This gene is recessive in nature; therefore, both the parents must pass on this defective gene to the child to make baby lactose intolerance. Babies with this type of lactose intolerance are not able to tolerate even their own mother's milk and exhibit symptoms shortly after birth (Diekmann et al. 2015).

7.3 Worldwide Distribution of Lactose Intolerance

According to reviews by Scrimshaw and Murray (1988) and Sahi (1994), the global prevalence of lactose maldigestion is above 50% in South America, Africa and Asia reaching almost 100% in some Asian countries such as China. In the USA, prevalence is 15% among Whites, 53% among Mexican-Americans and 80% in the Black population. In Europe, it varies from around 2% in Scandinavia to about 70% in Sicily. Australia and New Zealand have prevalence of 6% and 9%, respectively (Sahi 1994).

Adult-type hypolactasia also known as lactase non-persistence is genetically determinate (Enattah et al. 2002; Troelsen 2005). Its prevalence in Western countries varies widely from 4% in Denmark (Busk et al. 1975) to around 50% in Northern Italy (Burgio et al. 1984). In North Indians the frequency of lactose maldigesters was reported to be 48.5% out of 200 subjects when measured by lactose hydrogen breath test (Rana et al. 2004).

Lactose intolerance appears to be hereditary, passed down from parents through genes. Specific population shows high levels of lactose intolerance, while others do not. The approximate rates of lactose intolerance in various populations around the world according to Swagerty et al. (2002) are given in Fig. 7.2.

In Finland the incidence of hypolactasia is about 17% (Sahi 1994). Here the need for low-lactose or lactose-free products has been exceptionally high due to the high consumption of milk. Lactose-free milk has strongly gained more popularity and has become one of the basic milks in Finland and other countries (Jelen and Tossavainen 2003).

7.4 Fate of Lactose in the Intestine

Inside a lactose deficient person's gastrointestinal system, lactose is left untouched until it reaches the large intestine (colon) where bacteria finally break them apart. This faulty metabolism leads to production of lactic acids and other chemicals

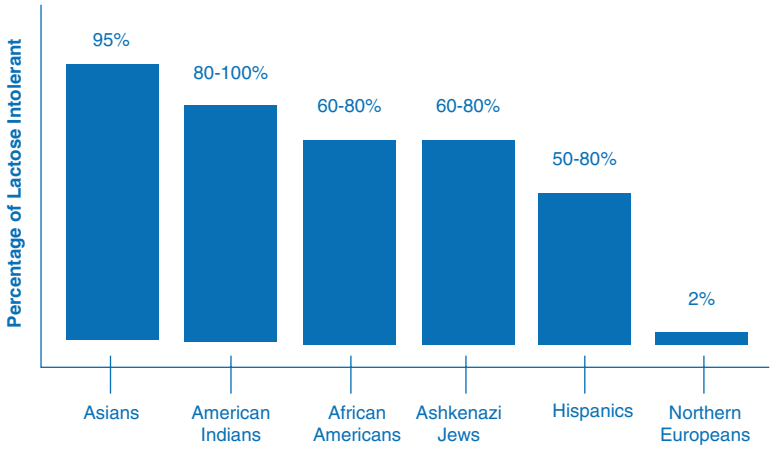

Fig. 7.2 The percentage of lactose intolerance at various parts of the world (Swagerty et al. 2002). The prevalence is above 50% in South America, Africa and Asia, reaching almost 100% in some Asian countries. In the United States, the prevalence is 15% among Whites, 53% among Mexican-Americans and 80% in the Black population. In Europe it varies from around 2%. Australia and New Zealand have prevalence of 6% and 9%, respectively. In general, it can be stated that about two thirds of the world adult population is lactase non-persistent

which cause distress. The signs and symptoms of lactose intolerance usually begin 30 min to 2 h after drinking or eating foods that contain lactose (Mayo Clinic Staff 2010). These symptoms include bloating, pain or cramps in the lower belly, rumbling stomach sounds, gas, loose stools, abdominal distention and nausea.

7.5 Treatment for Lactose Intolerance

At present, a different range of treatments is suggested to deal with lactose intolerance, depending on the patients. The most common method for very sensitive individuals is to eliminate diet. Patients can avoid lactose-containing foods which include dairy and non-dairy products. However, dairy products are a major source of calcium and are necessary for good health and strong bones. Calcium deficiency may lead to osteoporosis and bone fractures.

The second choice is to consume lactose-reduced dairy products. However the current treatment to remove lactose from milk is by addition of lactase enzyme. Following lactase treatment, the taste of the milk is drastically changed to sweeter because the lactose is hydrolysed to glucose and galactose (Harju et al. 2012). Furthermore, the commercial products are expensive, and thus cost limits public acceptance. So, to overcome the limitations, the enzyme can be immobilized and reused.

7.6 Lactose-Free Milk

The milk which doesn't contain lactose is simply termed as lactose-free milk. The lactase enzyme has been added to it, breaking down the lactose into digestible glucose and galactose. Lactose-free milk is available for individuals who are unable to break down lactose, the natural sugar found in milk. Lactose-free milk is a convenient way for lactose-intolerant individuals to consume milk and its beneficial nutrients. As a result, lactose-free milk may be a little sweeter than regular milk. The composition of lactose-free and low-lactose milk is summarized in Table 7.1. Europe is a worldwide leader in the lactose-free market. Sales of lactose-free products are expected to increase 75% between 2012 and 2016 reaching €529 M by the end of this period (Prescott 2012; Valio 2013).

7.7 β-Galactosidase Overview

7.7.1 Lactic Acid Bacteria

Lactic acid bacteria comprise a heterogeneous group of non-sporulating Gram-positive organisms which ferment sugars and produce lactic acid (Ozkaya et al. 2001). Genera of lactic acid bacteria include, among others, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus* (Kandler and Weiss 1986). With over 100 species and subspecies, the genus *Lactobacillus* represents the largest group within the family Lactobacillaceae. All lactic acid bacteria are anaerobes; however they are facultative anaerobes, and they can grow in the presence of oxygen. Some strains produce H_2O_2 through flavoprotein oxidase systems and eliminate H_2O_2 by their catalase or peroxidase. Lactic acid bacteria use lactose as their main source of carbon to produce energy (Madigan et al. 1997). Owing to some of their metabolic properties, lactic acid bacteria play an important role in the food industry, because they significantly contribute to the flavour and texture and in many cases to the nutritional value of the food products (McKay and Baldwin 1990). *L. plantarum* survives even at low pH of the stomach

Table 7.1 Composition of lactose-free milk and pasteurized low-fat milk (Harju 2007)

% Composition	Lactose-free milk	Pasteurized low-fat milk
Protein	3.3	3.3
Carbohydrates	3.1	4.8
Lactose	<0.01	4.8
Ash	0.7	0.7
Fat	1.5	1.5
Calcium (mg/100 g)	120	120
Energy (kJ)	160	193

and duodenum, resisting the effects of bile acids in the upper small intestine when ingested and temporarily colonizing the gastrointestinal tract by binding to the intestinal and colonic mucosa.

7.7.2 β-Galactosidase

β-Galactosidases (EC 3.2.1.23) are present in a wide variety of organisms including plants, animals and microorganisms and are known to catalyse both hydrolytic and transglycosylation reactions. Microbial β-galactosidases have a prominent position in terms of their role in the production of various industrially relevant products like biosensor, lactose-hydrolysed milk, the production of galacto-oligosaccharides for use in probiotic foodstuffs, etc. The enzyme can be obtained from microbial cells such as bacteria, fungi or yeast with variable properties depending on the species (Richmond et al. 1981; Hubber et al. 1994; Hung et al. 2001). Although, the most studied β-galactosidase is produced by *E. coli*, possible toxic factors associated with coliforms make it unlikely that crude isolates of this enzyme, which may be permitted in food processes (Laxmi et al. 2011). The major β-galactosidase enzymes of commercial interest are isolated mainly from the yeast *Kluyveromyces lactis*, *K. fragilis*, *K. marxianus*, *Candida kefyr* and *Saccharomyces cerevisiae* and the fungi *Aspergillus niger* and *A. oryzae* (Neri et al. 2008). On the basis of amino acid similarities**,** β-galactosidases have been separated into four glycoside hydrolase (GH) families: 1, 2, 35 and 42 (Henrissat and Davies 1997; Hidaka et al. 2002; Nakkharat and Haltrich 2006; Wang et al. 2009).

7.7.3 Sources of β-Galactosidase

The enzyme lactase (β-galactosidase) belongs to the group of sugar-converting enzymes in the family of hydrolases as well as other hydrolytic enzymes, for instance, lipases, esterases, peptidases etc. β-Galactosidase can be obtained from a wide variety of sources such as microorganisms, plants and animals; yet, according to their sources, their properties vary distinctly (Mahoney 1998). Enzymes of plants and animal origin have little commercial value, but several microbial sources of β-galactosidase are of vast technological interest. Microorganisms offer various advantages over other avaiLactic acid bacteriale sources such as easy handling, higher multiplication rate and high production yield. As a result of commercial interest in β-galactosidase, a large number of microorganisms (Finocchiaro et al. 1980; Joshi et al. 1989; Cho et al. 2003; Berger et al. 1995) have been assessed as potential sources of this enzyme.

β-Galactosidase can be produced by a large number of bacteria. The enzyme from *Escherichia coli* serves as a model for understanding the catalytic mechanism of β-galactosidase action, but it is not considered suitable for use in foods due to

toxicity problems associated with the host coliform (German 1997). Hence, the β-galactosidase from *E. coli* is generally not preferred for use in food industry (Joshi et al. 1989). Yeast has been considered as vital source of β-galactosidase from industrial point of aspect. With neutral pH optima, these are well suited for hydrolysis of lactose in milk and are widely accepted as safe for use in foods. But much work has been carried on the production of β-galactosidase from different yeast strains for its potential use (Panesar et al. 2010).

At the industrial level, β-galactosidases are attractive enzymes due to their hydrolase and transferase activities (Mahoney 1998). Indeed, these enzymes are used for the production of oligosaccharides related to their transglycosylation activity allowing the transfer of galactose hydroxyl groups to the disaccharide lactose (Rycroft et al. 2001). The produced galacto-oligosaccharides are very promising prebiotic agents, due to their effect on the decrease in clostridia numbers and on short-chain fatty acid generation (Rycroft et al. 2001). As a result of their hydrolytic activity, β-galactosidases are mainly used in the food industry to reduce the lactose concentration in milk products, with the aim of overcoming lactose intolerance, a worldwide problem (Scrimshaw and Murray 1988). The decrease in lactose concentration which permits milk consumption by lactose-intolerant individuals was the first probiotic effect identified and was exploited in *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) strains, the two lactic acid bacteria used for yoghurt production (Guarner et al. 2005).

7.7.4 Structure and Reaction Mechanism of β-Galactosidase

The enzymatic product of the LacZ gene, β-galactosidase, catalyses the hydrolysis of β-D-galactosides, such as lactose, into their component sugars by hydrolysis of the terminal nonreducing β-D-galactose residues. β-Galactosidase catalyses the hydrolysis of the terminal β-D-galacto-pyranoside moiety resulting in the formation of the yellow (λmax 420 nm) compound ortho-nitrophenol. Since milk is an opaque liquid, it is difficult to work with because there is no noticeable change. There is a substance called ortho-nitrophenyl-β-galactoside which can be used as a substitute for the milk. Ortho-nitrophenyl-β-galactoside is a colourless liquid. Lactase splits ortho-nitrophenyl-β-galactoside into ortho-nitrophenol and galactose. Ortho-nitrophenol is a clear yellow material, so we can observe a colour change from clear to yellow and use a spectrophotometer to measure how much product is being produced. The enzyme does not distinguish between lactose and ortho-nitrophenyl-β-galactoside and cleaves the oxygen bridge between the two sides of the molecule, resulting in the products galactose and the o-nitrophenol. The compound ortho-nitrophenol absorbs light at 420 nm, whereas the precursor molecule ortho-nitrophenyl-β-galactoside does not (Lederberg 1950). Therefore, the increase in light absorbance at 420 nm can be used to monitor β-galactosidase when ortho-nitrophenyl-β-galactoside is used as a substrate (Fig. 7.3). β-Galactosidase catalyses the breakdown of the substrate lactose, a disaccharide sugar found in milk into two

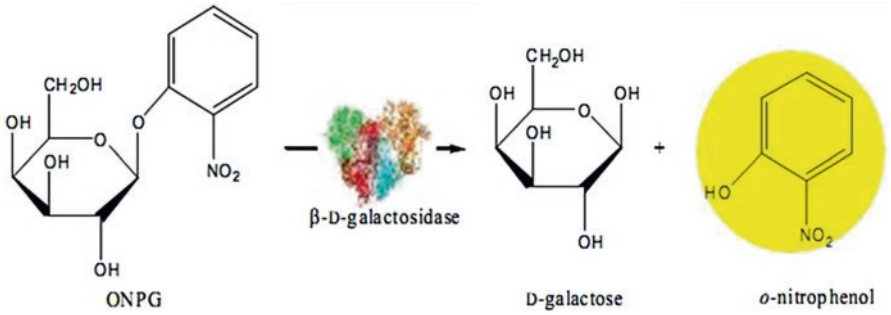

Fig. 7.3 Hydrolysis reaction of ortho-nitrophenyl-β-galactoside in the presence of β-D-galactosidase. When the β-galactosidase cleaves ONPG (*o*-nitrophenol group), ortho-nitrophenol is released. ONPG is colourless, while the product compound has a yellow colour and absorbs light (λmax = 420 nm). Therefore, enzyme activity can be measured by the rate of appearance of yellow colour using a spectrophotometer

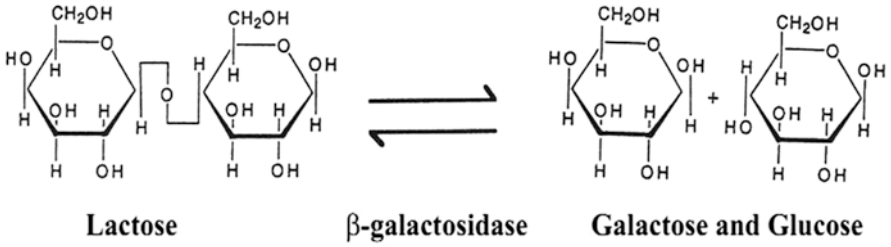

Fig. 7.4 Enzymatic hydrolysis of lactose to glucose and galactose by β-galactosidase. β-galactosidase catalyses the breakdown of the substrate lactose, a disaccharide sugar found in milk, into two monosaccharide sugars, galactose and glucose. The oxygen bridge connecting the two sides of the lactose molecule is cleaved through the addition of a water molecule

monosaccharide sugars, galactose and glucose (Fig. 7.4) (Matthews 2005). Deactivation mechanisms can be complex, since β-galactosidase has highly defined structures, and the slightest deviation from their native form can affect their specific activity. Better knowledge of β-galactosidase stability under the operating conditions could help optimize the profitability of enzymatic processes. The activity and thermal stability of enzymes are influenced by diverse environmental factors such as temperature and pH which can strongly affect the specific three-dimensional structures or spatial conformation of the protein (Jurado et al. 2004). Ustok et al. (2010) studied biochemical and thermal properties of β-galactosidase enzymes produced by pure and mixed cultures of *Streptococcus thermophilus* 95/2 (St 95/2) and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 (Lb 77). The inactivation energies of β-galactosidase from Lb 77, St 95/2 and mixed culture (Lb 77 and St 95/2) were 51.3, 44.0 and 48.3 kcal mol^{-1}, respectively.

