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Zhen Fang *Editor*

Pretreatment Techniques for Biofuels and Biorefineries

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Pretreatment Techniques for Biofuels and Biorefineries

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ISSN 1865-3529 ISSN 1865-3537 (electronic)
ISBN 978-3-642-32734-6 ISBN 978-3-642-32735-3 (eBook)
DOI 10.1007/978-3-642-32735-3
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2012953996

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Pretreatment is the first and the most crucial step for effectively using biomass and for developing new routes to produce biofuels and value-added products. Pretreatment is a process intensive step and, for example, it is the single most expensive processing step in cellulosic ethanol production, making up approximately 20–40 % of the product cost. Although there are many research articles that focus on pretreatment techniques, it was felt by the authors that there was a lack of a comprehensive source where one could turn to understand the many possible methods and their range of application.

This text includes 19 chapters contributed by world-leading experts on pretreatment methods for biomass. It gives an extensive coverage for different types of biomass (e.g. molasses, sugar beet pulp, cheese whey, sugarcane residues, palm waste, vegetable oil, straws, stalks and wood), for different types of pretreatment approaches (e.g. physical, thermal, chemical, physical–chemical and biological) and for methods that show subsequent production of biofuels and chemicals such as sugars, ethanol, extracellular polysaccharides, biodiesel, gas and oil. In addition to traditional methods such as steam, hot-water, hydrothermal, diluted acid, organosolv, ozonolysis, sulfite, milling, fungal and bacterial, microwave, ultrasonic, plasma, torrefaction, pelletization, gasification (including biogas) and liquefaction pretreatments, novel techniques (e.g. nano- and solid-catalysts, organic electrolyte solutions and ionic liquids) are introduced and discussed.

Each chapter was strictly reviewed externally by experts in biofuels listed in the Acknowledgement. The chapters are categorized into seven parts:

- Part I: Biopretreatment
- Part II: Thermal Pretreatment
- Part III: Chemical Pretreatment
- Part IV: Physicochemical Pretreatment
- Part V: Gasification, Liquefaction and Biogas
- Part VI: Novel Pretreatment Techniques
- Part VII: Treatment of Different Types of Biomass

This book offers a review of state-of-the-art research and provides guidance for future paths for developing pretreatment techniques of biomass for biofuels in the fields

of biotechnology, microbiology, chemistry, materials science and engineering. It is our intention to provide a systematic introduction to pretreatment techniques. It is an accessible reference book for students, researchers, academicians and industrialists in biorefineries.

Acknowledgement

First and foremost, I would like to thank all the contributing authors for their many efforts to insure the reliability of the information given in the chapters. Contributing authors have really made this project realizable.

Apart from the efforts of authors, I would also like to acknowledge the referees listed below for carefully reading the book chapters and giving constructive comments that significantly improved the quality of the book:

Prof. Nicolas Abatzoglou (Université de Sherbrooke, Canada), Dr. Muhammad T. Afzal (Univ. of New Brunswick, Canada), Dr. Taku Michael Aida (Tohoku Univ., Japan), Dr. Zainal Alimuddin Zainal Alauddin (Universiti Sains Malaysia), Prof. Jacques E. Amouroux (LGPPTS/UPMC/ENSCP, France), Dr. Anju Arora (Indian Agricultural Research Institute), Dr. Hassane Assaaoudi (McGill Univ., Canada), Dr. Hassan Azaizeh (Tel Hai College, Israel), Prof. Nicolas Brosse (Nancy Univ., France), Prof. Rafael B. Mato Chaín (Universidad de Valladolid, Spain), Dr. Pascale Champagne (Queen's Univ., Canada), Prof. Hongzhang Chen (Chinese Academy of Sciences), Prof. Andrzej G. Chmielewski (Warsaw Univ. of Technology), Prof. Jae-woo Chung (Gyeongnam National Univ. of Science and Technology, South Korea), Dr. Daniel Ciolkosz (Pennsylvania State Univ.), Dr. Louis J. Circeo (Applied Plasma Arc Technologies, LLC, Atlanta), Dr. Rudolf Deutschmann (Lakehead Univ., Canada), Dr. Tim Dumonceaux (Agriculture and Agri-Food Canada), Dr. Animesh Dutta (Univ. of Guelph, Canada), Prof. Elena Efremenko (The M.V. Lomonosov Moscow State Univ.), Prof. Xu Fang (Shandong Univ., China), Prof. Toshi Funazukuri (Chuo Univ., Japan), Prof. Pag-asa Gaspillo (De La Salle Univ., Philippines), Dr. Anli Geng (Ngee Ann Polytechnic, Singapore), Prof. Ashwani K. Gupta (Univ. of Maryland), Prof. Rick Gustafson (Univ. of Washington), Prof. Michikazu Hara (Tokyo Institute of Technology), Prof. Adriaan van Heiningen (Univ. of Maine), Dr. Kazuhiko Ishikawa (Advanced Industrial Sciencr and Technology, Japan), Dr. Keikhosro Karimi (Isfahan Univ. of Technology, Iran), Prof. Hansie Knoetze (Stellenbosch Univ., South Africa), Prof. Gunnur Kocar (Ege Univ., Turkey), Prof. Ramesh Chander Kuhad (Univ. of Delhi South Campus, India), Dr. Christopher Lan (Univ. of Ottawa), Mr. Jean-Rémi Lanteigne (École Polytechnique de Montréal, Canada), Dr. Jean-Michel Lavoie (Université de Sherbrooke, Canada), Dr. Jianjun Li (Chinese Academy of Sciences), Dr. Yebo Li (Ohio State Univ.), Prof.

Yin Li (Chinese Academy of Sciences), Dr. Lin Lin (Jiangsu Univ., China), Prof. Yun Liu (Beijing Univ. of Chemical Technology), Dr. Poupak Mehrani (Univ. of Ottawa), Dr. Felipe Alatriste Mondragon (Instituto Potosino de Investigación Cientifica y Tecnologica, Mexico), Dr. Antonis Mountouris (National Technical Univ. of Athens), Dr. Naim Najami (The Academic Arab College of Education, Israel), Prof. Yonghao Ni (Univ. of New Brunswick), Prof. Lucia García Nieto (Universidad de Zaragoza, Spain), Dr. Abdul-Sattar Nizami (Univ. of Toronto), Dr. Melek Özkan (Gebze Institute of Technology, Turkey), Prof. Igor Polikarpov (Universidade de São Paulo, Brazil), Prof. Xinhua Qi (Nankai Univ., China), Dr. Wensheng Qin (Lakehead Univ.), Dr. Armando T. Quitain (Kumamoto Univ., Japan), Dr. R. Michael Raab (Agrivida, Massachusetts), Dr. Mala Rao (National Chemical Lab., India), Prof. Joseph P. Roise (North Carolina State Univ.), Dr. Guus van Rossum (Univ. of Twente, the Netherlands), Prof. Roger Ruan (Univ. of Minnesota), Prof. Elio Santacesaria (Complesso Universitario di Monte S.Angelo, Italy), Dr. Anton Sonnenberg (Wageningen UR, the Netherlands), Dr. Andy Soria (Univ. of Alaska), Dr. Wolfgang Stelte (Technical Univ. of Denmark), Dr. Chia-Hung Su (Ming-Chi Univ. of Technology, Taiwan, ROC), Dr. Lee Keat Teong (Universiti Sains Malaysia), Mr. Xiao-fei Tian (Chinese Academy of Sciences), Prof. Montserrat Zamorano Toro (Universidad de Granada, Spain), Mr. Satriyo Krido Wahono (Indonesian Institute of Sciences), Dr. Haisong Wang (Chinese Academy of Sciences), Dr. Chunbao (Charles) Xu (Western Univ., Canada), Dr. Jing Yang (Southwest Forestry Univ., China), Dr. Wennan Zhang (Mid Sweden Univ.), Dr. Xiao Zhang (Washington State Univ.), Prof. Xiao-yu Zhang (Huazhong Univ. of Science and Technology, China), Dr. Y.-H. Percival Zhang (Virginia Tech), Dr. Xuebing Zhao (Tsinghua Univ., China), Dr. Junyong Zhu (USDA Forest Service).

I am also grateful to Ms. June Tang (Associate Editor, Springer) for her encouragement and guidelines during my preparation of the book.

Finally, I would like to express my deepest gratitude towards my family for their kind cooperation and encouragement, which help me in completion of this project. Especially, I would like to dedicate this book to my Mother (Ms. Shubi Yu) for her love and support during her final illness. She passed away at 9:30 am on April 9, 2012 in Taining, Fujian.