Several researchers have already determined some of the properties and kinetic parameters of β-galactosidase, such as the deactivation rate constants (kd), half-life

(t1/2), deactivation energy and the kinetic constants (Km and Vmax) (Jurado et al. 2004; Ustok et al. 2010; Ladero et al. 2000). However, there are few papers in the literature considering the thermal and kinetic properties that compare crude and purified enzymes (Braga et al. 2013).

7.8 Immobilization

Enzyme immobilization is a technique that not only enhances the stability of enzyme but also helps in the separation of enzyme from the product, thereby minimizing the chances of product contamination. Immobilization could be a key to improve enzyme stability, recovery and reuse of expensive catalysts. In addition, utilization of immobilized enzymes permits to greatly simplify the design of industrial reactors and to facilitate the control of process parameters.

Immobilization of β-galactosidase can be achieved by various methods such as adsorption, covalent attachment, chemical aggregation, entrapment and encapsulation. Compared with soluble β-galactosidase, immobilized β-galactosidase may provide many advantages in production of lactose-reduced dairy commodities, such as high enzyme reusability, high yield, improvement of thermal stability, continuous operation, controlled product formation, high reactor productivity and no contamination of product by the enzyme with simplified and efficient processing (Kishore et al. 2012). Enzymes are too expensive to be discarded after single use, which makes their commercial exploitation uneconomical. Thus, immobilization of enzymes could be an essential crucial requirement of industrial utilization.

7.8.1 Overview of Enzyme Immobilization

Immobilized enzymes are generally more stable, and there are many potential applications that range from chemical synthesis to biotechnology and medicine (Liang et al. 2000). Immobilization enables use of the enzymes in continuous, fixed-bed operation. Furthermore, immobilization leads to a more convenient handling of the enzyme, minimizing or eliminating protein contamination of the product (Sheldon 2007) Therefore, immobilization of enzymes could be, in short time, a requirement of industrial utilization.

The potential advantages associated with the immobilization of biocatalysts can be summarized as follows (End and Schöning 2004):

- Higher stability with regard to temperature, pH and catalyst poisoning
- Repeated use of biocatalysts
- Higher resistance to shear stress and contamination
- Increased reaction rate due to high catalyst concentration (for certain reactor types)

7.8.2 Methods for Immobilization of β-Galactosidase

Based on the nature of interaction that leads to immobilization, immobilization methods are classified as physical and chemical methods. A different approach is to classify immobilization methods as irreversible and reversible (Guisán 2006). Immobilization has shown to improve the stability of β-galactosidase, reusages, and reduces the processing time in food and other industries.

7.8.2.1 Physical Methods

The physical immobilization technique can be divided into adsorption and entrapment. It can be further divided into non-covalent adsorption and ionic adsorption.

7.8.2.1.1 Adsorption

Physical adsorption is considered as the simplest method of immobilization in which an enzyme is immobilized onto a water-insoluble carrier and the biocatalysts are held on the surface of the carriers by physical forces (van der Waals forces). Still, additional forces are involved in the interaction between carrier and biocatalyst principally hydrophobic interactions, hydrogen bridges and heteropolar (ionic) bonds (Hartmeier 1986).

7.8.2.1.2 Encapsulation

Encapsulation is one simple immobilization method realized under mild conditions, commonly with minimum denaturation of the enzyme. By potentially taking the inherent advantages of both bio-polymeric and inorganic materials, polymer–inorganic hybrid carriers have been increasingly applied in enzyme encapsulation. β-Galactosidase microencapsulation in lipid vesicles has been delivered for treating lactose intolerance, but there was a problem of contact between the enzyme and substrate (Walde and Ichikawa 2001; Monnard 2003; Nogales and Lopez 2006).

7.8.2.1.3 Entrapment

Enzyme entrapment is typically achieved using a polymer network such as an organic polymer or sol–gel and is usually performed in situ (Sheldon 2007). Entrapment protects enzymes by preventing direct contact with the environment, thereby minimizing the effects of gas bubbles, mechanical sheer and hydrophobic solvents, but has the drawback of mass transfer limitations and low enzyme loading.

7.8.2.1.4 Ionic Adsorption

Ionic adsorption was the oldest immobilization method that has been used at industrial scale (Chibata 1978). Ionic attachment to non-ionic surfaces can also be realized through a polyvalent metal cation. Chelation of a transition metal by both the carrier surface and the enzyme results in binding to the surface. Braga et al. (2013) immobilized β-galactosidase on Eupergit C, and immobilization kinetics were investigated.

7.8.2.2 Chemical Methods

The chemical immobilization technique can be divided into covalent binding and cross-linking.

7.8.2.2.1 Covalent Binding

Covalent binding is probably the most complex immobilization method, as allows a large number of attachment possibilities between supports and enzymes. The immobilization of enzymes by covalent attachment to a solid carrier involves formation of a covalent bond between amino acid side-chain residues of the protein with reactive groups on the support surface. The major advantage of covalent binding is stabilization of the immobilized enzyme. Covalent attachment is the preferred method if enzyme value is high, minimal protein leaching from the support is required or rational control of the biocatalyst properties is desired. Polyurethane powder from hexamethylene diisocyanate and butanediol has also been exploited to immobilize *Penicillium canescens* β-galactosidase by covalent binding between the amino groups of enzyme and the isocyanate groups in polyurethane powder (Budriene et al. 2005). β-galactosidase from *Kluyveromyces lactis* was covalently immobilized onto a magnetic polysilosane-polyvinyl alcohol using glutaraldehyde as activating agent.

β-Galactosidase was covalently immobilized onto a gold-coated magnetoelastic film via a self-assembled monolayer of ω-carboxylic acid alkylthiol containing terminal carboxylic group (Ball et al. 2003). Verma et al. (2012) have recently developed an elegant method in which β-d-galactosidase from *Kluyveromyces lactis* was covalently immobilized to functionalized silicon dioxide nanoparticles and maximum lactose hydrolysis by immobilized β-d-galactosidase was achieved at 8 h.

7.8.2.2.2 Cross-Linking

Cross-linking means construction of three-dimensional enzyme structures by linking the enzyme molecules covalently. Cross-linking of enzyme aggregates or crystals, using a bifunctional reagent, allows preparation of carrierless macroparticles.

The main advantages of the cross-linking immobilization method are (i) more concentrated enzyme activity in the catalyst than in case of carrierbound enzymes, (ii) high stability and (iii) lower production costs, as the (potentially) expensive carrier is no more needed. Main drawbacks of this method are the difficult control of aggregates size, difficult substrate accessibility to the cores of aggregates and lack of mechanical strength of cross-linked enzyme (Zhao et al. 2006).

7.9 Nanoimmobilization

Nanomaterials always have advantages and preferences over bulk materials in terms of their miniature size, large surface area with high enzyme loading capacity and aqueous suspendability for uniform distribution throughout reaction mixture. Nanostructures are very attractive for enzymatic immobilization processes, since they possess ultimate characteristics to equilibrate principal factors which determine biocatalysts efficiency, including specific surface area, mass transfer resistance and effective enzyme loading. Enzymes immobilized on nanoparticles may show high stability in a wide range of temperature and pH, compared to free enzymes. A lot of materials are used at nanosize in processes of immobilization, like silica, chitosan, gold, diamond and metals, including graphene and zirconium (Cipolatti et al. 2014). Therefore, nanomaterials are getting more attentions for utilization as a support material for efficient enzyme immobilization. Its exploitation can be achieved in production of lactose-reduced dairy commodities at industrial scale, which could be used for harmless consumption by lactose-intolerant individuals. Nanotechnology has significantly improved and modernized several technology and industrial sectors including medicine, food safety and many others. Nanomaterials are at the leading edge of the rapidly developing field of nanotechnology. Recently, applications of semiconductor metal oxide nanoparticles are getting more extensive covering different fields such as optoelectronics, catalysis, medicine and sensor devices. Among the metal oxide nanostructures, ZnO nanoparticles have extensively been investigated for several technological applications such as catalysis, gas sensing (Lin et al. 1998), cancer treatment, chemical absorbent (Singhal et al. 1997), antibacterial and UV-blocking properties (Wang et al. 2005, 2009; Yadav et al. 2006) and cosmetic and pharmaceutical industries (Li et al. 2007). In the recent past, metal oxide nanoparticles have been exploited as a potential candidate in immobilizing industrially important enzyme**.** Apart from this, Zn compounds have been currently listed as GRAS, i.e. generally regarded as safe by the US Food and Drug Administration (21CFRI82.8991).

7.9.1 Physical Synthesis

In physical synthesis, two methods are employed. They are planetary ball milling and pyrolysis. It is generally known as mechanical alloying and widely used for nanoparticles preparation. It is capable to produce solid–solid, solid–liquid and solid–gas chemical reactions. It can be used for large-scale production. The important parameters for mechanical milling process was milling time, milling speed, charge ratio, temperature nature of milling atmosphere, chemical composition of the powder mixture, milling machine, etc. In this method the particles are ground by a size-reducing mechanism and air classified to get the oxidized nanoparticles (Goya 2004). In pyrolysis, an organic precursor is hurried through an orifice with high pressure and burned. Then the ash is air classified to get the oxidized nanoparticles. In both of the above-mentioned methods, the major disadvantage is high consumption of energy to maintain pressure and temperature. It is a costly method (Grimm et al. 1997).

7.9.2 Chemical Synthesis

In wet-chemical methods, the nanoparticles were produced in a liquid medium containing reduced agents like sodium borohydride or potassium bitartrate or hydrazine. The agglomerations of nanoparticles are protected by adding stabilizing agents like sodium dodecyl benzyl sulphate or polyvinylpyrrolidone. Chemical methods are low cost, but the major disadvantage is usage of toxic solvents results in the formation of hazardous by-products (Thakkar et al. 2010).

7.9.3 Biological Synthesis

Among the various methods reported for synthesis of nanoparticles, biological method is of great interest because in physical and chemical methods, the use of expensive chemicals as reducing and capping agents and toxic solvents along with the tedious process control, as well as heating at high temperature at reduced pressure, limits their biological applications. To overcome these issues, there is great demand for biogenic synthesis of nanoparticles. Among the microorganisms, prokaryotic bacteria have received the most attention in the area of biosynthesis of nanoparticles (Singh et al. 2014).

7.9.4 Nanoparticles in Enzyme Immobilization

Recently, immobilization of industrially important enzymes onto nanomaterials with improved performance has paved the way to myriad of application-based commercialization (Wolf et al. 2003; Qayyum et al. 2011). Nanostructure materials exhibited interesting properties such as large surface to volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability that make them potential candidate materials to play an important role in enzyme immobilization (Yoon et al. 2000). The large surface area of nanomaterials provided better matrix for the immobilization of enzymes leading to increased enzyme loading per unit mass of particles. Moreover, the multipoint attachment of enzyme molecules to nanomaterials surface reduces protein unfolding resulting in the enhanced stability of the enzyme attached to the surface of nanoparticles (Jia et al. 2003). Nanostructured materials with ability to control size and shape enable better interaction with the enzyme, increase immobilization efficiency and enhance the long-term storage and recycling stability of the enzyme (Kim et al. 2008).

A novel and efficient immobilization of β-d-galactosidase from *Aspergillus oryzae* has been developed by using magnetic Fe_3O_4–chitosan (Fe_3O_4–CS) nanoparticles as support (Pan et al. 2009). Kishore et al. (2012) reported that β-galactosidase was covalently attached onto functionalized graphene nanosheets for various analytical applications based on lactose reduction. Numerous conventions about the immobilization of proteins on nanoparticles have been created up to this point. Consequently, it is imperative to consider compound immobilization utilizing nanoparticles.

Zinc oxide (ZnO) is listed as 'generally recognized as safe' (GRAS) by the US Food and Drug Administration (21CFR182.8991). Transition metal oxide nanostructures and semiconductors have generated considerable interest as next-generation technologies (Wang 2004). Zinc oxide (ZnO), a multi-tasking metal oxide, is considered to be one of the best metal oxides that can be used at a nanoscale due to its unique optical and electrical properties (Vayssieres et al. 2001; Konenkamp et al. 2002), making it a potential substance with wide applications in optoelectronics, many industrial areas and pharmaceutical and cosmetic industries. The low cost and non-toxicity of ZnO uniquely make it suitable for many purposes.