May 30, 2012 Zhen Fang
Kunming

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Part I
Biopretreatment

Chapter 1
Biological Pretreatment of Lignocellulosic Biomass for Enzymatic Saccharification

Nandhagopal Narayanaswamy, Pratibha Dheeran, Shilpi Verma and Sachin Kumar

Abstract Biological delignification is an attractive approach for pretreatment of lignocellulosic biomass. This approach is very cost effective, low-energy requirement, environment friendly, low formation of toxic materials such as furfural, hydroxymethylfurfural, etc. Biological approach has been demonstrated using direct microorganism as well as using enzymes extracted from microbes. The microbial treatment includes fungi such as white-rot fungi, brown-rot fungi and soft-rot fungi, and bacteria. Both of brown-rot and soft-rot fungi principally degrade the plant polysaccharides with minimal lignin degradation, while white-rot fungi are capable of complete mineralization of both the lignin and the polysaccharide components. This chapter presents a brief review of the relevant and updated literature on biological pretreatment of lignocellulosic biomass. Various approaches used by different researchers for biological delignification of lignocellulosic biomass, including microbial and enzymatic approaches, mode of action, effect of biological pretreatment on lignocellulosic biomass, effect of biological pretreatment on enzymatic hydrolysis, have been included in this chapter. The chapter also provides a glimpse of the gaps, which need to be studied.

Keywords Lignocelluloses · Pretreatment · Lignin · Biological delignification · Fungi · Bacteria

1.1 Introduction

In view of environmental and fossil fuel security concern, the future energy economy will probably be based on a broad range of alternative energy resources such as wind, water, sun, nuclear fission as well as biomass. Extensive use of fossil fuels in the

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Z. Fang (ed.), *Pretreatment Techniques for Biofuels and Biorefineries,*
Green Energy and Technology,
DOI 10.1007/978-3-642-32735-3_1,

last century has greatly depleted the energy reserves. Presently, the petroleum-based fuels—gasoline, aviation turbine fuels, and diesel—all liquid fuels, and compressed natural gas (CNG) are almost excessively used in the transportation sector. The increasing rate of consumption of fossil fuels has raised severe problems including the issues of depletion of energy resources, increase in fuel prices, and global climate change. The major attraction of the use of renewable energy fuels is the reduction of environmental impacts that are associated with the use of the fossil fuels [1]. Therefore, an imperative technology is required to ward off the apprehensive problems of meager fossil fuels and its negative impact on environments. Finally, researchers are looking for the economical way to produce alternative fuels and energy preferably from abundantly available biodegradable or eco-friendly and renewable raw materials such as biomass or renewable resources such as sun, wind, water, etc. These resources have a vital role and equal contribution in the energy sector [2].

Among the potential bioenergy resources, lignocellulosic biomass has been identified as a cheap and effective feed-stock for the production of biofuels such as bioethanol, biobutanol, and biogas. Lignocellulosic biomass is available about 180 million tons per year from agriculture and other sources [3, 4]. Lignocellulose is the most abundant renewable and natural resource, which have a promising role in renewable energy sector and have fetched many researchers toward a new road map to the biofuels production. Biofuels such as ethanol, butanol, hydrogen, biogas, etc. from lignocellulosic biomass and non-food sources have caught worldwide attention. The lignocellulosic biomass has increased its attention because these raw materials do not compete with food crop and is less expensive than conventional feed-stock like sugarcane, corn, etc. In general, lignocellulosic feed-stocks are observed as promising alternative sources because it consist massive amount of carbohydrates [5]. All lignocellulosic biomass predominantly comprise cellulose, hemicellulose, and lignin, but in a different ratio with respect to distinct biomass [6]. However, lignocellulosic materials are naturally recalcitrant and have more complex structure [3, 5]. Lignocellulosic biomass for the production of biofuels includes forest residues such as wood; agricultural residues such as sugarcane bagasse, corn cob, corn stover, wheat, and rice straws; industrial residue such as pulp and paper processing waste; municipal solid wastes; and energy crops such as switch grass [7–11].

Cellulose is the most abundant organic compounds on the earth and this polysaccharide consists of linear chain of several hundred to 10 thousands recurring D-glucose units with molecular formula $(C_6H_{10}O_5)\,n$, linked by $\beta(1 \rightarrow 4)$ glycosidic bonds. Cellulose is a structural component of a primary cell wall in green plants and algae. Naturally, cellulose can be found in two different forms in the plant materials, consists of parts with crystalline structure and amorphous structure. The crystalline celluloses are well organized, which are tightly bundled and bound together by strong inter chain hydrogen bonds while this is less pronounced in amorphous cellulose.

Hemicelluloses, the second most abundant natural polymer on the earth [3, 12], are the heterogeneous polymers consisting of pentoses (D-xylose, D-arabinose),

hexoses (D-glucose, D-mannose, and D-galactose), and sugar acids. Hemicellulose is a connector between cellulose and lignin, and it leads to more rigidity. In hardwood, hemicelluloses are dominantly found as xylan, whereas in softwood glucomannan are most common [3, 12, 13]. Xylans are commonly found as heteropolysaccharide in many plants with backbone chain of 1,4-linked β-D-xylopyranose units. Along with xylose unit, xylan may comprise arabinose, glucornic acid or its 4-O-dimethyl ether, acetic acid, ferulic, and *p*-coumaric acids. Xylan can be simply extracted in an acid or alkaline environment but in the case of glucomannan requires stronger acid or alkaline environment [3, 12]. Hemicellulose is also an economically important natural polymer as it contains ample amount of pentose sugar, which can be used as a substrate in food, pharmaceutical, and biofuels industries.

Lignin, the third largest available biopolymer in nature [3, 12], is a heterogenous and irregular arrangement of phenylpropanoid polymer that reduces the chemical and enzymatic degradation to maintain the recalcitrant and insoluble properties of lignocellulose. Three phenylpropionic alcohols primarily exist as monomers of lignin (i) coniferyl alcohol, (ii) coumaryl alcohol, and (iii) sinapyl alcohol. In general, herbaceous plants such as grasses, rice, and wheat straws have the lowest contents of lignin, while in softwoods lignin content is found to be higher. Lignin is the major rate-limiting component in the carbon recycling reaction, as its oxidation rate is naturally very slow [14, 15]. Furthermore, lignin has an important role in conducting water in plant stems and giving physical strength to the plants.

The main routes to produce fuels from biomass (biofuels) include fermentation of sugars to alcohol, gasification and chemical synthesis, and direct liquefaction. The biological process for converting lignocellulose to biofuels requires: (1) delignification to liberate cellulose and hemicelluloses from the matrix; (2) depolymerization of the carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars [16–19]. All these processes comprise the same main components: hydrolysis of the hemicellulose and the cellulose to monomer sugars, fermentation, and product recovery. The main difference between the process alternatives is the hydrolysis steps, which can either be accomplished by an acid or by enzymes [20].

Lignocellulosic materials need to be saccharified to produce fermentable sugars. This is an intensive process involving a combination of pretreatment and either chemical (acid hydrolysis) or enzymatic hydrolysis [20–22]. In the chemical process, the hydrolysis of sugar polymers in lignocellulosic material is catalyzed by an acid, whereas in the enzymatic process, enzymes are used for hydrolyzing cellulose and hemicellulose to sugar monomers [23–25]. Several factors influence the yields of the monomeric sugars from the lignocellulosic matter and the by-products during hydrolysis. These factors include biomass particle size, liquid-to-solid ratio, type and concentration of acid used, temperature, reaction time, length of the macromolecules, porosity of the biomass, degree of polymerization of cellulose, configuration of the cellulose chain, association of cellulose with other protective polymeric structures within the plant cell wall such as lignin, pectin, hemicellulose, proteins, and mineral elements, etc. [26–28].

Enzymatic hydrolysis offers major advantages over other chemical routes (e.g., acid hydrolysis) such as higher yields, minimal by-product formation, low-energy requirements, mild operating conditions, and low-chemical disposal costs [29]. Hydrolysis of cellulose to glucose in aqueous media catalyzed by the cellulase enzyme suffers from slow reaction rates due to high crystalline structure of cellulose, degree of polymerization, pore volume, acetyl group bound to hemicellulose, surface area, hydrophobicity, and biomass particle size, which make the penetration of enzymes to the active sites very difficult [30–32]. The enzymatic hydrolysis without pretreatment yields sugars which is $<20\,\%$ of the theoretical quantity, whereas $>90\,\%$ of the theoretical quantity of sugars are obtained with enzymatic saccharification after pretreatment [33, 34]. Therefore, pretreatment is a necessary and prudent step to break the crystalline structure of the lignocelluloses, the removal of lignin to expose the cellulose and hemicellulose molecules for efficient enzymatic conversion, and saccharification of feed-stock [5, 31, 35–39].