7.9.5 Recent Research About Immobilization of β-Galactosidase

Immobilization is not a new method, and it is widely used in other industries especially in food, pharmaceuticals and biotechnology. β-Galactosidases have been immobilized by several methods onto a variety of matrices, including entrapment, cross-linking, adsorption, covalent binding or a combination of these methods (Table 7.2). β-Galactosidase from *Lactobacillus plantarum* HF571129 was

Table 7.2 Immobilized β-galactosidase and its methods

Source of enzyme	Method of immobilization	Supports used	Equipment/ bioreactor	Conversion (%)	References
Saccharomyces fragilis	Covalent coupling	Corn grits	Packed-bed reactor	Hydrolysis rates (50% within 3 h)	Siso et al. (1994)
Kluyveromyces lactis	Entrapment	Calcium alginate beads	Laboratory scale bioreactor with recirculation	99.5% of hydrolysis (30 h)	Becerra et al. (2001)
Kluyveromyces fragilis	Covalent coupling activated by epichlorohydrin	Cellulose beads	Fluidized-bed reactor	>90% conversion in 5 h	Roy and Gupta (2003)
Aspergillus oryzae	Entrapment	Alginate-gelatin beads	–	–	Freitas et al. (2011)
Aspergillus oryzae	Bioaffinity	Con A-cellulose	Packed-bed reactor	90% lactose hydrolysed (after 10 days)	Ansari and Husain (2010)
β-Galactosidase *(P. sativum)*	Cross linker	AuNp	–	–	Dwevedi et al. (2009)
Kluyveromyces lactis	Cross linker	Polysiloxane–polyvinyl alcohol magnetic (mPOS–PVA)	Batch	90% lactose hydrolysed (after 120 min)	Neri et al. (2008)
β-Galactosidase *(Cicer arietinum)*		Graphene nanosheets			Kishore et al. (2012)
Kluyveromyces lactis	Covalent	Chitosan nanoparticles	Batch	75% lactose hydrolysed	Klein et al. (2013)
Bacillus circulans	Covalent	Epoxy-activated acrylic	Packed bed	90% lactose hydrolysed	Torres and Viera (2012)
Aspergillus oryzae	Covalent	Magnetic Fe_3O_4–chitosan nanoparticles	Batch	50% lactose hydrolysed	Pan et al. (2009)
Kluyveromyces lactis	Covalent	Silicon dioxide nanoparticles	Batch	50% lactose hydrolysed	Verma et al. (2012)

immobilized on zinc oxide nanoparticles (ZnO NPs) using adsorption and cross-linking technique (Selvarajan et al. 2015). The mechanism adsorption of ZnO NPs and β-galactosidase on the surface of calcium alginate-starch beads and cross-linked with glutaraldehyde and epichlorohydrin is given in Fig. 7.5.

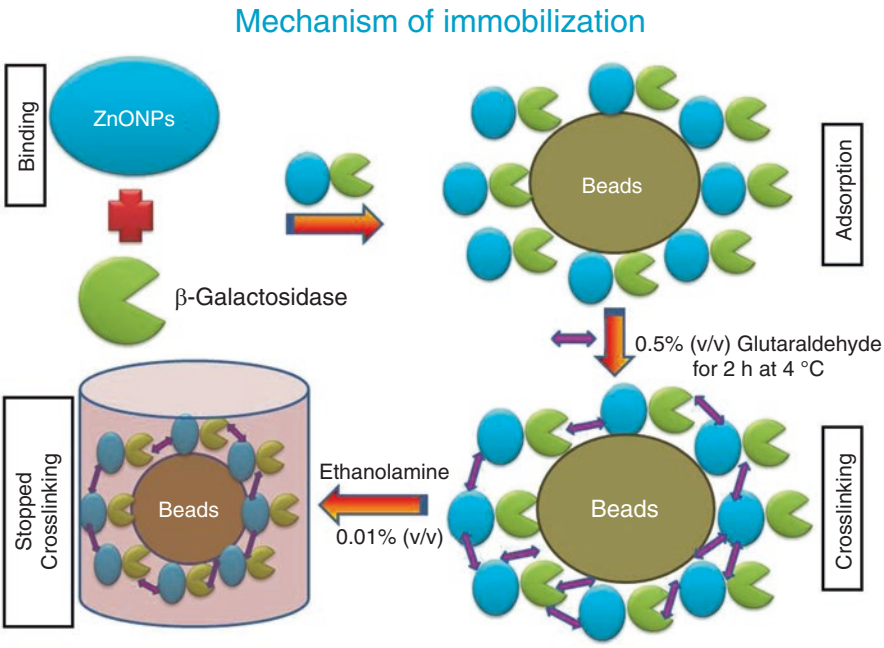

Fig. 7.5 Proposed mechanism of immobilization process. (Selvarajan et al. 2015)

7.9.6 Applications of β-Galactosidase

There are two main biotechnological applications in the lactose hydrolysis by β-galactosidase (Gekas and Lopez-Leiva 1985). The first application is in the utilization of whey because both glucose and galactose have greater fermentation potential (Kosaric et al. 1985). The second major application is in the production of lactose-free milk (Kretchmer 1972). Cheese manufactured from hydrolysed milk ripens more quickly than that made from normal milk. Treatment of milk and milk products with β-galactosidase to reduce their lactose content seems to be an appropriate method to increase their potential uses and to deal with the problems of lactose insolubility and lack of sweetness. Furthermore, this treatment could make milk, a most suitable food, avaiLactic acid bacteriale to a large number of adults and children that are lactose intolerant. Moreover, the hydrolysis of whey converts lactose into a very useful product like sweet syrup, which can be used in various processes of dairy, confectionary, baking and soft drink industries (Petzelbauer et al. 1999). Therefore, lactose hydrolysis not only allows the milk consumption by lactose-intolerant population but can also solve the environmental problems linked with whey disposal.

Packed-bed reactors are habitually used for kinetic studies of heterogeneous catalysed reactions. The employment of these reactors in biological processes could allow the application of innovative technology to hydrolyse lactose present in milk and milk products, in a commercial affair. For reaction system with product inhibition, the process efficiency in a packed-bed reactor is larger because the inhibition effect decreases due to low difference between substrate and product concentrations (Lilly and Dunnill 1976). The economic importance of enzymatic hydrolysis of lactose was increased from the 1960s. Lactose hydrolysis gives solubility increases from 18 to 55% at 80% conversion, and the sweetness raises up to 70% related to sucrose. Thus the production of self-sweetening products or products with less sucrose addition would be possible by using lactose-hydrolysed milk. In addition, the hydrolysis of lactose in dairy products increases their sweetness and eliminates the 'sandy defect' arising during lactose crystallization at low temperatures (Sheik Asraf and Gunasekaran 2010; Oliveira et al. 2011).

The food industry is responding to consumer demands by offering lactose-hydrolysed milk and lactose-free milk products in which some lactose has been removed physically and the rest is hydrolysed to obtain the same sweetness as ordinary milk (Harju 2003; Jelen and Tossavainen 2003; Zadow 1993). Several microbial sources of beta-galactosidase and reactor types have been used for the purpose of economic production of low-lactose milk. Lactose hydrolysis in plug flow reactor gives higher conversion compared to continuous stirred-tank reactor although the latter has good mixing and lower construction cost.

7.10 Conclusion

In this review, authors have tried their best to accumulate all the information regarding lactose intolerance, nanoimmobilization and application of β-galactosidase. The term lactose intolerance refers to the symptoms experienced when the dose of lactose exceeds the digestive capacity of intestinal lactase. Enzyme immobilization on nanoparticles allows researchers to take advantage of the fascinating properties of nanotechnology. The activity of immobilized enzymes is influenced by the methods and procedures of immobilization. Recently, more attention has been paid to the nanoimmobilization of enzymes on metal oxide nanoparticles.

Acknowledgements The authors are thankful to the Management, VIT University, Vellore, for providing the facilities and constant encouragement for this work.

Compliance with Ethical Standards

Conflict of Interest All the authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 8
Microbial Organic Compounds Generating Taste and Odor in Water

Dharumadurai Dhanasekaran, Saravanan Chandraleka, Govindhan Sivaranjani, and Selvanathan Latha

Contents

D. Dhanasekaran (✉)
Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

S. Chandraleka
Department of Chemistry, Urumu Dhanalakshmi College, Tiruchirappalli, Tamil Nadu, India

G. Sivaranjani · S. Latha
Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
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Abstract Odor compounds are mainly due to the presence of many volatile and semivolatile components with diverse chemical and physicochemical properties. These compounds are generally present within complex matrices. Odorous compounds in the soil have been subjected to scientific analysis for the determination of odor compounds produced by microorganisms. These compounds are also known as volatile organic compounds (VOCs). They are present in natural sources such as soil, air, freshwater, and marine water ecosystems, and they produce unpleasant musty odors or earthy odors.

VOCs have been isolated from Actinobacteria species, and these compounds play main roles in biological function. Common VOCs are alkanes, alkenes, alcohols, esters, ketones, sulfur compounds, and isoprenoid compounds. Geosmin and 2-methyl-isoborneol are naturally occurring compounds that have a very strong earthy taste and odor, and they can be simply detected by the human nose. Little is known about the fundamentals of microbial volatile odor compounds that contribute undesirable tastes or odors in water, soil, and aquaculture products. To address this knowledge gap, we have investigated the microbial community causing undesirable odors and tastes in water. The present review describes the microbial origin of odor compounds, particularly those caused by Actinobacteria. It also describes their distribution, occurrence, and chemical nature; detection of odor compounds; and biological methods used to remove undesirable odors from water.

8.1 Introduction to Taste and Odor Problems

Taste and odor problems cause common concerns about water quality for water utilities (Lalezary et al. 1986) and are relevant to the consistency and safety of drinking water. Mostly, taste and odor problems pose no risks to human health, but they raise consumer concerns regarding water safety. However, these problems are easy to remedy through control of dosage or filtration. Many volatile organic compounds (VOCs) causing odor problems can be identified from Actinobacteria cultures. In some investigations, the presence of odorous compounds from actinobacterial metabolites has coincided with the observation of aerial mycelium and spores (Bentley and Meganathan 1981). The most prevalent of such consumer complaints involve earthy–musty odors, which are primarily the result of two odor-causing compounds—geosmin (trans-1,10-dimethyl-trans-9 decalol; $C_{12}H_{22}O$) and 2-methyl-isoborneol ($C_{11}H_{20}O$)—in drinking water obtained from surface water sources. Removal of geosmin and 2-methyl-isoborneol is challenging because of their low odor threshold. Although taste- and odor-causing compounds do not cause health problems, their persistence causes a negative impression that the water is unsafe. In view of such aesthetic water quality concerns, more research is required to verify their abundance and capability to be metabolically active in several locations in India and other countries. Little is known about the fundamentals of microbial volatile odor compounds that contribute tastes or undesirable odors to water, soil, and aquaculture products. To address this knowledge gap, we have investigated microbial communities causing undesirable odors and tastes in water. The present

review describes the microbial origin of odor compounds, particularly from Actinobacteria; their distribution, occurrence, and chemical nature; methods for detection of odors in water, soil sediments, and aquaculture; and odor removal methods with biological treatments involving biofiltration, activated carbon, and advanced oxidation processing (AOP).

8.2 Principles of Odor Compounds

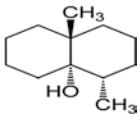
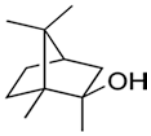
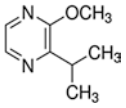
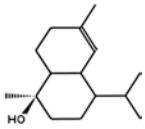
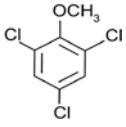
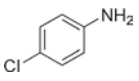
Natural odor compounds are found in soil, water, and certain specific exotic plant species. An attractive alternative method for flavor and fragrance synthesis is based on de novo microbial processes (fermentation) or bioconversion of natural precursors, using microbial cells and enzymes (biocatalysis). For the past 60 years it has been well known that microorganisms produce odor and taste chemicals. The characteristic flavor of any compound is mainly due to the presence of many volatile and nonvolatile components with diverse chemical and physicochemical properties. These compounds are generally present within complex matrices. Actinobacteria, particularly *Streptomyces* species are the main producers of odor compounds specifically 2-methyl-isoborneol and geosmin. However, different *Streptomyces* species have different abilities to produce 2-methyl-isoborneol and geosmin; therefore, some species may produce more odorous compounds than others.

8.3 Characteristics of Taste and Odor Compounds

The terms "taste" and "odor" are used jointly in the vernacular of water technology. As mentioned earlier, taste and odor problems in water supplies are concerned almost entirely with odors. Occurrences of tastes and odors at a water plant or in a water system are generally unpredictable. The odors caused by dead organic matter can be classified as vegetable odors and odors of decomposition. These smells vary in character in different waters and in different seasons.

Volatile compounds are easily transported through the air and, in most cases, dissemination of VOCs from their point of origin leads to atmospheric dilution of the substances (Bennett and Inamdar 2015). Approximately 1000 microbial VOCs have been identified to date (Piechulla and Degenhardt 2014). Bacterial VOCs also contribute to the ability of bacteria to interact with their environment. Indeed, several volatile compounds have been shown to influence growth, differentiation, stress resistance, and/or behavior in fungi, plants, or invertebrates (Wenke et al. 2012; Davis et al. 2013). Beyond such interactions with a wide range of eukaryote organisms, recent studies have revealed the roles of odor compounds in bacterial interactions in various environments, including soil, animal and plant microbiota, and biofilms. Geosmin, as mentioned earlier, is an odor-producing compound produced by certain species of Actinobacteria in water. Actinobacteria-derived taste and odor compounds are listed in Table 8.1.