Physical, physico-chemical, chemical, and biological processes have been studied for the pretreatment of lignocellulosic materials [40–42]. Enzymatic hydrolysis of lignocellulosics can be significantly enhanced by physical, chemical, and biological pretreatments of the lignocellulosic materials to remove and modify the lignin and hemicellulose and to reduce the fiber crystallinity. The physical and chemical pretreatment including grinding, organosolv process involving extraction with hot aqueous ethanol, ozonolysis, acid/alkaline treatment, oxidative delignification, carbon dioxide explosion, hydrogen peroxide, ultrasonic irradiation, ammonia fiber expansion, wet explosion, and acid or SO_2-catalyzed steam explosion, ammonia fiber explosion (AFEX) and biological pretreatment have been followed and optimized up to certain levels [5, 31]. The objective of physical pretreatment or mechanical pretreatment is generally used to reduce the particles size, crystallinity, and degree of polymerization; and consequently it leads to increase the surface area for enzyme and/or chemical accessibility. In thermal pretreatment method, various methods have been investigated such as steam explosion/steam pretreatment, liquid hot water, etc.

Chemical pretreatment is another important technique that has been commonly followed by many industries like paper and pulp industries for few decades. This treatment is mostly used by the researchers, which includes catalyzed steam explosion, acid/alkali treatment, ammonia fiber/freeze explosion (AFEX), ionic liquid pretreatment, organosolv, and pH-controlled hot water treatment. All the above treatments require different chemicals and different operating conditions [3, 43].

Biological pretreatment have been studied elaborately by various researchers because this technique is very cheap, less energy consuming process, and the refulgent area of research. In this method, microorganisms or enzymes are used as catalyst in order to modify lignin and to degrade the hemicellulosic content in the biomass. Several white-rot fungi and brown-rot fungi, such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Postia placenta*, *Phanerochaete carnosa*, *Gloeophyllum trabeum* and *Trametes versicolor* have been studied for pretreatment of biomass such as wheat and rice straws, corn stover and switch grass [31, 44]. An overview of biological pretreatment and its applications are shown in Fig. 1.1.

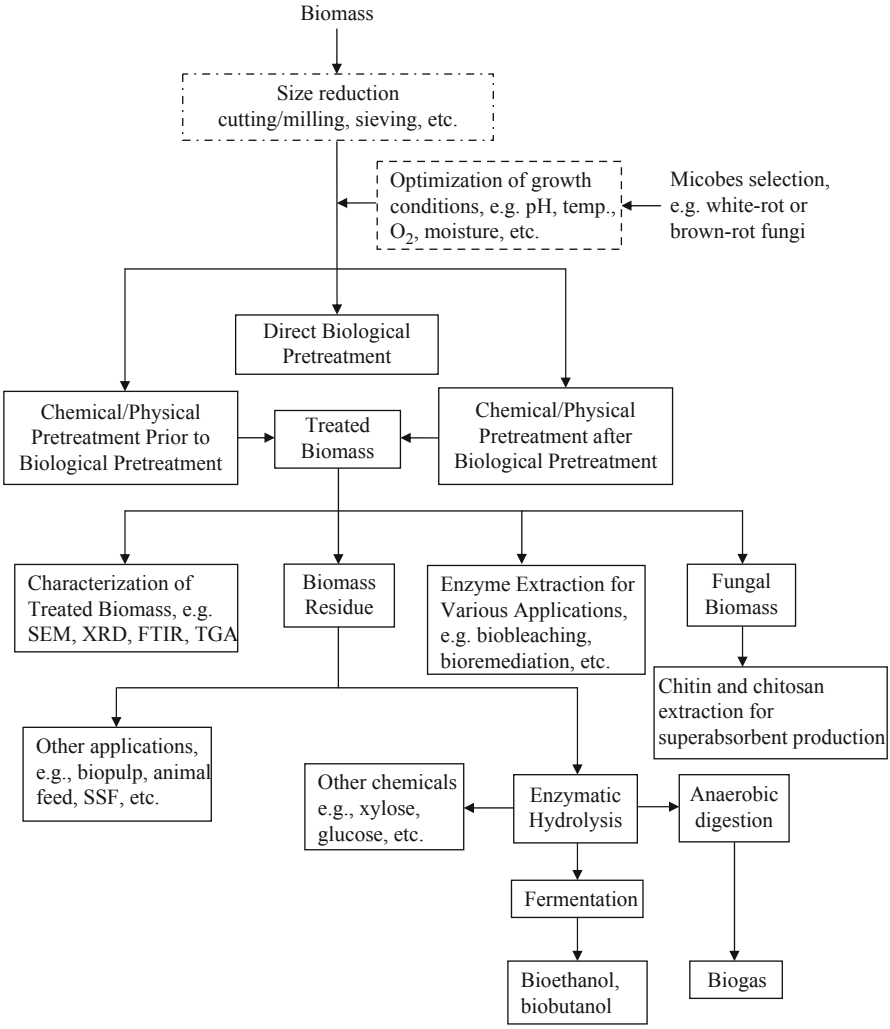

Fig. 1.1 Overview of biological treatment and its applications

All the pretreatment methods, except biological method, require expensive equipment that have demand of high energy depending on which the process to be carried out. Furthermore, these techniques often result in effluent and residue that tremendously have negative impacts on environments, inhibit the enzymatic reaction and the growth of microorganisms, which suppose to ferment the product of enzymatic saccharification [5, 31]. Indeed, biological pretreatment method using white-rot fungi has increased its attention because of the following inherent advantages, (i) safe and environmental-friendly method; (ii) low-energy consumption and cost effective; (iii) selective degradation; (iv) in some cases treated biomass directly can be used for

enzymatic conversion or fermentation; (v) increase the cellulose digestibility of many types of forage fiber and agricultural wastes [45].

This pretreatment retains many special features itself, that is why now researchers looked into biological route to achieve desired target. So far, many research papers have already been reported that the biological pretreatment has been tested and established beyond its level. Organo-solvent (ethanol, methanol, butanol, ethylene glycol, *n*-butylamine, etc.) also used along with biological treatment to enhance the degradation of internal lignin seal, removing hemicelluloses and disturbing crystalline nature of cellulose [46].

Despite all these advantages, however, biological pretreatment is a very slow process; and moreover some important components (hemicelluloses and cellulose) of biomass are also consumed either by same microorganism or by some foreign invaders. Low-saccharification rate (35–40 %) is found when compared with chemical and physical treatment methods [47]. Main objective of this chapter is to discuss various biological pretreatment methods, advantages, disadvantages, and to come out with the key to resolve the barriers in biological treatment.

1.2 Overview of Biological Pretreatment Methods

Falkowski et al. [48] reported that lignin may accumulate in terrestrial ecosystems for decades, on longer time scales most of these molecules are oxidized, so that the accumulation of organic carbon in soils is a miniscule fraction of the total carbon fixed by the ecosystem. Lakes may also store substantial amounts of organic matter in sediments. Various microorganisms have been used by many researchers and this zero pollution approach has received good attention as it helps to enhance the fermentation and enzymatic saccharification rate without much capital investment.

1.2.1 Bio-oxidation or Bio-mineralization of Lignin

In the 1920s, a small amount of research was conducted on biodegradation of lignin and few concepts about lignin were recapitulated in the 1930s (i) lignin is among the plant cell wall polymers that is more resistant to biodegradation, but can be degraded, (ii) white-rot fungi degrade lignin in wood, and (iii) could not be delignified (without loss of wood carbohydrates) [49]. Waksman et al. [50] investigated the lignin degradation in compost and soil environment. In 1951, Gottileb and Pelczar reported that white-rot fungus, *Polyporus versicolor*, used Braun's native lignin as the growth substrate. This finding revealed that lignin could be used as a sole carbon and energy source for white-rot fungi. In addition to white-rot fungi, other groups of fungi were found to degrade lignin, partially, namely basidiomycetous litter-decomposing and brown-rot fungi as well as soft-rot fungi in the 1950s [51, 52]. The first lignin compound was studied and reported in the 1960s; [53–56] and biodegradation assays based on ^{14}C-lignin were developed in the 1970s and it was revealed how lignin

was optimally degraded under laboratory conditions [57, 58]. The white-rot fungus, *P. chrysosporium*, was used as experimental organism in USA and in other laboratory, *Sporotrichum pelverulentum* was chosen for lignin bio-degradation studies [59, 60]. Previously, *T. versicolor* was a well studied experimental fungus [53, 61].

In the late 1970s and the early 1980s, very important concepts were investigated in the physiology of lignin degradation by *P. chrysosporium*. The most important discoveries were (i) low nitrogen requirement and lignin mineralization takes place during secondary metabolisms; (ii) highest mineralization was found at 100 % oxygen, thus, lignin degradation is oxidative and agitation having detrimental effects in lignin degradation; (iii) veratryl alcohol formation takes place during lignin oxidation [58]. Some other fungi, for example, *Phlebia radiata* [49, 62] were also found to readily degrade lignin and lignin model compounds in a similar way. The first extracellular enzymes involved in lignin degradations were discovered in 1983–1984 [63–65] and named as lignin peroxidases (LiPs). The catalytic mechanism of LiPs, based on initial one-electron oxidation of the lignin model compounds followed by subsequent breakdown reactions via radical cation intermediates, was experimentally verified [66, 67].