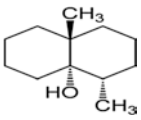
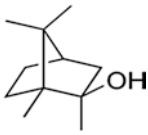
Table 8.1 Taste and odor description, chemical nature, and structure of earthy, woody, musty, and moldy odor–causing volatile organic compounds (*VOCs*) derived from the actinobacterial genera *Actinomadura*, *Micromonospora*, *Nocardioides*, and *Streptomyces*

Taste and odor description	Bacteria	Compound	VOC structure
Earthy	*Actinomadura* sp., *Micromonospora* sp., *Nocardioides* sp., *Streptomyces* sp.	Geosmin	
Musty	*Micromonospora* sp., *Nocardioides* sp.	2-Methyl-isoborneol	
Earthy, musty	*Streptomyces* sp.	Geosmin, 2-methyl-isoborneol	
Moldy, musty	Actinobacteria	2-Isopropyl 3-methoxypyrazine	
Woody, earthy	Actinobacteria including *Streptomyces* sp.	Cadin-4-ene-1-ol	
Moldy, musty	Actinobacteria including *Streptomyces* sp.	2,4,6-Trichloroanisole	
Moldy, musty	Actinobacteria	Chloroanisole	

8.3.1 Geosmin

Geosmin is an organic compound, first identified in Actinobacteria by Gerber and Lechevalier (1965), with a molecular formula of $C_{12}H_{22}O$ and a molecular weight of 182.3 g/mol. The term "geosmin" means "earth odor." The molecular structure of this compound shows a bicyclic tertiary alcohol. It is produced both intracellularly and extracellularly, and it is released into the water when these microbes die. In acidic conditions, geosmin decomposes into odorless substances such as argosmin;

Table 8.2 General characteristics of geosmin and 2-methyl-isoborneol: physical and chemical properties and odor threshold (Juttner and Watson 2007)

Characteristic	Geosmin	2-Methyl-isoborneol
Molecular formula	$C_{12}H_{22}O$	$C_{11}H_{20}O$
Molar mass (g/mol)	182.3025	168.2759
Boiling point at 101.325 kPa (°C)	270–271	207–209
Flash point (°C)	103.89	83.33
Vapor pressure (Pa)	5.56	6.76
Odor threshold (μg/L)	0.015	0.35
Structure		

hence, vinegar and other acidic ingredients are used in fish recipes to reduce the muddy flavor. This earthy-smelling compound is also observed in cured meat, dried beans, canned mushrooms, and other root crops (Lloyd and Grimm 1999; Maga 1987). This compound is also responsible for an earthy taste and odor problems in drinking water supplies (Table 8.2). The odor threshold concentration for geosmin is 1–10 ng/L at 45 °C (McGuire et al. 1981; Rashash et al. 1997).

8.3.2 2-Methyl-Isoborneol

2-Methyl-isoborneol is a bridged aliphatic structure, which was first found as a natural metabolite of Actinobacteria and named "methylisoborneol" by Gerber (1969). In addition, Rosen et al. (1970) determined that 2-methyl-isoborneol was produced by Actinobacteria in natural waters. It was subsequently shown to be produced as a secondary metabolite by different species of Cyanobacteria and Actinobacteria. 2-Methyl-isoborneol is characterized by an earthy–musty odor, which can be detected by people at very low concentrations (Table 8.2). The odor threshold concentration of 2-methyl-isoborneol is 5–10 ng/L, and its molecular formula and molecular weight are $C_{11}H_{20}O$ and 168.28 g/mol, respectively.

8.3.3 Chemical Compounds Behind the Smell of Rain

After the first rain, we smell a very exclusive and pleasant earth odor. This earthy odor is known as geosmin. The Gram-positive *Eubacterium,* Actinobacteria and, in particular, *Streptomyces*—which also represent the normal flora in soil and water—are the causative agents of geosmin odor. Actinobacteria, including *Streptomyces*

are adapted to dry, desiccated, or moist climate conditions. During rain, they readily form spores which are stress resistant and can survive under desiccation or extreme heat. Geosmin is chemically dimethyl-9-decalol. It is contained in the spore coat of soil bacteria. When raindrops strike the ground, soil containing spores is spread into the air. The spores are microscopic, circular, and more lightweight than the soil particles. They remain suspended as a soil–water aerosol, and when we inhale the aerosol, we smell the geosmin present in the spores. Because these bacteria generally do not form spores in moist soil, which is formed after first the rain, they relapse back to their filamentous vegetative form. They are present as spores in the soil only before the start of rain, when the weather is already dry or is warm and damp, so there are no spores present in wet conditions and therefore no geosmin odor present in the environment.

The most important thing is that these bacteria are found in all types of soils, and in fresh and aquatic water sources, all over the world. Therefore, every *Homo sapiens* smells enormous amounts of these odor compounds (Fig. 8.1). Chater et al. (2002) have sequenced the genome (of 8000 genes) of the *S. coelicolor* A3 strain, which produces many chemicals, including geosmin. Simons (2003) has suggested that camels can detect the smell of geosmin released by *Streptomyces* miles away on wet ground, and can track the geosmin to find an oasis; in return, the camels carry away and disperse the spores of *Streptomyces*.

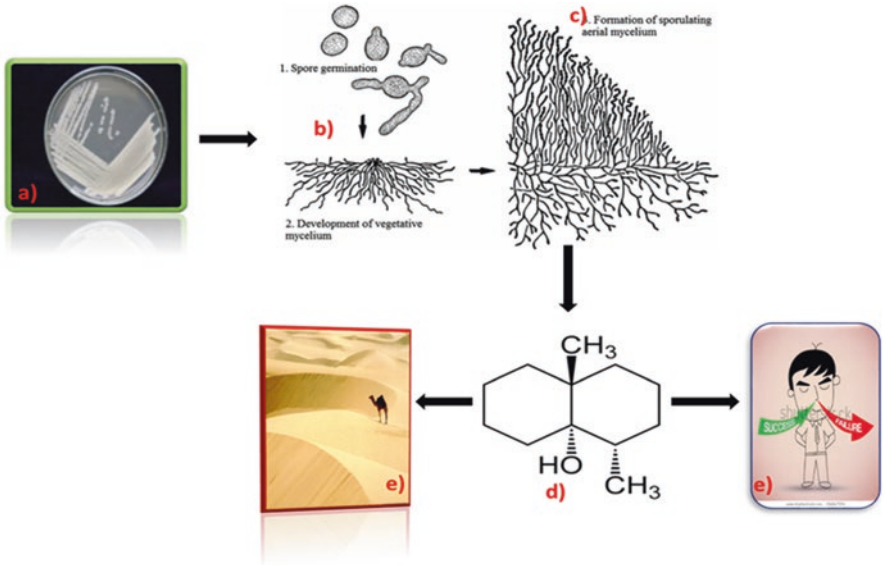


Fig. 8.1 Detection of geosmin odor by mammals: (**a**) *Streptomyces* culture; (**b**) spore germination; (**c**) aerial mycelium formation; (**d**) chemical structure of geosmin; (**e**) mammalian inhalation of geosmin

8.4 Odor-Producing *Streptomyces* in Different Habitats

Geosmin and 2-methyl-isoborneol are produced by members of *Streptomyces* found in soil and water sources such as lakes, reservoirs, and running water. In addition, there are several other biological sources that are often overlooked, notably those that originate from terrestrial ecosystems (Table 8.3).

Table 8.3 *Streptomyces* species that produce geosmin and 2-methyl-isoborneol

Streptomyces strains	Compounds	Reference
S. odorifer	2-Methyl-isoborneol	Gaines and Collins (1963)
S. antibioticus IMRU 3720	Geosmin	Gerber and Lechevalier (1965)
S. fradiae IMRU 3535	Geosmin	Gerber and Lechevalier (1965)
S. griseus LP-16	Geosmin	Gerber and Lechevalier (1965)
S. alboniger 12464	Geosmin	Gerber (1967)
S. lavendulae 3440 1-Y	Geosmin	Gerber (1967)
S. viridochromogenes 94	Geosmin	Gerber (1967)
S. griseoluteus IMRU 3718	Geosmin	Rosen et al. (1968)
S. antibioticus Nr. 5234	2-Methyl-isoborneol	Medsker et al. (1969)
S. griseus ATCC 10137	2-Methyl-isoborneol	Medsker et al. (1969)
S. odorifer ATCC 6246	2-Methyl-isoborneol	Piet et al. (1972)
S. odorifer ATCC 6246	Geosmin	Piet et al. (1972)
S. albasporeus, S. filipinensis, S. resistomycificus	Geosmin, 2-methyl-isoborneol	Kikuchi et al. (1973)
S. paraecox ATCC 3374	2-Methyl-isoborneol	Medsker et al. (1968)
S. lavendulae CBS 16245	2-Methyl-isoborneol	Gerber (1979)
S. tendae	Geosmin	Dionigi et al. (1992)
S. halstedii	2-Methyl-isoborneol, geosmin	Schrader and Blevins (2001)
Streptomyces sp.	2-Methyl-isoborneol, geosmin	Klausen et al. (2005)
S. malaysiensis	2-Methyl-isoborneol, geosmin	Tung et al. (2006)
Streptomyces sp.	Geosmin, 2-methyl-isoborneol	Zuo et al. (2009)
Streptomyces sp.	Geosmin	Schrader and Summerfelt (2010)
Streptomyces sp.	Geosmin, 2-methyl-isoborneol	Petersen et al. (2014)

8.4.1 *Odor-Producing* Streptomyces *in Freshwater*

The presence of substances that impart disagreeable taste and odor to drinking water is one of the principal causes of complaints from consumers. These substances may be present because of artificial or natural processes, and often result from microbial growth and metabolism. Surface waters—including reservoirs, natural lakes and rivers, and water tanks—are important sources of drinkable water throughout the world but often contain sporadic matter with an earthy–musty odor and flavor.

The major causes of odor problems associated with drinking water supplies are biological activity in water sources, especially that of Actinobacteria (*Streptomyces*, *Nocardia*, and *Microbispora* species) and Cyanobacteria (*Oscillatoria*, *Anabaena*, and *Aphanizomenon* species) (Juttner and Watson 2007). Zaitlin et al. (2003) investigated the role of Actinobacteria in the production of odorous compounds from the Elbow River—an important drinking water source for the city of Calgary in Ontario, Canada—and the results showed that the Elbow River had a high concentration of Actinobacteria, with a mean count of 256 colony-forming units (CFU) per milliliter. Actinobacteria, including *Streptomyces* have also been found of soft deposits in drinking water distribution pipes, at counts of 1.5×10^3 CFU L^{-1} (Zacheus et al. 2001).

However, it is still debatable as to whether Actinobacteria are capable of active growth in open water; the evidence suggests that they are active on submerged substrates. Isolates of *Streptomyces* capable of high levels of geosmin production have been found in association with zebra mussels, although it was not determined whether this bacterium was associated with a specific tissue or fecal/biofilm material (Zaitlin et al. 2003). Actinobacteria, including *Streptomyces* produce geosmin and 2-methyl-isoborneol, which lower the quality of surface water used for drinking. Combined microautoradiography and catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) analysis have been used to study the distinctiveness and activity of Actinobacteria in a freshwater environment, and 1.3×10^8 Actinobacteria per liter were found in a reservoir (Nielsen et al. 2006).

8.4.2 *Odor-Producing* Streptomyces *in Aquaculture*

Muddy–earthy–musty odors are generally known to be associated with wild-caught freshwater fish (Tucker 2000; Howgate 2004), although the occurrence of such odors has also been reported for a diverse range of freshwater aquaculture species (Lovell 1983; Yamprayoon and Noomhorm 2000; Robertson et al. 2005; Petersen et al. 2011). The source of muddy–earthy–musty flavors in freshwater fish is commonly acknowledged as originating from two compounds: geosmin and 2-methyl-isoborneol. Geosmin and 2-methyl-isoborneol are metabolites of certain groups of algae, Actinobacteria, and Cyanobacteria (Tucker 2000), and are found in various water sources such as lakes, reservoirs, and running water (Juttner and Watson 2007).

Wohl and McArthur (1998) studied samples of aquatic vegetation from three stream sites located within the Savannah River site in South Carolina, USA, and identified 32 distinct Actinobacteria strains. More than 45% of the Actinobacteria colonies isolated were *Streptomyces*. Of the 32 distinct strains identified, 34% were strains of *Streptomyces*, while *Pseudonocardia*, *Nocardia*, *Micromonospora*, and *Actinoplanes* each accounted for an additional 10% of the diversity, so a high level of *Streptomyces* occurred in the river and produced the musty odor. Klausen et al. (2005) found that the existence of the odorous geosmin and 2-methyl-isoborneol in freshwater environments indicated that odor-producing Actinobacteria were present in one oligotrophic and two eutrophic freshwater streams, as well as in aquaculture connected to those streams, in Denmark. Sequencing of 16S ribosomal RNA (rRNA) genes in eight bacterial isolates with typical Actinobacteria morphology from those streams and ponds demonstrated that most of them belonged to the genus *Streptomyces*.

The lowest geosmin concentrations were measured in the oligotrophic Funder Stream (1.0–2.4 ng L^{-1}), while higher concentrations occurred in the eutrophic Holtum and Vorgod Streams (2–6 ng L^{-1}, except for one finding of 12 ng L^{-1} in June 2003). 2-Methyl-isoborneol concentrations of 2 ng L^{-1} in the Vorgod Stream (in December 2002) and 9.6 ng L^{-1} in the Holtum Stream were measured.