In the 1990s, in addition to pivotal studies on catalytic and enzymatic properties of the lignin-modifying peroxidases as well as their molecular biology, the major areas of research were dealt with the potential applications of white-rot fungi and their enzymes in biopulping (biomechanical pulping), pulp bleaching, and other applications. The most promising fungi for biopulping are so-called selective lignin degraders, that is, fungi that degrade larger amounts of lignin relative to carbohydrates such as *C. subvermispora* [49].

Generally, wood basidiomycetous fungi that cause white-rot in wood, called white-rot fungi, are the major lignin degraders in nature, which specifically degrades the lignin in different woody and straw or lignocellulosic biomass such as corn stover, wheat straw, paddy straw, sugarcane trashes, various wood materials, etc. [68]. The concept behind this biological degradation of lignin is secretion of several lignolytic enzymes by these white-rot fungi. Lignolytic enzymes such as LiP, manganese peroxidase (MnP), laccase (Lac), and versatile-peroxidase (VP) have a vital role in many applications like biopulping, biobleaching, biofuels, xylose, and enzyme production [32, 68, 69]. After this breakthrough discovery, many researchers conducted various experiments on molecular biology studies on lignolytic enzyme coding gene, for example, cloning and sequencing of LiP gene [70], cloning and sequencing of MnP gene [71, 72], heterologous expression of Lac [73], homologous expression of peroxidases [49, 74], 3D structure of Lac [75], and cell-free mineralization of ^{14}C-labeled synthetic and natural lignins by MnP [76]. The following microbial diversity has been described by various researchers for lignin degradation:

1.2.1.1 White-Rot Fungi

Wood-rotting basidiomycete fungi are usually divided into white-rot and brown-rot fungi. As mentioned earlier, several white-rot fungi are involved in lignin

biodegradation such as *P. chrysosporium*, *C. subvermispora*, *Phlebia subserialis*, *Echinodontium taxodii*, etc. [31, 32, 44, 77]. Majorly, white-rot fungi grow well on hard woods such as birch and aspen. On the other hand, certain species *Heterobasidion annosum*, *Phellinus pini*, and *P. radiata* grow well on soft woods such as pine and spruce [32]. However, the feasibility of biological pretreatment is still in its infancy because of the extremely long treatment time as well as the difficulty in selectively degrading lignin [5, 78, 79].

The growth of fungi on lignocellulosic biomass results in a loss of dry matter. During the fungal growth, all the main components (cellulose, hemicelluloses, and lignin) are consumed in part by the fungus for its growth and metabolic activities. The loss and the selective degradation of lignin is greatly depends upon the strain which is taking the course of degradation. For example, *Flammulina velutipes*, *Fomes marginatus*, and *Laetiporus sulfurous* decompose wheat straw very slowly or poorly, hence, these white-rot fungi are unsuitable for biological delignification. Some other fungi *Ganoderma applanatum*, *Poria* sp., and *Trametes gibbosa* grow well on wheat straw, but they degrade the hemicellulose and cellulose; therefore, these strains are also not found suitable for biodelignification. Although it is very difficult to remove lignin alone from the lignocellulose, some unique fungal species such as *Stropharia rugosoannulata*, *Hapalopilus rutilans*, *P. ostreatus*, *C. subvermispora*, *Lentinula edodes*, and *Pleurotus eryngii* have high affinity with lignin; and they are able to consume lignin faster than non-lignin content of biomass. Therefore, these strains are good delignifier and can be used efficiently in biological pretreatment of lignocellulose [80, 81].

White-rot fungi are more commonly found on angiosperm than on gymnosperm wood species in nature [82]. Generally, syringyl (S) units of lignin are more selectively degraded whereas guaiacyl (G) units are more resistant to degradation. The transmission electron microscopy revealed that *C. subvermispora* and *Pleurotus eryngii* partially removed the middle lamella while *P. radiata* apparently removed the lignin from secondary cell walls, when these fungi were grown on straw [83]. In fibers, the middle lamella contains a high concentration of G lignin, while, secondary walls contain a high proportion of S lignin. Various environmental conditions like cultivation time, pH, nutrient ingredients (nitrogen source), and oxygen level have been optimized by many researchers in order to achieve the maximum degradation of lignin [84]. Lignin degradation by white-rot fungi occurs through the action of lignin degrading enzymes such as peroxidases (LiP and MnP) and phenol oxidase (Lac) [5, 78, 85]. These enzymes are regulated by carbon and nitrogen sources [16]. Almost all white-rot fungi produce Lac and MnP, but only some of them produce LiP [32].

White-rot fungi degrade lignin in biomass with two different mode of degradation, named as selective and non-selective degradation. In non-selective degradation, all three components (lignin, cellulose, and hemicellulose) were almost degraded equally, whereas in selective decay mostly hemicellulose and lignin were degraded [32]. Some white-rot fungi species remove lignin without loss of cellulose from LCCs and cause white-mottled or white-pocket type of rot and those species referred as selective delignifier, for example, *Phellinus nigrolimitatus* [32, 86]. More than

1,500 species of white-rot fungi are able to decompose lignin with little consumption of cellulose [87]. There are also some fungi that are able to degrade the same wood with both types of attack selective and non-selective [49]. Good examples of such fungi are *G. applanatum* and *H. annosum*. The selective delignifiers have a pivotal role in biopulping, biobleaching and bio-fuel industries. However, the ratio of lignin–hemicellulose–cellulose decayed by a selected fungus can differ enormously; and even different strains of the same species, for example, *P. chrysosporium* and *C. subvermispora*, may act in another way on the same kind of wood. *C. subvermispora* was found to be one of the most lignin removers from woody materials, but grew poorly on rice straw [88]. Furthermore, the comparative studies of *C. subvermispora* and *P. chrysosporium* revealed that *C. subvermispora* genetic inventory and expression patterns exhibit increased oxidoreductase potential and less cellulolytic capability relative to *P. chrysosporium* [89]. Some examples of white-rot fungi, which posses selective degradations, are *Pycnoporus cinnabarinus*, *P. ostreatus*, *P. eryngii*, *P. radiata*, *Phlebia tremellosus*, *P. subserialis*, *P. pini*, and *Dichomitus squalens* [32, 86, 90]. The selective delignifiers have a potential role in pretreatment of various lignocelluloses in order to attain the considerable amount of feed-stock for the biofuel production.

Some species remove lignin more readily than carbohydrates [86]. Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones form as decay progresses and large voids filled with mycelium. This type of rot is referred to as non-selective or simultaneous rot [86]. *T.* (syn. *Coriolus, Polyporus) versicolor* and *Fomes fomentarius* are typical simultaneous-rot fungus [32, 61]. Therefore, the use of non-selective fungi is greatly limited by its non-selective degradation of plant cell walls and it may be used in biological pretreatment to some extent.

1.2.1.2 Brown-Rot Fungi

Most of the brown-rot fungi degrade cellulose and hemicellulose more rapidly than lignin in woods. But the lignin is modified up to certain level and left as modified brown lignin residue, hence collectively called as brown-rot fungi. Many brown-rot fungi such as *Serpula lacrymans*, *Coniophora puteana*, *Meruliporia incrassata*, *Laetoporeus sulphureus*, and *G. trabeum* are used in various investigations [91, 92]. Most of the brown-rot fungi prefer soft-wood to hard-wood as substrate, for example *S. lacrymans* (dry-rot fungus) and *C. puteana* are the most harmful fungi occurring in wood in temperate region.

Brown-rot fungi have a unique mechanism to break down the wood. In contrast to white-rot fungi that de-polymerize the cell wall carbohydrates only to the extent that they utilize degraded product in fungus metabolism, brown-rot fungi accumulates the de-polymerized cell wall cellulose and hemicellulose since the fungus does not utilize all the products in the metabolism [61]. Early in the decay process, these brown-rot fungal hyphae penetrate from one cell to another through existing pores in wood cell walls. The hyphae start penetration from the cell lumen, where they are in close connection with the S3 layer. The brown-rot affects the S2 layer of the wood cell wall first [49].

Although brown-rot fungi consume economically important materials in biomass, the potential biotechnology application of brown-rot fungus is used to produce cattle feed from pine dust through solid-state fermentation. The brown-rotted lignin is used as an adhesive as it reacts more rapidly than native lignin due to increased phenolic-hydroxyl groups, for example, to replace phenol-formaldehyde flake board resin [49]. *G. trabeum* is the most extensively used fungus for treatment of wood chips. For example, Monrroy et al. [46] pretreated bioorganosolv process of *Pinus radiata* wood chips by using bioorganosolv process. They used *G. trabeum* for 3 weeks followed by organosolv treatment with various ratios of ethanol–water mixture at pH 2 and optimized H factor (factor that combine time and temperature in one variable). They found significant improvement in solvent accessibility and H factor was found to be decreased from 6,000 to 1,156 for obtaining 161 g ethanol/kg of *P. radiata* wood. Another example, Ray et al. [93] pretreated Scots pine (*Pinus sylvestris*) sapwood by *C. puteana* for 35 days and they found that glucose release from the wood increased by four to five folds after 10 days exposure with minimum loss of weight (5 %) and maximum sugar release occurred 15 days after exposure to *C. puteana* with 9 % weight loss.

To some extent, brown-rot fungi have similar pathways to degrade the lignocellulose as white-rot fungi. The wood decay mechanisms of both types of fungi rely on radical formation, low pH, and the production of organic acids such as oxalic acid. The radical formation would maximize the solubility of lignin in alkali and the decay process is an oxidation reaction, hence decay can be enhanced by high oxygen supply. However, many proposed mechanisms are not fully proven experimentally [49].

1.2.1.3 Soft-Rot Fungi and Other Microfungi

Blanchette [86] has described two kinds of soft-rot: type I consisting of biconical or cylindrical cavities that are formed within secondary walls and type II refers to an erosion form of degradation. For example, *Daldinia concentrica* is the most efficient fungus of type II group, which primarily affect hardwood. Nilsson et al. [94] found 53 % weight loss in birch wood within 2 months. During early stage of classification of different wood-rotting fungi, *Xylariaceous ascomycetes* from genera such as *Daldinia, Hypoxylon*, and *Xylaria* have often been regarded as white-rot fungi, but today these fungi are categorized to soft-rot fungi as they cause typical type II soft-rot in wood. In coniferous wood (e.g., pine wood), the weight loss was very low and it has been thought that these type of woods have more guaiacyl units in middle lamella, which inhibit the growth of soft-rot fungi [49].

Some microfungi (*Penicillium chrysogenum*, *Fusarium oxysporum*, and *Fusarium solani*) identified in a forest soil sample are able to mineralize grass lignins upto 27 % [49]. However, most of the soft-rot and microfungi consume readily economically important carbohydrates during invading and have very less applications in biological pretreatment.

1.2.1.4 Bacteria and Actinomycetes

In biological pretreatment process, bacteria and actinomycetes are not as efficient as white- and brown-rot fungi. Very few bacteria, such as filamentous bacteria belonging to the genus *Streptomycetes* are well known degraders of lignin, have been studied for pretreatment. These bacteria have been found to have some role in final mineralization of lignin. Non-filamentous bacteria *Pseudomonas* degrade very little amount of lignin. Since these bacteria do not have extra cellular oxidoreductase, which is one of the very essential enzymes for delignification and cannot be utilized in biological pretreatment. Actinomycetes are bacteria which form multicellular filaments; thus, they resemble fungi, also produce extracellular peroxidase as white-rot and brown-rot fungi, for example LiP-type enzyme. *Streptomyces* sp. EC1 produces peroxidase and cell-bound demethylase requiring H_2O_2 and Mn^{2+}, both have been produced at relatively high levels in the presence of Kraft lignin or wheat straw [49]. Bacteria actinomycetes *Streptomyces viridosporus* have also been studied up to some extent [95]. Godden et al. [96] studied activity of peroxidase and catalase in six actinomycetes strains.

Thermophilic actinomycetes have been isolated from a wide range of natural substrates, for example from desert sand and compost. The genera of the thermophilic actinomycetes isolated from compost include *Nocardia*, *Streptomyces*, *Thermoactinomyces*, and *Micromonospora*. Actinomycetes degrade lignin as their primary metabolic activity and at high nitrogen levels compared to white-rot fungi, most of which degrade lignin via their secondary metabolism [97].

The lignin-degrading actinomycete species examined till date have been shown to oxidatively de-polymerize lignin. The primary degradative activity of actinomycetes is solubilization of lignin, with low levels of mineralization compared with the white-rot fungi. The depolymerization reactions produce a modified water-soluble, acid precipitable polymeric lignin as the principal lignin degradation product. The range of actinomycete species capable of metabolizing lignin is still unknown. Moreover, the strains examined thus far solubilize lignin to an acid-precipitable polymeric lignin-like product.

1.3 Enzymes Involved in Lignin Degradation or Mineralization

Enzymes face several challenges in the degradation of macromolecular lignin [49]. As mentioned earlier, this substrate is a large heterogeneous polymer and very difficult to degrade by microbes. Indeed, lignin does not contain enzymatically hydrolysable linkages and is stereo-irregular. For lignin degradation, the enzymes or agents must be oxidative. Many extracellular enzymes involved in lignin degradation are, as mentioned earlier, LiPs (LiPs, ligninases, EC 1.11.1.14), manganese peroxidases (MnPs, Mn-dependent peroxidases, EC 1.11.1.13) and Lacs (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). Further, some accessory enzymes are also involved in hydrogen peroxide production. Glyoxal oxidase (GLOX) and aryl alcohol oxidase

(AAO) (EC 1.1.3.7) belong to this group. LiPs and MnPs are heme-containing glycoproteins, which require hydrogen peroxide as an oxidant [49, 98].

1.3.1 Lignin Peroxidase (LiP)

Tien et al. [63] discovered LiP in the extracellular medium of *P. chrysosporium* grown under nitrogen limitation. The enzyme uses H_2O_2 as co-factor or mediator for activity and is capable of oxidizing and/or cleaving lignin and lignin model compounds. This was supposed to be the key reaction of lignin degradation. Very few fungi are found to produce extracellular LiP [98]. *P. chrysosporium*, *T. versicolor*, *Bjerkhandera* sp., and *T. cervina* are some fungi, which can produce LiPs [32]. Indeed, LiP was found to play only a minor role in lignin degradation by *T. versicolor*, at least as measured by bio-bleaching of kraft pulp [99].

LiPs are monomeric homo-protein and glycol protein belonging to oxidoreductase family, which specifically act on peroxide as an acceptor (peroxidases). These enzymes have molecular weight of 40 kDa and isoelectric points (pI) ranging from 2.8 to 5.3. The absorption spectrum of the native enzyme in *P. chrysosporium* has a very distinct maximum at 406–409 nm due to the presence of a single heme group, where Fe^{3+} pentacoordinates with four heme tetrapyrrole nitrogen and a histidine of LiPs (protoporphyrin IX) [32, 98]. The interaction of LiPs with its substrate follows ping-pong mechanism [100]. As shown in Fig. 1.2, LiPs are oxidized by H_2O_2 to two-electron oxidized intermediates (LiP I) along with iron ions as Fe^{4+} and free radical residues on tetrapyrolle. LiP I then oxidises the donor substrate by one electron, where the donor substrate, VA (3,4-dimethoxybenzyl alcohol, VA) yields second intermediate LiPs complex (LiPs II) in which iron ion is found in same oxidation state, that is, Fe^{+4}, but there is no free radical residue on tetrapyrolle of heme and a radical cation. LiP II then oxidises a second molecules of donor substrate (VA), confers another radical cation and native form of LiP. Here the reformation of native LiP mainly depends upon the LiP II reduction step, which is a rate limiting step in catalytic cycle. Because the reduction of LiP II is a relatively slow process and LiP II is less potent than LiP I complex. Consequently, LiP II complex is long available for reaction again with H_2O_2 leads to inactivation of enzyme and forms LiP III complex (Fig. 1.2), which is characterized as a complex between LiP and superoxide. The catalytic cycle of LiP is described in Fig. 1.2. VA radical cations act as redox mediators and are capable to reduce LiP III complex back to its native form, LiP. In this LiP catalytic cycle reaction, VA radical cations ($VA^{\bullet+}$) are usually restored back after its oxidation reaction with non-phenolic compounds of lignin.