Schrader et al. (2005) isolated geosmin and 2-methyl-isoborneol in recirculating aquaculture systems. Certain species of Cyanobacteria are responsible for these problems in pond-cultured fish. In these ponds, Actinobacteria, including *Streptomyces*, were isolated from the settling unit of recirculating aquaculture system (RAS) 1 and the standpipes of RAS 1 and RAS 5. Geosmin levels were significantly higher in biosolid samples (mean values from six RASs: 9200 ng/kg and 36,400 ng/kg in the settling unit and the standpipe, respectively) than in water samples from the settler inflow and outflow (2.3 ng/L and 2.8 ng/L, respectively) and the side-drain inflow and outflow (2.8 ng/L and 3.2 ng/L, respectively).

8.4.3 *Odor-Producing* Streptomyces *in Soil*

Soil is a complex, nutrient-poor, and highly heterogeneous environment consisting of both water- and air-filled pores (Young et al. 2008). Due to the physical properties, such as low molecular weight, lipophilicity, high vapor pressure, and low boiling points, soil contains a number of soildwelling bacteria (Actinobacteria), which produce compounds such as geosmin. They secrete it into the surrounding soil, and it is then disturbed by rainfall, spreading through the air. Geosmin is associated with *Streptomyces* spores, which are present in huge numbers in many soils. We can safely assume that a time traveler from today who visited the planet as it was about 440 million years ago would find the smell of the soil familiar, as the earliest land plants collaborated with the first *Streptomyces* to generate protocompost.

VOCs released from soil can be derived from abiotic processes (Warneke et al. 1999), but most of the compounds that are emitted are likely to be products of root

or microbial (i.e., bacterial and fungal) metabolism (Bunge et al. 2008; Mayrhofer et al. 2006). Some of the most common types of VOCs emitted from soils and litters include monoterpenes, alcohols, and ethers (Stotzky and Schenck 1976; Leff and Fierer 2008), but the types and quantities of VOCs released during microbial decomposition are highly variable and influenced by the nature of the substrate. Such differences could be driven by changes in various soil characteristics, including the microbial community composition, microbial biomass, carbon substrate characteristics, redox status, nutrient availability, and moisture status. VOCs may also act as a carbon source for microorganisms, increasing soil carbon dioxide production and decreasing nitrogen mineralization rates (Paavolainen et al. 1998; Mackie and Wheatley 1999; Amaral and Knowles 1997).

Zuo et al. (2009) studied Actinobacteria, which are major producers of the typical odorous compounds geosmin and 2-methyl-isoborneol in terrestrial soil environments. Most Actinobacteria can produce spores, which can survive under extreme conditions and are dispersed extensively by wind and water flow (Goodfellow and Williams 1983). Many reports have shown that episodes of high terrestrial runoff may introduce Actinobacteria and their secondary metabolites (geosmin and 2-methyl-isoborneol) into surface waters, resulting in odors (Zaitlin et al. 2003). Recently, Forbes and Perrault (2014) isolated volatile compounds from soil and air samples; a total of 249 VOCs of interest were detected, many of which were present in soil samples (60%) and in air samples (17%).

8.4.4 Odor-Producing Streptomyces *in Sediment*

Sediments are an important reservoir of nutrients that are potentially available for *Streptomyces* fabrication, with their abundance often being correlated with sediment nutrient status. Sediments and muds in freshwater environments have, for some time, been recognized as a possible habitat for Actinobacteria growth and odor and flavor production (Adams 1929; Thaysen 1936; Issatchenko and Egorova 1944; Bays et al. 1970). Furthermore, sterilized sediment has been found to produce geosmin (460 ng kg^{-1}) after inoculation with *S. albidoflavus.*

In addition to those sources of geosmin and 2-methyl-isoborneol, Actinobacteria have also been found to produce musty odors in sediments (Schrader and Blevins 1993). However, systematic investigations into the abundance and taxonomy of the Actinobacteria that are responsible for geosmin and 2-methyl-isoborneol production in sediments are still lacking. Sugiura and Nakano (2000) studied 40 isolates of Actinobacteria species from the sediment of Lake Kasumigaura in Japan, which has a mean depth of 4.0 m, and found that they produced both geosmin (approximately 60 mg kg^{-1} of dry weight (dw)) and 2-methyl-isoborneol (approximately 50 mg kg^{-1} dw) in cultures grown in BS medium. In 2006, Tung et al. studied odor-producing *Streptomyces* isolated from the mud of the Feng-Shen reservoir, and the results showed that *S. malaysiensis* (identified by using M liquid cultures) produced geosmin concentrations of up to 4.5 ng mg^{-1} and

2-methyl-isoborneol concentrations of up to 2.4 ng mg^{-1}. Similarly, Zuo et al. (2010) reported high levels of geosmin occurring in the sediments of the Xionghe reservoir, which has a mean depth of 13.2 m. Up to 5280.1 ng kg^{-1} dw of geosmin was detected in the sediment, and eight strains of *Streptomyces* isolated from the sediment were confirmed as producers of geosmin and 2-methyl-isoborneol, detected by headspace solid-phase microextraction–gas chromatography–mass spectrometry (HSPME-GC-MS) analysis. On the basis of in situ analysis and the production of odorous compounds by the isolated Actinobacteria, it was concluded that the geosmin in sediments was produced by species of *Streptomyces*. The concentrations of geosmin in the overlying water were significantly correlated with those in the sediments ($r = 0.838$, $p < 0.05$). The geosmin in the overlying water was released from the sediments and, consistent with the findings of in vitro studies, the percentage release was between 21.4% and 51.4% over 12 d.

8.5 Biosynthesis of Geosmin

Bentley and Meganathan (1981) were the first researchers to investigate the biosynthetic pathway of geosmin and 2-methyl-isoborneol metabolism, using radio-gas chromatography. The original results reported by Bentley and Meganathan favored the mevalonate pathway for *Streptomyces* on the basis of the production of labeled geosmin and 2-methyl-isoborneol from labeled acetate. These researchers proposed that geosmin and 2-methyl-isoborneol were synthesized through an isoprenoid pathway (also known as the terpenoid or mevalonate pathway), with 2-methyl-isoborneol having a monoterpene precursor (geranyl pyrophosphate) and geosmin a sesquiterpene precursor (farnesyl pyrophosphate). Figure 8.2 shows a simplified biosynthetic pathway for the formation of 2-methyl-isoborneol and geosmin in *Streptomyces* and myxobacteria (Friedrich and Watson 2007).

The latter pathway may function exclusively in the synthesis of geosmin and other isoprenoids in some groups such as myxobacteria and may contribute to geosmin production in the stationary growth phase of *Streptomyces*. Several studies have also confirmed the same pathway for *Streptomyces* and Cyanobacteria. Dionigi et al. (1992) studied the effects on the growth and metabolism of the geosmin-producing Actinobacteria *S. tendae* and revealed that farnesol can inhibit the geosmin synthesis process, in turn suppressing geosmin-producing species.

The terpenes produced in *Streptomyces* species give the impression of being derived from either the mevalonate-dependent or mevalonate-independent pathways. Cane and Watt (2003) and Gust et al. (2003) identified a germacradienol synthase enzyme (Cyc 2 protein) from *S. coelicolor* that is needed for the biosynthesis of geosmin. Singh et al. (2009) experimentally proved that important production of an intracellular pool of acetyl coenzyme A (acetyl-CoA) after deletion of the doxorubicin biosynthetic pathway led to improved growth and longer survival of the cell culture. Likewise, greater accumulation of acetyl-CoA led to biosynthesis of

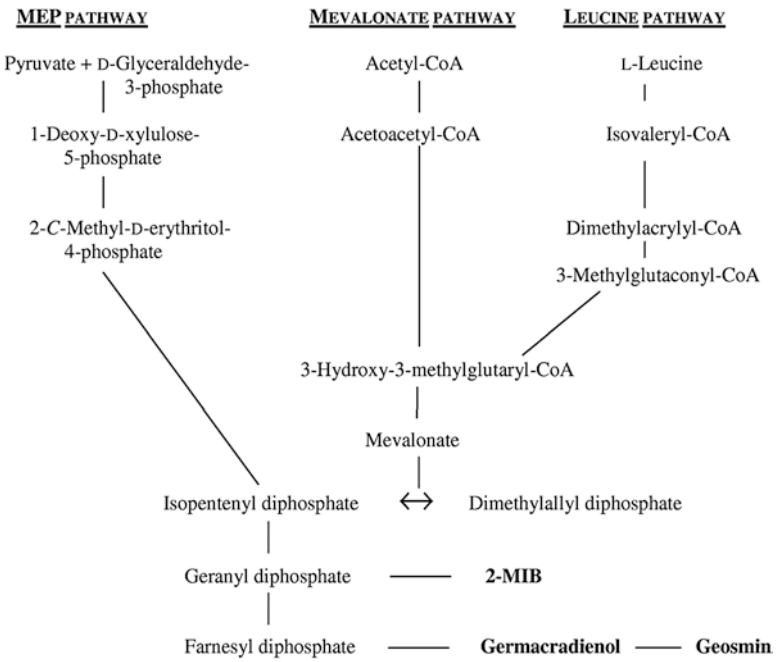


Fig. 8.2 Biosynthetic scheme for the formation of 2-methyl-isoborneol and geosmin in *Streptomyces* and myxobacteria

geosmin in *S. peucetius*. As the concentration of geosmin synthase increased, production of geosmin increased in tandem in the presence of adequate acetyl-CoA and the rate of enzyme activity rose in direct proportion to the increase in the substrate concentration.

However, a more recent study with *S. coelicolor* revealed that germacradienol synthase is a multifunctional enzyme with the N- and C-terminal domains each harboring a distinct functional active site (Jiang et al. 2007). The N-terminal is responsible for catalyzing the cyclization of farnesyl diphosphate (FPP) to germacradienol, and C-terminal catalyzes the conversion of germacradienol to geosmin. Likewise, Komatsu et al. (2008) found that of six *Streptomyces* species that were tested, *S. ambofaciens, S. coelicolor* A3, *S. griseus*, and *S. lasaliensis* produced 2-methyl-isoborneol. The regions containing monoterpene cyclase and methyltransferase genes were amplified by using PCR from *S. ambofaciens* and *S. lasaliensis*, respectively, and their genes were heterologously expressed in *S. avermitilis*, which was naturally deficient in 2-methyl-isoborneol biosynthesis by insertion and deletion; all exoconjugants of *S. avermitilis* produced 2-methyl-isoborneol.

8.5.1 Environmental Conditions That Favor Geosmin Production

Being a secondary metabolite, geosmin is produced by *Streptomyces* during secondary mycelial growth coinciding with sporulation. This has been demonstrated by the inhibition of geosmin production by *Streptomyces* mutant strains that are incapable of aerial mycelium development, as well as by normal growth on media not conductive to sporulation (Bentley and Meganathan 1981; Dionigi et al. 1992). In the presence of aerial mycelium and spores to correspond with the excretion of terpenoid compounds, whereas nondifferentiating strains either did not excrete such compounds or released them only to a limited extent. The aerial mycelium, which ultimately produces spores, develops from the substrate mycelium accompanied by lysis of the substrate hyphae. During this transition phase, *Streptomyces* species are particularly susceptible to competition from other organisms, and many secondary metabolites (i.e., antibiotics) appear in this growth phase, with the production of geosmin and 2-methyl-isoborneol by *Streptomyces* cultures exhibiting more morphological differentiation (Scholler et al. 2002; Tung et al. 2006).

As the secondary mycelial stage of growth is obligatorily aerobic, *Streptomyces* require the presence of oxygen for geosmin and 2-methyl-isoborneol production. Schrader and Blevins (1999) reported that increased geosmin production occurred in *Streptomyces* cultures in the presence of higher concentrations of atmospheric oxygen. Sunesson et al. (1997) observed that the carbon dioxide concentration also affected geosmin production by *S. albidoflavus*, with an elevated concentration (10% carbon dioxide atmosphere) being observed to decrease geosmin production. However, Schrader and Blevins (1999) observed more geosmin production in cultures of *S. halstedii* grown in a 10% carbon dioxide atmosphere than in those grown in 5% or ambient carbon dioxide concentrations. Despite being neutrophiles, these bacteria have been detected in both moderately acidic (pH 5) and alkaline (pH 9) aquatic environments (Jiang and Xu 1996).

Blevins et al. (1995) showed that *S. halsetdii* grew optimally in a neutral pH range (6–7) but, interestingly, the highest geosmin production occurred at pH 9 and in the extensive range of pH 6 to 11. Similar observations were reported by Yagi et al. (1987). Certainly, temperature is an important parameter affecting the metabolic activity of *Streptomyces*, which are predominately mesophilic and exhibit optimum growth between 25 °C and 30 °C (Goodfellow and Williams 1983). Likewise, Wood et al. (1985) determined that the minimum temperature for geosmin production by *S. albidoflavus* in nutrient-amended reservoir water was 15 °C and that all documented cases of earthy odor problems occurred when the water temperature exceeded this. Recently, Zuo et al. (2010) established that some sediment isolates of *Streptomyces* could grow slowly and produce relatively low concentrations of geosmin at 4 °C and 10 °C.

8.5.2 Purpose of Geosmin Biosynthesis

The production of geosmin and 2-methyl-isoborneol coincides with *Streptomyces* morphological differentiation and sporulation, suggesting that the possible biological purpose of these metabolites is related to the reproductive phase of the life cycle of these bacteria (Bentley and Meganathan 1981; Dionigi et al. 1992). Similarly, Scholler et al. (2002) and Tung et al. (2006) reported that volatile metabolite compounds isolated from actinobacterial species played a main role in biological function. However, many other secondary metabolites, they may serve as a defense strategy, to antagonize rival microorganisms in times of harsh conditions (e.g., nutrient limitation) when their reproductive growth is initiated to ensure the survivability of the next generation of germinating spores. The low-level toxicity of geosmin and 2-methyl-isoborneol to higher-order organisms is evident from numerous studies (Nakajima et al. 1996; Burgos et al. 2014).