As in this catalytic cycle reaction, VA plays an important role. Three major functions of VA have been investigated so far. Firstly, VA acts as a mediator in electron-transfer reaction. Secondly, VA is a good substrate for compound II, therefore VA is essential for completing the catalytic cycle of LiP during the oxidation of terminal substrates. Furthermore, if the inactive LiP III complex forms, the intermediate $VA^{\bullet+}$ will be capable of reducing LiP III complex back to its native form LiP

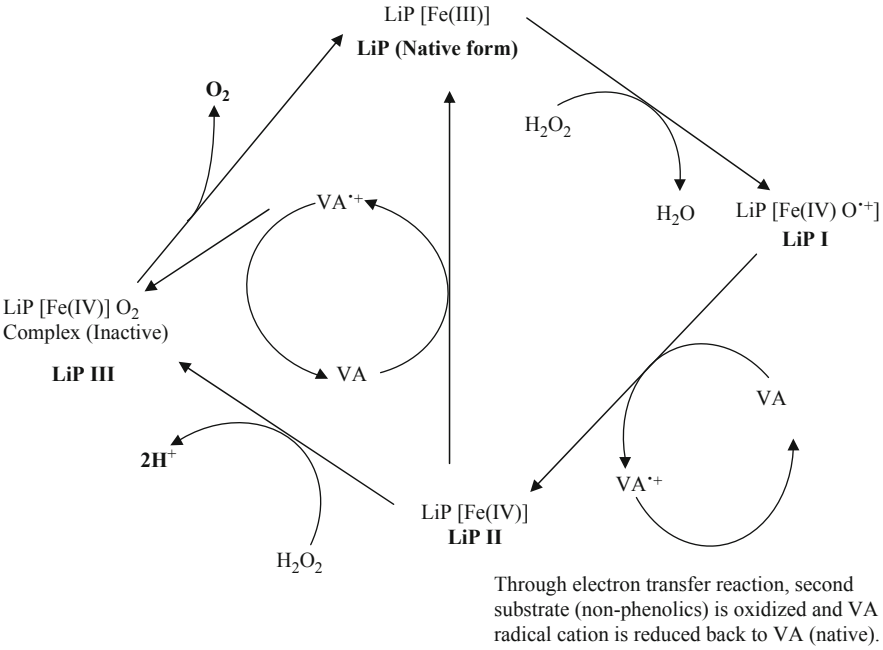

Fig. 1.2 Catalytic cycle of LiP [32, 98]

(Fig. 1.2). Thirdly, VA prevents the H_2O_2-dependent inactivation of LiPs by reducing LiP II complex back to its native form LiP. Almost all the white-rot fungi synthesize VA via *de novo* glucose pathway during early stage of secondary metabolism in parallel with LiP production [98].

LiPs oxidize non-phenolic and phenolic units of lignin by removing one electron and creating free radicals, which lead to chemically decompose the polymer. LiP has been shown to oxidize fully methylated lignin, lignin model compounds as well as various polyaromatic hydrocarbons. LiPs cleave selectively Cα–Cβ bond, aryl Cα bond, aromatic ring opening and demethylation in the lignin molecule [32, 98].

1.3.2 Manganese Peroxidase (MnP)

Manganese peroxidase (EC 1.11.1.13, Mn(II):hydrogen-peroxide oxidoreductase, MnP) also require H_2O_2 as an oxidant in the Mn-dependent catalyzing reaction in which Mn^{2+} is converted to Mn^{3+} by MnP. Mn^{3+} then oxidizes phenolic rings to phenoxyl radicals, which leads to decomposition of compounds. Both LiPs and MnPs are heme-containing glycoproteins [49, 101, 102]. But LiPs are not as widespread as MnPs, and major difference between MnPs and LiPs in lignin degradations are as LiPs generally oxidize nonphenolic lignin substructures and MnPs oxidize phenolic

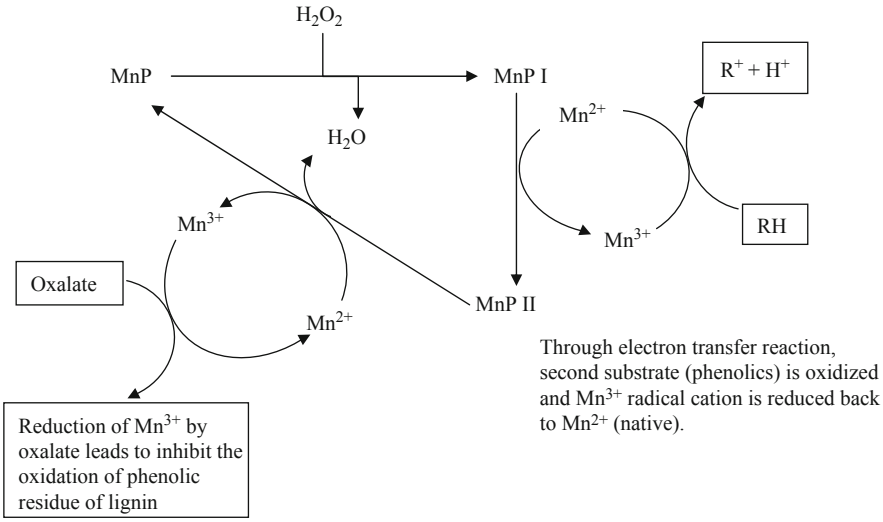

Fig. 1.3 Catalytic cycle of MnP [32, 98]

rings of lignin [49]. MnPs have an important role in lignin depolymerization, chloro-lignin, and demethylation of lignin. Therefore, MnPs have a very essential role in biological pretreatment of lignocellulosic biomass. So far, many researchers have reported that *P. chrysosporium*, *Pleorotus ostreatu*, *Trametes* sp., and several other species, which belong to *Meruliaeiae*, *Coriolaceae*, and *Polyporaceae* produce MnP [32].

MnPs contain one molecule of heme as iron protoporhyrin IX and comprise with 357 amino acid residues, three sugar residues (Glc Nac, Glc Nac at Asn 131, and a single mannose at Ser 336), two structural calcium ions, a substrate Mn^{2+} and 478 solvent molecules. For MnP, the acidic amino acids, aspartic acid, and two glutamic acids have been proposed as manganese-binding residues [32, 98]. MnPs act on its substrate almost similar to LiPs action. Thus, the native form of MnP is oxidized by addition of H_2O_2 to form MnP I complex (Fig. 1.3). Then this catalytic cycle involves in the oxidation of Mn^{2+} to Mn^{3+} by MnP I and MnP II complexes. Finally, Mn^{3+} oxidizes the lignin compounds by diffusing into the lignified cell wall and attacks it from inside. Indeed, MnP I can directly involve in the oxidation of phenolic compounds such as 2,6-dimethoxyphenol, guaiacol, and phenolic tetrameric lignin model compounds. This oxidation reaction clearly elucidates that MnP oxidizes the phenolic part of the lignin indirectly via Mn ions. But MnP naturally does not oxidize aromatic compounds of lignin directly as LiP. Because they do not have tryptophan residue, required for electron transfer to non-phenolic substrates [98, 103]. Recently, MnPs have been isolated from *Bjerkandera* sp. BOS55 and *P. eryngii* that are found to be oxidized Mn^{2+} as well as aromatic compounds [98]. Hence, it is very clear that addition of Mn^{2+} may play further enhancement in the bio-oxidation of phenolic compounds of lignin and may induce MnP production in fungi.

1.3.3 Laccase (Lac)

Laccases (Lac, EC 1.10.3.2, benzendiol: oxygen oxydoreductase) belong to blue copper protein or oxidase family. Lac has been found in fungi, bacteria, and plants. The major producers of Lac are of fungi kingdom, whose diversity can be found in soil, phytopathogenic, and freshwater inhabiting ascomycetes and basidiomycetes [104]. Lac is generally larger than peroxidases as it has a molecular weight of approximately 60 kDa and pI 3–6 [49]. Optimum pH for better Lac activity is found to be 3–5 [105]. Lac catalyzes four single-electron oxidations of aromatic amines and phenolic compounds such as phenolic substructure of lignin, which coincide with the reduction of O_2 to H_2O [32, 98]. Indeed, it can also oxidize nonphenolic compounds under certain conditions, for example, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) [106], 1-hydrobenzotriazole (1-HBT) [107], and violuric acid [108]; natural mediators such as 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol [109], and 3-hydroxyanthranilate [110]. Therefore, the natural mediator should be produced by organisms for the complete oxidation of lignin.

Lac is produced by almost all the white-rot fungi. Generally, it has several Lac encoding genes and secrete as multiple isoforms [49, 106]. Lac contains four copper atoms of three distinct types per enzyme, and each type has a different role in the oxidation of substrate [98]. Type I copper directly involves in the reaction with the substrate. The type I copper gives a maximum absorbance at a wavelength of 610 nm, which gives to the enzyme a typical blue color. The type II copper and the two type III copper cluster are found in triangular forms. Copper II and III complexes involve in the binding, the reduction of O_2 and the storage of electrons originating from the reducing substrates. The type II copper does not have absorbance in a visible range, while the type III copper has a maximum absorption at 330 nm, hence copper II and III complexes do not have any color [98]. The entire crystalline structure of Lac containing four copper atoms in the active site has been studied from *T. versicolor* and *C. maxima* [111, 112]. Bourbonnais et al. [106] reported that the white-rot fungus *T. versicolor* produces two laccase isozymes (I and II).

For effective biological pretreatment of lignocellulose, various white-rot fungi can be used in addition to copper ions in order to induce the secretion of Lac enzymes. In some special cases, Lac can also be induced by addition of aromatic compounds like VA and 2-5 xylidine [32]. Although Lac generally oxidizes phenolic residues of lignin, it also oxidizes non-phenolic compounds of lignin with addition of ABTS as discussed earlier. Therefore, Lac action can be induced further by addition of some special catalyst in the biological pretreatment. For some fungi such as *C. subvermispora* and *Ganoderma lucidum* the Lac production could be increased in the presence of lignocellulosic materials. Recently, some bacterial Lacs have also been characterized from *Azospirillum lipoferum*, *Bacillus subtilis*, *Streptomyces lavendulae*, *Streptomyces cyaneus*, and *Marinomonas mediterranea* [113].