Zaitlin and Watson (2006) maintain that the high concentrations used in these studies, which greatly exceeded those typically encountered in freshwater environments, may be encountered by organisms near sources of microorganisms or at the microscale level in sediments, soil, and biofilms. Similarly, Watson (2003) reported that the water industry has tended to treat geosmin and 2-methyl-isoborneol as metabolic waste products, but it seems unlikely that they would play no adaptive biological role, given the complexity and energetic costs of their biosynthesis and the ubiquity of these compounds in nature. Terpenoids have a possible biological function as antimicrobial compounds, as well as playing alternative adaptive roles in the life of *Streptomyces*. More recently, these and many other secondary metabolites have been re-examined for their potential bioactivity, to understand the triggers, mode, and dynamics of their production (Watson 2003; Watson and Cruz-Rivera 2003).

8.6 Geosmin Detection

Previous analytical methodologies for the analysis of geosmin and 2-methyl-isoborneol have included closed-loop stripping and conventional purge-and-trap techniques. Closed-loop stripping involves large sample volumes and an adsorbent bed, which must be eluted properly for accurate compound quantization. With a purge-and-trap technique, large enough sample volumes cannot be easily analyzed to obtain the sensitivity required without encountering technological challenges. One current and effective way to measure the concentrations of taste and odor compounds in raw and finished water sources is solid-phase microextraction (SPME) followed by GC-MS detection (Fig. 8.3). The SPME method utilizes a SPME fiber that is exposed to the headspace of the sample being evaluated. Target compounds from the sample are adsorbed onto the fiber coating and then thermally desorbed from the fiber in a heated injection port.

This method has recently been accepted and published as standard method 6040 D for analysis of taste and odor compounds. The method is sensitive to odor

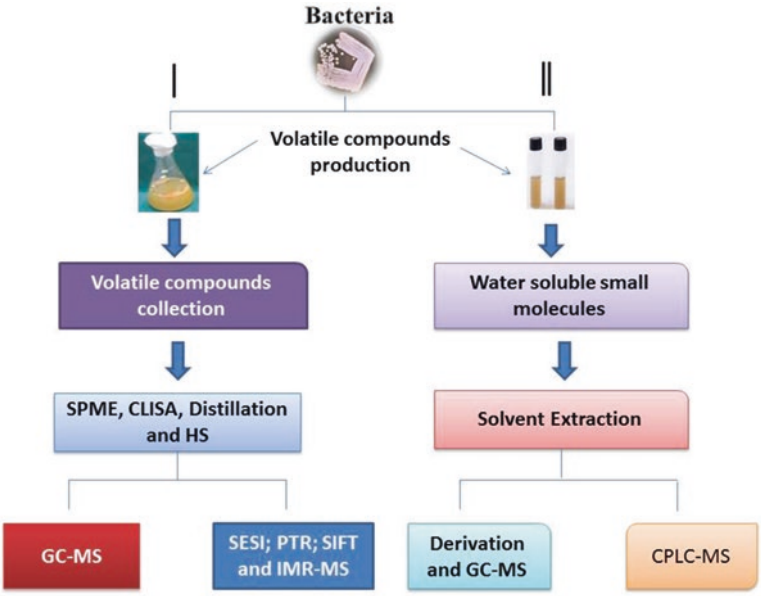


Fig. 8.3 Geosmin production and laboratory analysis of volatile organic compounds (*VOCs*) and water-soluble small molecules by solid-phase microextraction (*SPME*), closed-loop stripping analysis (*CLISA*), and gas chromatography–mass spectrometry (*GC-MS*), Headspace-gas *chromatography* (*HS-GC*), secondary electrospray ionization-mass spectrometry (*SESI-MS*), selected ion flow tube mass spectrometry (*SIFT-MS*), proton transfer reaction mass spectrometry (*PTR-MS*), Ion molecule reaction mass spectrometry (*IMR-MS*)

compounds down to low single-digit parts per trillion (ppt), reporting limits using quadrupole or ion-trap MS.

Recent technology for geosmin detection includes GC-MS, which allows highly sensitive measurement of metabolites at levels as low as parts per trillion. This method, however, can require large sample volumes and intensive sample concentration procedures such as liquid–liquid extraction, closed-loop stripping analysis (CLISA)—which requires complex equipment—simultaneous distillation extraction, or purge-and-trap techniques, all of which can result in low sample throughput due to lengthy protocols (Watson et al. 2000). The high-resolution mass spectrometers required for detection of low concentrations are extremely costly. Sensory analysis of geosmin by human assessment is a simple method for detection but relies upon the sensing capabilities of the individual and has several limitations, with the lack of quantification being paramount. Hence, sensing of geosmin is strictly qualitative and is not a suitable technique for measuring concentrations in drinking water sources. Enzyme-linked immunosorbent analysis (ELISA) uses antibodies to detect geosmin and provides a rapid field test for geosmin detection; however, it is costly and its detection threshold (1 μg L^{-1}) is too high to be of any practical value.

8.6.1 Analytical Methods Involved in Geosmin Detection

Analytical methods are designed to separate, isolate, identify, and quantify analytes of interest within a sample. There are various techniques, and there have been various reviews on the separation of these components, specifically in mammals. With regard to characterizing odorous compounds, the most frequently implemented analytical techniques are gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), gas chromatography–flame ionization detection (GC-FID), gas chromatography–time-of-flight mass spectrometry (GC-TOF-MS), nano–liquid chromatography–mass spectrometry (nano-LC-MS), matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS), electrospray ionization–mass spectrometry (ESI-MS), gel electrophoresis, thin-layer chromatography (TLC), and gas liquid chromatography (GLC).

In GC—the most widely used analytical tool—a mixture of VOCs is separated into individual VOCs and semi-VOCs, which are eluted out of the GC column at different times. This allows quantification and qualification of the compounds within the mixture. Another reason for the common implementation of GC is that it can analyze volatile compounds that can be detected via the olfactory system. Use of GC-MS to identify compounds is more efficient than use of other detectors because it has an extensive library available (the US National Institutes of Standards and Technology (NIST) electron ionization–mass spectrometry (EI-MS) database), with over 200,000 entries for comparison matching.

In our recent study, a total of 26 actinobacterial isolates were used for the screening of odor-producing compounds. Out of 26 isolates, 13 were isolated from soil samples collected at three different locations in Tiruchirappalli, Tamil Nadu, India. On the basis of morphological appearance, the actinobacterial isolates were identified as *Streptomyces*, *Actinopolyspora*, *Saccharopolyspora*, and *Actinomadura* species (Fig. 8.4). Further, the actinobacterial isolates were screened for their ability to produce odor metabolites. Among the 26 tested actinobacterial cultures, only isolate SD7 exhibited excellent odor production, with a total score of 4.0, while the *Streptomyces* species DDBH005 showed good odor production, with a score of 3.14. Nine other isolates revealed moderate odor production, with scores in the range of 2.0–2.86, while 15 isolates demonstrated poor odor production, with scores in the range of 1.12–1.86. On the basis of the olfactory analysis and growth profile of *Streptomyces* species, cultures of *Streptomyces* species SD2 and LD23 were selected as potent odor producers and subjected to detection of the genes responsible for odorous compound production, as well as identification of odorous metabolites. Amplification of the geoA gene was performed using the primers 245F and 551R, in which no bands were obtained for both *Streptomyces* species SD2 and LD23. This could be due to the nonexistence of the geoA gene in *Streptomyces* species SD2 and LD23 or incompatibility of the selected primers for amplification of the geoA gene. Further, the GC-MS analysis results illustrated that both isolates contained the volatile odor compound 2-methyl-isoborneol.

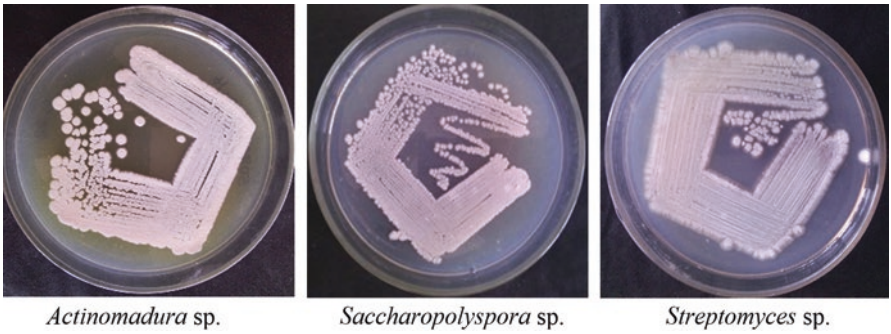


Fig. 8.4 Cultural morphology of odor-producing Actinobacteria isolates in starch casein agar

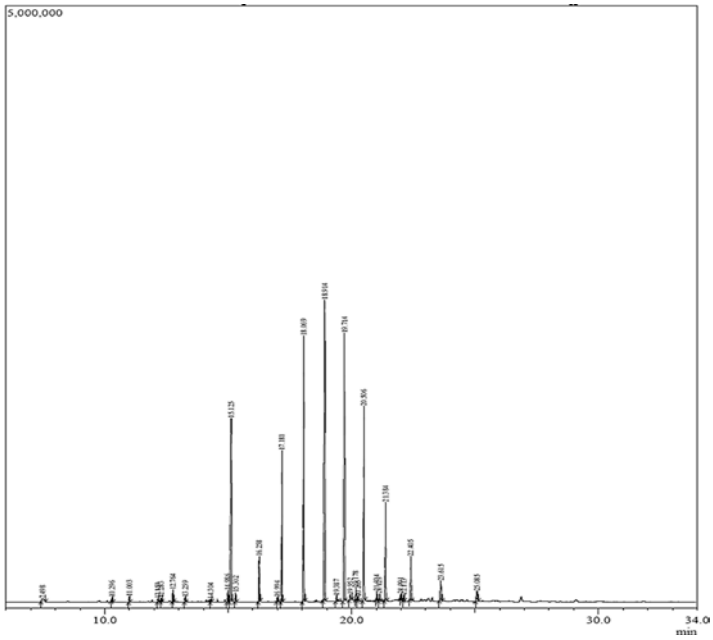


Fig. 8.5 Gas chromatography–mass spectrometry (*GC-MS*) of odor compounds derived from *Streptomyces* species SD2: 2-methyl-isoborneol (retention time 7.498 min) and germacradienol (retention time 9.89 min)

GC-MS analysis was performed to determine the presence of odor compounds in *Streptomyces* species SD2, in which the presence of hydrocarbon-derived compounds was observed, indicating the existence of individual volatile compounds in high proportions. Figure 8.5 shows the GC-MS spectrum of *Streptomyces* species SD2, with two main peaks with retention times of 7.498 min and 9.89 min, corresponding to 2-methyl-isoborneol and germacradienol, respectively. The molecular mass of 2-methyl-isoborneol was found to be 168.2 g/mol, whereas that of germacradienol was 223 g/mol.

8.7 Treatment of Odor-Causing Compounds

When taste and odor problems occur in drinking water, the general water treatment process cannot remove the whole amounts of the compounds, because of the extremely low odor thresholds of geosmin of (15 ng/L) and 2-methyl-isoborneol (35 ng/L). Lalezary et al. (1986) found that conventional water treatment technologies—consisting of breakpoint prechlorination, coagulation, sedimentation, and postchlorination—are not effective in reducing geosmin and 2-methyl-isoborneol in potable water to below their odor thresholds. For this reason, advanced water treatment processes are required to remove geosmin and 2-methyl-isoborneol compounds. These advanced technologies include biological treatment, AOP, chlorination, and some integrated systems (Table 8.4).

8.7.1 Biofiltration

Biofiltration is one of the methods most commonly used to remove geosmin and 2-methyl-isoborneol from drinking water. The main biofiltration systems used for geosmin and 2-methyl-isoborneol removal are activated carbon, slow sand filtration, and ultra/nanofiltration. The rates of removal of geosmin and 2-methyl-isoborneol by biofiltration are dependent on the biofilter media, biomass, temperature, and contact time. Some soil and aquatic bacteria are capable of biodegrading 2-methyl-isoborneol and geosmin, though there is no evidence of significant removal. The temperature of the water, which is typically between 10 °C and 20 °C, does not have a significant impact on removal of geosmin and 2-methyl-isoborneol.

Table 8.4 Water treatment methods for odor removal. Geosmin and 2-methyl-isoborneol in water can be removed by ozonation/biofiltration, granular activated carbon/sand biofiltration, oxidation/powdered activated carbon, and rapid mix/flocculation/sedimentation methods

Treatment technology	Findings	References
Ozonation/biofiltration	By-product (nonbiodegradable) from ozonation can be used by the bacteria as a substrate; this enhances the geosmin and 2-methyl-isoborneol removal efficiency of the biofilter	Nerenberg et al. (2000)
Oxidation/powdered activated carbon	70% geosmin and 2-methyl-isoborneol removal efficiency	Jung et al. (2004)
Granular activated carbon/sand biofiltration	86% geosmin and 52% 2-methyl-isoborneol removal efficiency	Elhadi et al. (2004, 2006)
Rapid mix/flocculation/sedimentation	70–90% geosmin and 2-methyl-isoborneol removal efficiency	Huck et al. (1995)
O_3/granular activated carbon	89% removal efficiency with single O_3 and >95% removal efficiency with combined O_3/granular activated carbon	Young et al. (1996)

8.7.2 Activated Carbon

Activated carbon is one of the methods most widely used to remove geosmin and 2-methyl-isoborneol in water utilities. Activated carbon can be categorized into two different systems depending upon its particle size: granular activated carbon and powdered activated carbon. In granular activated carbon, the activated carbon is used as a granular medium above the sand/gravel media filter for the removal of geosmin and 2-methyl-isoborneol from the water passing through it. Powdered activated carbon is basically used in the rapid mix stage, reacts with contaminants in the water, and is finally removed as sludge after the filtration process. Both granular activated carbon and powdered activated carbon are commonly used and are known to be effective for control of geosmin and 2-methyl-isoborneol. Although the removal of geosmin and 2-methyl-isoborneol by activated carbon reduces their levels to below their odor threshold concentrations, the complex procedure and high cost of activated carbon make the method challenging to implement in conventional drinking water treatment plants.