1.3.4 Versatile-Peroxidase (VP)

Versatile-peroxides (VP) are found in various *Bjerkandera* species and *Pleurotus* species [114]. VA can oxidize both phenolic and non-phenolic compounds of lignin as well as Mn^{2+} [32]. The catalytic mechanism is similar to LiP [101]. For example, VP oxidize nonphenolic model compounds such as veratrylglycerol β-guaiacyl ether and results to the formation of veratraldehyde. VP also oxidize Mn^{2+} to Mn^{3+}, VA to veratraldehyde and *p*-dimethoxybenzene to *p*-benzoquinone [32].

1.3.5 Peroxide-producing Enzymes

In lignin biodegradation, hydrogen peroxide (H_2O_2) plays an important role and the rate of oxidation of lignocellulose entirely depends upon the availability of H_2O_2 [98]. Therefore, white-rot fungi have to produce some accessory enzymes for H_2O_2 production in order to support the ligninolytic oxidative reaction of LiPs and MnPs. Such enzymes are glyoxal oxidase (GLOX) found in *P. chrysosporium* and many other white-rot fungi and AAO. Naturally, fungi secrete the GLOX substrates, which are reduced into H_2O_2. For example, *P. chrysosporium* produces glyoxal and methylglyoxal as natural extracellular metabolites. In some cases, the product of lignin oxidation reaction may also undergo a reduction reaction by GLOX, for example, arylglycerol β-aryl ether structure of lignin is oxidized by LiPs to glycolaldehyde and this cleavage product acts as a substrate for GLOX [115].

On the other hand, AAOs produce H_2O_2 through another route in some white-rot fungi. Chlorinated anisyl alcohols are secreted as extracellular metabolites in LiPs producing strains *Bejrkandera* species and secreted metabolites are further reduced to H_2O_2 by specific AAO. Many alkoxybenzyl alcohols are LiP substrates, but not chloroanisyl alcohol. Therefore, this enzymatic mechanism of H_2O_2 production clearly elucidates that fungus separates its lignolytic and H_2O_2-generating pathways [32].

1.3.6 Cellobiose Dehydrogenase (CDH) in Ligninolysis

Cellobiose dehydrogenase (CDH; EC 1.1.99.18; cellobiose: [acceptor] 1-oxidoreductase) is an extracellular flavocytochrome secreted by several wood-degrading fungi (white-rot and brown-rot fungi) under cellulolytic culture conditions. It oxidizes soluble cellodextrins, mannodextrins, and lactose efficiently to their corresponding lactones by a ping-pong mechanism using a wide spectrum of electron acceptors including quinones, phenoxyradicals, Fe^{3+}, Cu^{2+}, and tri-iodide ion [116]. CDH activity was first discovered by Ulla Westermark and Karl-Erik Eriksson as a cellobiose-dependent reduction of quinones in the two white-rot fungi *T. versicolor* and *P. chrysosporium*. This enzyme has been isolated from the

white-rot fungi *P. chrysosporium*, *T. versicolor*, *P. cinnabarinus*, *Schizophyllum commune*; the brown-rot fungus *Coneophora puteana*; and the soft-rot fungi *Humicola insolens* and *Myceliophtore thermophila* (*Sporotrichum thermophile*) [117]. Interestingly, no CDH activity has been reported so far from cultures of *C. subvermispora,* even though it is a selective delignifier [118].

Recently, it was found that CDH has shown to participate in the ligninolytic metabolism of white-rot fungi in the presence of H_2O_2 [118]. Henriksson et al. [119] have summarized the findings of various researchers regarding the CDH activity in ligninolysis that it reduce Fe^{3+} to Fe^{2+} and cellobiose or cello-oligosaccharides to H_2O_2. In the presence H_2O_2, the reaction favors the formation of Fenton's reagent that trigger the production of hydroxyl radicals. This hydroxyl radical is highly reactive and known to attack lignin and cellulose. Further, Henriksson et al. [117] have discussed the following hypothesis/theory about the CDH activity:

- CDH supports the lignin degradation by reducing the aromatic radicals, which is produced from lignin oxidation reaction by LiP and Lac. Enzymatic reaction is a reversible reaction; therefore lignin degraders may favor the polymerization of the radicals in vitro condition. CDH may inhibit polymerization by reducing the radicals created by LiP and Lac.
- CDH supports MnP.
- CDH reduces toxic quinones to phenols that can be used as redox mediators by lignolytic enzymes.
- CDH reduces compound II of lignolytic peroxidases and thus, complete the catalytic cycle in the absence of peroxidase substrate.
- CDH degrades and modifies cellulose, hemicelluloses, and lignin by generating hydroxyl radicals in a Fenton type reaction.

All the above theory/hypothesis is not yet proved practically and still unclear concepts. Although, the hypothesis is unclear, the last point about generation of hydroxyl radicals gives plausible explanations for many of the characteristic properties of CDH and it may be the most attractive suggestion for the function of CDH [117]. Further, Dumonceauxa et al. [120] suggested that CDH is not important in lignin degradation, at least for *T. versicolor* delignifying and concluded that it is possible that some other enzyme masked the effect of the lack of CDH by performing reductive reactions. Hence, the CDH-deficient mutant can still degrade or modify the lignin in a similar manner as the wild type but does not degrade cellulose [121].

1.3.7 Low-Molecular Weight Compounds Involved in Lignin Degradation (Mediators)

As per theoretical and practical views, it is elucidated that enzymes Lac and peroxidase (LiPs and MnPs) are larger than the pore size of the cell wall, and they cannot have direct contact with the lignin. Various low-molecular weight compounds are

found in white-rot fungi, which play an important role in ligninolytic enzyme system of white-rot fungi. It has been studied the role of mediators or co-factors in various in vitro studies, which revealed optimum concentration of H_2O_2, lignin, O_2, and suitable mediators [122]. Therefore, for effective biological removal of lignin components from lignocellulosic biomass, the fungi and/or bacteria should not fail to produce these mediators or co-factors.

1.3.7.1 Veratyl Alcohol

As discussed earlier, the role and importance of VA is very essential in bioligninolysis. It is generally synthesized de novo from glucose via shikimate pathway at the early stage of secondary metabolism in parallel with the LiPs production. The biosynthetic pathway for VA was performed with ^{14}C isotope trapping experiments in the ligninolytic fungus *P. chrysosporium* (ATCC 34541); and concluded that the pathway proceeds as follows: Phenylalanine → cinnamic acid → Benzoate/Benzaldehyde → VA [115, 123]. In *P. chrysosporium*, VA production is induced by nitrogen-limitation, whereas in *Bjerkandera* sp., the nitrogen element does not have any significant regulatory effect on the VA biosynthesis [124]. Furthermore, LiPs action on non-phenolic residue of lignin can be enhanced by addition of VA in biological pretreatment of lignocellulose. As per Hammel et al. [115], VA protects LiPs against H_2O_2-mediated inactivation reaction (rate limiting step) in the LiPs catalytic cycle reaction (Fig. 1.2) and it has been proposed that VA act, in vivo as a stabilizer for the enzymes.

1.3.7.2 Manganese

Naturally, all wood materials and residues contain manganese elements, which are present sometime in high concentration depending upon the type of the wood materials, varying from 10 mg/kg to100 mg/kg of dry wood. The importance of Mn^{2+} can clearly be found during the fungal decay on woody materials as it accumulates in the form of MnO_2 precipitates. Indeed, the insoluble Mn^{4+} species deposits at the tip of new fungal hyphae in the early stages of infestation and growth [125]. As mentioned earlier, Mn^{2+} stimulates the production of MnP and enhances the degradation of lignin components during oxidation reaction, where Mn^{3+} is generated by MnP and acts as a mediator for the oxidation of various phenolic compounds. Therefore, addition of Mn^{2+} increases the biological oxidation rate in biological pretreatment of lignocellulose. On the other hand, addition of Mn^{2+} inhibits the action of LiPs and its production [98, 125]. Hence, it is very essential to optimize the concentration of Mn^{2+} in order to achieve better biological pretreatment. Indeed, in decaying wood, naturally a manganese concentration gradient is established, allowing soluble forms of manganese (Mn(I1) and Mn(III)) to diffuse into regions of low manganese concentration [126].