8.7.3 Advanced Oxidation Processing

AOP using ozone and other oxidants, combined with ultraviolet/vacuum ultraviolet, has been shown to be effective in the removal of geosmin and 2-methyl-isoborneol. Most such processes use ozone as the main oxidant to remove geosmin and 2-methyl-isoborneol. However, the oxidation reaction is known to produce disinfection by-products, which can cause birth defects and cancer. Currently, the use of ozone combined with other technologies, commonly ultraviolet radiation, is known to be effective. Lundgren et al. (1988) removed more than 95% of geosmin and 2-methyl-isoborneol by using 7 mg/L of ozone in water with 50 ng/L of 2-methyl-isoborneol and geosmin. Koch et al. (1992) used ozone dosages of 1, 2, and 4 mg/L with hydrogen peroxide (0.2 mg/mg) and improved 2-methyl-isoborneol removal by 20%.

Altogether, it is concluded that the soil Actinobacteria *Streptomyces* species have the ability to produce odorous compounds. Though odor production can be determined by olfactory analysis, quantitative measurements can be obtained only by dynamic instrumentation. Identification of the gene(s) responsible for production of these odor compounds requires detailed study with different gene-specific primers.

8.8 Conclusions

Geosmin and 2-methyl-isoborneol have been identified as the main taste- and odor-causing compounds in drinking water sources such as rivers, lakes, and water dams. Although these two compounds have not been associated with any serious health

effects, the taste and odor resulting from their presence in the water supply are considered unsafe by consumers. Evidence for the widespread distribution, abundance, and activity of *Streptomyces* and other Actinobacteria in natural and man-made aquatic environments has been appraised. Cultivars of these bacteria isolated from soil, freshwater habitats including sediment, vegetation, the water mass, or more specialized substrates readily demonstrate geosmin-producing abilities and 2-methyl-isoborneol–producing abilities in vitro, and they may indeed be potent sources of earthy–musty odors. To elucidate the contribution of these bacteria to this aesthetic water quality problem, more research is required to verify their abundance and capability to be metabolically active in such habitats. Some conventional technologies are used to treat or remove geosmin and 2-methyl-isoborneol in water sources. Coagulation, sedimentation, chlorination, and ozonation have been found to be effective for their treatment. Globally, odor- and flavor-producing microorganisms significantly reduce drinking water quality in many cities, and there is a great need for alternative management practices to reduce taste and odor compounds. The first step in the development of new water treatment procedures will be to identify the dominant odor producers.

Acknowledgements D.D. thanks the University Grants Commission (UGC), New Delhi, India, for financial support under the Raman Fellowship for Postdoctoral Research in the USA (F. no. 5-29/2016(IC) Dt.10.02.2016) and the Department of Science & Technology–Fund for Improvement of Science & Technology Infrastructure (DST-FIST) Programme (ref. no. SR/FIST/LS1-013/2012) dated August 13, 2012. S.L. is indebted to the Department of Science and Technology–Innovation in Science Pursuit for Inspired Research (DST-INSPIRE), New Delhi, India, for financial support in the form of a Junior and Senior Research Fellowship (DST Award Letter no. IF110317/DST/INSPIRE Fellowship/2011/Dt.29.06.2011).

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Chapter 9
Nanoparticles for Soil Remediation

Avipsha Sarkar, Sombuddha Sengupta, and Shampa Sen

Contents

Abstract Soil pollution refers to the fall of soil quality due to the introduction of "xenobiotic" compounds which alter the composition of soil. This "altered" soil can be toxic to life and can have detrimental effects. The contamination level is generally read as a direct measure to the rate and amount of industrialization as well as acts as an indication as to how much of the "contaminant" is released into the environment. The main areas of soil pollution are generally near effluent and/or waste disposal sites of industries. Irrespective of where the effluent/waste is dumped, the damage to the ecosystem due to these human activities tends to threaten life to an extreme point.

In this chapter we shall be elucidating the main polluting factors of the soil along with the deficiency of normal macroscopic techniques in handling them. We shall also highlight the various nano-remediation techniques along with the different types of nano-materials used simultaneously elucidating why they are considered to be the "remediation of the future."

A. Sarkar · S. Sengupta · S. Sen (✉)
Department of Biotechnology, School of BioScience and Technology, VIT University, Vellore, Tamilnadu, India

K M Gothandam et al. (eds.), *Nanoscience and Biotechnology for Environmental Applications*, Environmental Chemistry for a Sustainable World 22,
https://doi.org/10.1007/978-3-319-97922-9_9

9.1 Introduction

The utility of soil has a broad spectrum. For countries whose main form of national revenue is agriculture, soil availability and arability are of prime importance. Soil pollution has been defined as the presence of pollutants, toxic substances, and contaminants in it, beyond a threshold limit which can be injurious or harmful to plants and animals. Polluted soil may affect humans by inducing specific diseases or lead to the development of ill health. Human interaction with soil for these xenobiotics to enter the system can be numerous. It can pass via consumption also referred to as geophagia, or it can come in contact during normal manual labor. Sometimes direct exposure to these contaminants can occur if it is in vapor form or via consumption of plant and animal products that have accumulated large amount of soil pollutants through biomagnification. Observations have shown that inhalation of certain soil contaminants has given rise to malignancies, especially while inhaling soil contaminated by asbestos from minerals (Oliver 1997).

There are different contaminants that are present in soil which vary from country to country. A study has suggested that in India heavy metals along with mineral oil are the most important soil contaminants. The approximation of the relative concentrations of these contaminants has been done on agricultural plots. Induction-coupled plasma mass spectroscopy (ICP-MS) was used for this purpose (Sanghi and Sashi 2001). An overview of the heavy metal contamination estimates has been depicted in Fig. 9.1.

Some elemental soil contaminants include aluminum, arsenic, fluorine, iodine, chromium, cadmium, zinc, and thallium (Wuana and Okieimen 2011). The concentration of these substances varies with human activity generally leading to pollution. When there exists an isolated subsistent community, the relationship between contaminants and etiology of diseases can be easily identified. For example, heart diseases may accompany poor acid soil, and this has been seen in the coastal regions of the United States. Hence it is established that soil pollution tends to affect several realms of the ecosystem. Unless we counteract this pollution, a dark future lies ahead.

Various physical (such as soil washing and air sparging), chemical (such as electrokinetic remediation and in situ chemical oxidation), and biological methods (such as use of microorganisms and enzymes) have been introduced to control this rampant form of pollution. However, there are certain factors that have to be considered for any computable aim of remediation that would cause a considerable impact on the process of decision-making. The decisions that guarantee "cheaper, smarter, and cleaner" remediation ideas include (IAEA, *Non-technical Factors Impacting on the Decision Making Processes in Environmental Remediation*; Gravrilescu et al. 2003; Suthersan and McDonough 2005; Khan et al. 2004; Burden and Sims 1999; http://www.uga.edu/srel/soil.htm):

- Effectiveness of remediation.
- The cost involved in the program of remediation.
- Cleanup targets.

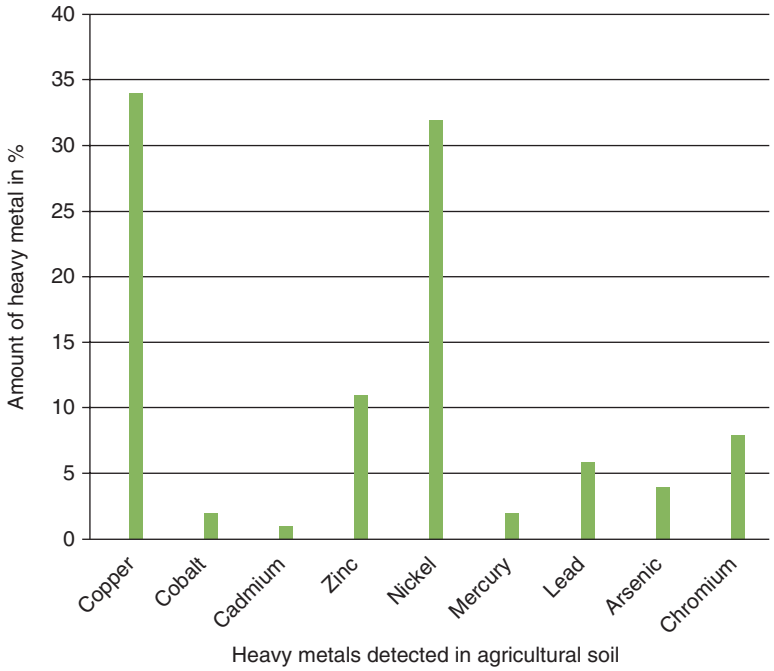

Fig. 9.1 A concise bar graphical representation of the main metal contaminants present in Indian soil displayed according to their amount in percentage

- The properties (physical and chemical) as well as the volume of polluted soils.
- Socioeconomic considerations.
- The application of technology should be done by experts.
- Concentration of the pollutants and their form.
- Collateral damage.
- Any selected usage of the cleaned site.

9.2 Existing Methods of Soil Remediation

Soil remediation is nothing but a method devised to remove the contaminants from the soil via several optimized methods. Soil remediation is generally aimed at removal of hydrocarbons (petroleum), heavy metals, pesticides, cyanides, volatiles, creosotes (these are carbonaceous products released during the distillation of several types of tars), and semi-volatiles.

The main types of soil remediation processes used presently are given below:

1. Bioremediation: This form of remediation generally involves the removal of soil contaminants making use of microorganisms. Both aerobic and anaerobic

microbes are used for this purpose. Some of the microorganisms used in this process of bioremediation are *Pseudomonas putida, Dechloromonas aromatica*, *Deinococcus radiodurans*, *Methylibium petroleiphilum*, *Phanerochaete chrysosporium*, etc. Bioremediation is essentially effective when the soil is contaminated by petroleum hydrocarbons (Jørgensen et al. 2000). But it is a site-specific procedure, and its viability study should be done so that it can be applied successfully in full-scale. The final goal of all the viability studies remains the same which is to exactly decide the cost and time needed to remove the hydrocarbons according to the standard cleanup norms. These studies give an idea about the potential of biological procedures to deal with the contamination problem (Balba et al. 1998).

2. Thermal soil remediation: This process makes use of a primary treatment unit also referred to as PTU. Here the contaminated soil has to be heated to temperatures of 650oF to 900oF. This results in the vaporization of the contaminants. The method is generally used for the treatment of hydrocarbon-contaminated soil (petrol spills). Water also tends to move out of the soil sample. The heat-treated soil is then discharged into a cooling unit which mainly has a mixer or an auger. Here water is added to introduce the initial moisture content of the soil and also to control dust (Kawala and Atamanc'zuk 1998).
3. Air sparging: As we know the soil is arranged in strata. In this process air at high pressure is introduced (sparged) into the strata to remove the organic vapors of the contaminants if trapped. The vapors are then removed via a carbon filter. The process depends on the pH of the soil, concentration of contaminate, and permeability of the soil (Marley et al. 1992).
4. Electrokinetic remediation: This procedure has a great potential to remove metals from soil in heavily contaminated areas that include heterogeneous soil and/or soil with little permeability at the particular sites. The process requires installation of wells encompassing the contaminated area. Electrodes are then introduced in the wells where a low voltage DC current is given. The contaminants moved toward their respective electrodes (cathodes or anodes) according to their charge as well as the direction of water flow due to the resultant electric potential (Pamukcu and Wittle 1992; Segall and Bruell 1992; Acar and Alshawabkeh 1993; Probstein and Hicks 1993; Eykholt and Daniel 1994). These are then extracted after collection at the respective electrodes (cathodes or anodes) and then finally treated.
5. Natural attenuation: This process involves the use of natural procedure to lower the environmental pollution concentration to a standard tolerable level. US EPA (Stokinger and Uranium 1981) defined this process as "use of natural processes to contain the spread of the contamination from chemical spills and reduce the concentration and amount of pollutants at contaminated sites." This can be named as bio-attenuation or intrinsic remediation (Nirdosh 1999; Mulligan and Yong 2004; Brady et al. 2003) and is known as the least invasive method of remediation.
6. Phytoremediation: This technology being eco-friendly and cost-efficient has raised interest among many specially because metal-hyperaccumulating plant

species are being used to remediate heavy metals from contaminated soils (He et al. 2015). The plants that can accumulate heavy metals at some concentrations are barley, sunflower, cruciferous plants, and brassica (Zhao et al. 2003; Koopmans et al. 2008).

7. Soil washing: This is an ex situ form of soil remediation which involves scrubbing of soil with water to remove contaminants. This method helps in remediation by either suspending the contaminants in the wash solution or concentrating them into a smaller volume of soil by particle size diffusion, attrition scrubbing, or gravity separation. It has shown a great prospect in the field of soil remediation as it can be used to aim at a wide range of pollutants like radio nucleotides, heavy metals, and organic pollutants among others. The process has the following steps: pretreatment (removing oversize material), separation (removal of contaminants by targeting a cutoff size of 63–74 microns), coarse-grained treatment (removing the residue from the separation step), fine-grain treatment (aiming at removing material finer than 63–74 micron), process water treatment, and residual management (Goutam and Gotmare 2016). The methods along with the contaminants that they target are shown in Table 9.1.

9.3 Limitations of the Existing Remedial Methods

However, there exists certain disadvantages to these methods. Bioremediation has a longer treatment time and hence, cannot be used as an immediate remediation method. Further, it is not suitable for a wide range of contaminants. Additionally,

Table 9.1 The various processes of soil remediation along with the contaminant they target

Sr. No.	Remediation method	Contaminants that it targets	References
1.	Bioremediation	Chlorinated solvents, polychlorinated biphenyls, benzene, toluene, ethylbenzene and xylene (BTEX), polyaromatic hydrocarbons, pesticides	Vidali (2001) and Lee and Banks (1993)
2.	Thermal soil remediation	Volatile and semi-volatile components, polynuclear aromatic hydrocarbons	Kawala and Atamanc'zuk (1998) and Carrigan and Nitau (2000)
3.	Air sparging	Volatile organic compounds	Johnson et al. (1993)
4.	Electrokinetic remediation	Heavy metals	Reddy and Chintamreddy (2003)
5.	Natural attenuation	Metals and organic contaminants	Mulligan and Yong (2004)
6.	Phytoremediation	Metals	Audet and Charest (2007)
7.	Soil washing	Organic contaminants, heavy metals, radio nucleotides	Goutam and Gotmare (2016)

field monitoring to check the rate or extent of biodegradation and performance evaluation is not possible. In case of thermal treatment of soil, the soil needs to be excavated; this adds to the processing cost. Moreover, setting up of preexisting conditions for processing also tends to add to the cost making it financially taxing when considered in a large scale. The process of air sparging is not suitable for stratified soils and cannot be utilized to treat closed aquifers. There are certain physicochemical as well as biological interactions that are yet to be properly understood. Further, it induces the migration of contaminants from the site of remediation to other zones, which is undesirable. Extensive pilot-scale testing and monitoring to ensure vapor control and limit contaminant migration is essential when it comes to air sparging. Even though electrokinetic remediation has been seen to be the most efficient of all the processes existing, it too has certain disadvantages. For example, the process is time-consuming and tedious. The problem with this process also arises if there is a large concentration of non-targeted ionic species. Further studies show that employment of this method changes the pH of the soil and can have adverse effect on the microbial community of the soil which can be detrimental. The electrolyte will be degraded under acidic conditions existing close to the anode. Stagnant or static zones should be maintained between the electrodes where movement is considerably slow (Sharma and Reddy 2004).

Hence an alternative method having minimum disadvantages is required to carry out efficient soil remediation.

9.4 Nanotechnology

Nanotechnology can be described as research and technology development at the atomic, molecular, or macromolecular levels. The length scale of these moieties are approximately one to one hundred nanometers in any dimension. The creation and use of structures, devices, and systems have novel properties and functions because of their small size and the ability to control or ‘‘manipulate matter on an atomic scale” (US EPA 2007).

When compared to large-sized particles, nano-materials are much more reactive due to bigger surface area per unit mass (Rickerby and Morrison 2007). This makes the usage of nano-materials more appropriate in case of environmental pollution control.

9.4.1 Nanotechnology and Soil Remediation

Engineered nano-materials have a greater potential for environmental pollution control because they are cost-effective and more reactive than the standard methods. The engineered nano-materials also enhance the option of in situ methods of treatment. Some examples of such engineered nano-material used in soil remediation are:

- Nanoscale calcium peroxide – used for degrading organic compounds like gasoline
- Nanoscale zerovalent iron – utilized for destroying organic compounds that are halogenated
- Nanoscale metal oxides – used for metal adsorption
- Other nanoparticles, such as carbon nanotubes, bionanoparticles, polymeric nanoparticles, etc. – used for the removal of aromatic and heavy metal contaminants

The above nano-implemented methods are quite novel. Intensive study regarding the impact of these engineered nano-materials on the surrounding environment and their mobility is required (Mueller and Nowack 2010).

9.5 Engineered Nano-materials for Soil Remediation

9.5.1 Nanoscale Calcium Peroxide

The use of chemical oxidation as a method of remediation for contaminated soil is a growing trend when compared to the traditional methods of in situ remediation. Common reagents used in this case are Fenton's reagent (mix of ferrous salts and hydrogen peroxide), sodium and potassium permanganate, ozone, calcium peroxide, etc. For this process of chemical oxidation, the use of calcium peroxide is the best choice as the release of the peroxide is at a slower pace. This results in longer time for attenuation of the remediation reagent. However, the only drawback of this method is that the effective speed of reaction in this case is very slow (Khodaveisi et al. 2011). The use of calcium peroxide nanoparticles however speeds up the reaction by increasing the surface to volume ratio. The production of this nanoparticle has also shown to reduce agglomeration of the individual moieties once they have been applied or released into the soil (Khodaveisi et al. 2011). Calcium peroxide nanoparticles have recently been applied to remediate soil contaminated with oil (Karn et al. 2009). Recent research and developments have also led to formulation of surface modification to prevent irreversible agglomeration of these nanoparticles (Khodaveisi et al. 2011). The technique to reduce agglomeration has been based on hydrolysis-precipitation procedure. Here $CaCl_2$ is used as a precursor along with PEG200 (Polyethylene glycol 200), which is a surface modifier. Transmission electron microscopic (TEM) analysis has shown production of nanoparticles of size ranging from 15 to 25 nm (Khodaveisi et al. 2011). Nanoscale calcium peroxide has been reported to have a beneficial role in removing aromatic substances from the soil (Khodaveisi et al. 2011). Recently two American companies have used these for the successful removal of gasoline, heating oil, and methyl tertiary butyl ether (MTBE) from soil (Mueller and Nowack 2010). The oxygen produced as a result of this degrading reaction between the nanoparticles and the contaminant facilitates in creating an aerobic environment suitable for bioremediation (Mueller and Nowack

2010). These nanoparticles have also been extremely useful in hydrocarbon degradation (Cassidy et al. 2008; Goi et al. 2011; Khodaveisi et al. 2011; Bianchi et al. 1994; Arienzo 2000; Hanh et al. 2005). Calcium peroxide nanoparticles have been documented to target contaminants residing in a wide pH spectrum (Arienzo 2000). Research has also shown that application of calcium peroxide nanoparticles reduces the remediation time by subsequent oxidation and alkalization of the soil environment (Malachouska-Joutsz and Niesler 2015).

9.5.2 Nanoscale Zerovalent Iron (nZVI)

Perhaps the most widely utilized nano-remediation technique is the use of nZVI in groundwater and soil remediation (Nowack 2008). nZVI has a number of potential benefits like its faster activity on contaminants and its capability to target a broad spectrum of soil and water contaminants (Zhang and Elliott 2006; Mueller et al. 2012; Zhang 2003; Tratnyek and Johnson 2006; Klimkova et al. 2008). The degradation of polycyclic aromatic hydrocarbons (PAHs) by these nZVIs adsorbed to soils has been reported at room temperature (Chang et al. 2005, 2007). However, when similar conditions were maintained and were implemented, only 38% of the polychlorinated biphenyls (PCBs) were destroyed. This was mainly accounted for their very strong sorption to the soil matrix (Varanasi et al. 2007).

According to Zhang et al. in 2003, these nanoparticles induced an increase in the pH, decrease in the reduction potential by rapid consumption of O_2, and production of hydrogen. The first field application of these nanoparticles was in the year 2000. These nanoparticles remained active in the soil for up to 8 weeks. In one study, Zhang et al. achieved a 99% treatment efficiency of TCE (trichloroethylene), a carcinogenic chemical by the application of nZVI.

9.5.3 Drawbacks of Using Nanoparticles for Soil Remediation

When these iron nanoparticles are released into the soil, it experiences a high degree of aggregation which impedes its movement. Coating these nanoparticles with a suitable polymer decreases their aggregation probability. Klaine (2008) suggested that despite the environment containing many natural particles at the nanoscale, manufactured nanoparticles may act differently. These materials are designed to have specific surface properties and chemistries that are not likely to be found in natural particles. Irrespective of their increased specificity and wide range of diffusion capacity, these nanoparticles display certain limitations. Ultra particulate testing and screening have shown that particles having a size less than 100 nm can display pulmonary toxicity. Further inhalation of Fe^0(s) nanoparticles could result in

the release of Fe(III), followed by oxidative damage due to generation of Fe(IV) (Keenan and Sedlak 2008). In vitro studies examining the response of the central nervous system to low concentrations of nano-Fe and nanomagnetite showed that these nanoparticles are taken up into cells and produce an oxidative stress response (Auffan et al. 2008). These studies indicate a potential for adverse health effects from exposure and uptake of Fe oxide nanoparticles into mammalian cells.

9.5.4 Nanoscale Metal Oxides

Manganese oxide (MnO_2) was known to be effective in the degradation of pharmaceutical chemicals which have been released into the water bodies, especially groundwater. But its relative importance and potential in remediation of soil-sorbed contaminants was never explored. It was Han et al., in the year of 2015, who synthesized, for the first time, a stabilized form of these manganese oxide nanoparticles using carboxymethyl celluloses (CMC) as a stabilizer. They then tested their effectiveness for degrading aqueous and soil-sorbed estradiol. This gave definitive proof about the probability of manganese oxide nanoparticles being used as a potential source of soil remediation. The scientists observed that in typical aquatic pH, which is around 6–7, CMC-stabilized MnO_2 exhibited faster degradation kinetics for oxidation of 17-β-estradiol than MnO_2 which were not stabilized. The reactive action becomes even more pronounced when used for treating soil-sorbed estradiol. This is mainly because of CMC having the ability to complex with metal ions and preventing the reactive sites from binding soil components which inhibits its activity. A retarded first-order rate model was able to interpret the oxidation kinetics for CMC-stabilized MnO_2. There are many factors which tend to inhibit the oxidation effectiveness of these nanoparticles. Desorption rate, soil-MnO_2 interaction, soil-released metals, and reductants are some of them. CMC-stabilized MnO_2 nanoparticles hold the potential for facilitating in situ oxidative degradation of various emerging contaminants in soil and groundwater (Han et al. 2015).

Cerium oxide has recently come into attention due to the wide applicability of cerium oxide nanoparticles. Cerium oxide nanoparticles were useful in prevention of the bioaccumulation of soil contaminants from industries of textile, food processing, pharmaceutical, detergent, pesticides, and leather tanneries (Pradhan and Parida 2010; Dahle and Arai 2015). Iron-cerium oxide in mixed oxide states, or as a multiferrite composite, is seen to have potential to remove such contaminants by affinity-based binding, resulting in the subsequent removal of heavy metals from the soil (Vivekananthan et al. 2014; Peng et al. 2015). Another reason for such mixed oxide formulation is to provide stability to these nanostructures (Vivekananthan et al. 2014). Cerium oxide nanoparticles being semiconducting in nature display photo-catalysis. This phenomenon is also important for the degradation of dyes which contaminate soil (Korsvik et al. 2007).

9.6 Other Nano-materials

Other classes of nano-material which are used in the process of soil remediation are carbon nanoparticles (carbon nanotubes, including single walled and double walled), polymeric nanoparticles (nanowire of polypyrrole, polyaniline, poly(3,4-ethylenedioxythiophene) dendrimers (PAMAM)), nanocomposites (nanocomposite of polyethylene oxide and polyethyleneimine; CNT epoxy composites consist of hydrocarbon polymer composites, conjugated polymer composites, CNTs with polycarbonates, fluoropolymers, polyethylene glycol, polyester polyamides, and so forth), and bionanoparticles (virus, plasmids, proteins, etc.) (Rizwan et al. 2014).

The brilliant properties of carbon-based nano-materials such as nanocrystals and carbon nanotube(s) (CNT(s)) have gifted us with new technologies to solve and identify a broad range of environmental applications: sorbents, high-flux membranes, depth filters, antimicrobial agents, environmental sensors, renewable energy technologies, and pollution prevention strategies (Rizwan et al. 2014; Mauter and Elimelech 2008). For example, CD-co-hexamethylene/toluene diisocyanate polyurethanes and CNT-modified equivalents have been developed and have been successfully applied in removing organic contaminants from soil to a considerable low level (Li et al. 2010).

Among polymeric nanoparticles, amphiphilic polyurethane (APU) NP are used for remediation of soil contaminated with polynuclear aromatic hydrocarbons (PAH). The particles are made of polyurethane acrylate anionomer (UAA) or poly(ethylene glycol). APU is stable and has a hydrophobic interior and hydrophilic exterior, facilitating their mobility through soil. The affinity of APU particles for soil contaminants such as phenanthrene (PHEN) can be controlled by changing the size of the hydrophobic segment used in the chain synthesis. The motility and dispersion of colloidal APU suspensions in soil are controlled by the charge density or the size of the pendent water-soluble chains that reside on the particle surface (Rizwan et al. 2014; Tungittiplakorn et al. 2004).

Studies from mine pools suggested that *Gundelia tournefortii, Centaurea virgata, Reseda lutea, Scariola orientalis, Eleagnum angustifolia*, and *Noaea mucronata* (plants growing in these pools) had a strong ability to accumulate heavy metals from soil, such as lead, copper nickel, zinc, etc. Out of these plants, *Noaea mucronata* was seen to have the highest ability to accumulate lead and other heavy metals. Nanoparticles were produced from these phyto-sources and are actively being used in nanobioremediation of the environment (Mohsenzadeh and Rad 2012).

9.7 Conclusion

Nanoparticles are seen to be potential entities for the remediation of soil. These particles have been seen to be extremely reactive and have high sorption capacity. There are however technicalities associated with this form of environment

remediation. The two most important being (i) the delivery to the targeted region and (ii) toxicity to animals and plants.

The Royal Society and the Royal Society of Engineering in 2008 summed up nano-remediation as: "While there have been no significant events that would lead us to suppose that the contemporary introduction of novel materials is a source of environmental hazard, we are acutely aware of past instances where new chemicals and products, originally thought to be entirely benign, turned out to have very high environmental and public health costs."

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https://doi.org/10.1007/978-3-319-97922-9