1.3.7.3 Oxalate

Two enzymes, oxaloacetase and glyoxylate oxidase that catalyze the hydrolysis of oxaloacetate and the oxidation of glyoxylate, respectively, are responsible for the biosynthesis of oxalate. An important aspect is that LiPs and MnPs are capable of decomposing oxalate in the presence of VA or Mn^{2+} [98,125]. The breakage of oxalate results in the formation of carbon dioxide and formate anion radical ($R\text{-}CO_2^{+-}$), which is further oxidized by O_2 to give CO_2 and superoxide (O_2^{+-} or HOO^{+-}) under aerobic conditions. The active oxygen species are suggested to directly participate in the oxidation of lignin. This reaction can be observed in oxidation of phenol red and kojic acid by MnP in the presence of Mn^{2+} and oxalate without exogenous addition of H_2O_2. This suggests that oxalate may be regarded as a passive sink for H_2O_2 production [98]. If the oxalate reduces the VA^{+-} and Mn^{3+} ions, the mineralization rate of lignin will be affected adversely (Figs. 1.2 and 1.3). As mentioned earlier, VA^{+-} and Mn^{3+} both should be reduced by phenolic and/or non-phenolic compounds of lignin for the effective degradation of lignin. For better biological treatment, it is important to conquer the excessive action of oxalate on VA^{+-} and Mn^{3+}.

1.3.7.4 2-Chloro-1,4-dimethoxybenzene

White-rot fungi produce a wide range of organohalogen metabolites. The most commonly produced halogens are chlorinated anisyl metabolites (CAM) and chlorinated hydroquinone metabolites (CHM). CAM has an important physiological function in lignin degradation, contributing as substrates for AAO involved in extracellular H_2O_2 production. Among CHM metabolites, chlorinated 1,4-dimethoxybenzene such as 2-chloro-1,4-dimethoxybenzene, 2,6-dichloro-1,4-dimethoxybenzene, tetrachloro-1,4-dimethoxybenzene, and tetrachloro-4-methoxyphenol are identified. 2-Chloro-1,4-dimethoxybenzene (2-Cl-1,4-DMB) is another substrate for LiP, indicating a possible active function in the wood decomposition process. Like VA, it can also act as a redox mediator [98, 127].

1.4 Effect of Biological Treatment on Lignocelluloses

Biological pretreatment of lignocellulosic biomass changes the physico-chemical characteristic of biomass. Among the changes, lignin degradation is the most attractive and most studied. For example, lignin loss in wheat straw was found 25 % after 1 week [128]; lignin loss in corn straw was up to 54.6 % after 30 days pretreatment with *T. vericolor* [129]; lignin loss increased from 75.67 % to 80 % when corn stalk treated with *Irpex lacteus* [130]; lignin extractability and glucose yield could be improved in canola straw with fungus strain *T. vericolor* and cellobiose dehydrogenase-deficient strain (m4D) [44]. Degradation of lignin by microbes is mainly due to a non-specific oxidative reaction, which leads to complete oxidation of lignin. Among bio-delignifier, white-rot fungus is one of the mostly studied microbes,

as discussed earlier, which has unique capability to cleave carbon–carbon linkages of lignin and oxidizes with the help of various lignolytic enzymes. The changes in terms of the ratio between *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of lignin were analyzed using pyrolysis-gas chromatography–mass spectrometry (Py-GC–MS) and concluded that the susceptibility of lignin units are in the following order: S > G > H. This order indicates that the biomass with S-rich lignin is more susceptible to fungal degradation than the biomass with other lignin units [32].

During fungal attack on biomass, hemicellulose and cellulose are also consumed and among biomass components, hemicellulose is easier to degrade. White-rot fungi such as *P. chrysosporium* [131], *P. citrinopileatus* and *P. florida* [132], *Trametes ochracea* and *E. taxodii* 2538 [77], *C. subvermispora* [133] have been found to degrade hemicellulose along with lignin loss (Table 1.1) and showed the multiple endoxylanase activity. This effect results in reduction of recalcitrance of lignocellulose but increases the risk of loss of cellulose or lowering the all sugar recovery in bioconversion process [130].

White-rot fungi also secrete cellulase enzyme with different specificity and synergistic characteristics during biological treatment of lignocellulose. Cellulase hydrolyzes β-1,4-linkage of cellulose to glucose and the hydrolyzed products are utilized by same fungi or other microbes. As mentioned earlier, non-selective white-rot fungi mineralize all lignocellulosic components equally. Selective white-rot fungi generally degrade negligible amount of cellulose and have promising role in the biological pretreatment of lignocellulose. Cellulose loss can be analyzed by X-ray diffraction (XRD) method in terms of crystallinity index (CrI). When the corn stover was treated with brown-rot fungi *Fomitopsis* sp. IMER2, the crystallinity degree of treated biomass could be increased from 33.22 % to 46.06 % and crystalline portion from 59.96 % to 94.96 % [134]; and it was found that the crystalline change of the treated biomass is due to *Fomitopsis* sp. IMER2 preferential degradation of the amorphous region of cellulose. In contrast, crystallinity decreased from 68.4 % to 64–65.9 % after the biological pretreatment of Japanese red pine (*Pinus densiflora*) with three white-rot fungi [5].

Further, Xu et al. [31] investigated the surface morphological changes during white-rot fungus *I. lacteus* CD2 attack on corn stover by scanning electron microscopy (SEM). SEM images showed some physical changes after biological treatment and resulted in irregular holes in the corn stover. The functional group changes and bond arrangement in the treated corn stover were analyzed by Fourier transform infrared (FTIR) spectroscopy [31], wheat straw biodegradation by *P. chrysosporium* [131] and bamboo culms (*Phyllostachys pubescence*), which was treated by *E. taxodii* 2538 and *T. versicolor* G20 [135]. The various characterization results, obtained by distinguished researchers, indicate that biological treatment increases the pore volume, pore size and remarkably enhance the surface area of the lignocellulose. A more-defined surface area obtained from wheat straw treated by *P. chrysosporium* supplemented with Tween 80 inorganic salts, indicating removal of lignin and making more accessible the surface of hemicellulose and cellulose [128]. Xu et al. [31] also indicated that biological treatment of corn stover with *I. lacteus* CD2 enhanced the pore size and pore volume of corn stover, resulted more accessible surface area for enzymatic saccharification.

Table 1.1 Effect of biological treatment on lignocellulosic components

Raw material	Strain name	Weight loss (%)	Lignin loss (%)	Cellulose loss (%)	Hemicellulose loss (%)	Reference
Softwood, *Pinus densiflora*	*Ceriporia lacerata*lacerate	9.5 ± 0.5	13.1 ± 0.4	8.0 ± 0.5	–	[5]
	Polyporus brumalis	9.9 ± 0.4	11.6 ± 0.3	10.6 ± 0.3	–	
	Stereum hirsutum	10.7 ± 0.7	14.5 ± 0.4	7.8 ± 0.3	–	
Sugarcane trashes	*Cellulomonas cartae*	15.5 ± 3.83	5.5 ± 0.26	25.4 ± 0.66	–	[13]
	Cellulomonas uda	24.3 ± 2.06	5.5 ± 0.25	21.8 ± 1.25	–	
	Bacillus macerans	17.5 ± 0.49	5.5 ± 0.22	30.4 ± 0.51	–	
	Zymomonas mobilis	17.9 ± 0.54	8 ± 0.51	26.8 ± 0.63	–	
Prosopis juliflora wood	*Pycnoporus cinnabarinus*	18.87 ± 1.11	8.87 ± 0.22	4.06 ± 0.18	–	[136]
Lantana camara wood	*Pycnoporus cinnabarinus*	15.4 ± 1.88	13.13 ± 1.32	2.34 ± 0.54[a]	–	
Chinese willow (*Salix babylonica*, hardwood)	*Echinodontium taxodii*	32.5 ± 1.7	45.6 ± 2.0	26.7 ± 0.2	50.8 ± 1.8	[77]
China-fir (*Cunninghamia lanceolata*, softwood)	*Echinodontium taxodii*	24.1 ± 0.9	39.8 ± 1.2	12.6 ± 0.1	31.4 ± 2.7	
Corn stover	*Ceriporiopsis subvermispora*	18.8	39.2	4.8 ± 0.25	28 ± 0.5	[137]
	Ceriporiopsis subvermispora	14.59 ± 0.28	31.33 ± 1.01	4.49 ± 1.29	22.45 ± 0.54	[138]
Bamboo clums	*Echinodontium taxodii*	10.58	24.28	1.64	28.46	[135]
	Flammulina velutipes	2.27	3.14	3.88	4.82	
	Ganoderma lucidum	12.1	10.56	12.83	15.16	
	Trametes ochracea	15.21	18.63	10.79	29.22	
	Trichaptum biforme	11.04	12.54	8.48	32.7	
Water hyacinth	*Pleurotus citrinopileatus*	31.9 ± 0.2	19.1 ± 0.4	30.1 ± 0.5	37.5 ± 0.1	[132]
	Pleurotus plorida	28.8 ± 0.4	19.7 ± 0.3	28.5 ± 0.8	30.5 ± 0.7	
Moso Bamboo	*Irpex lacteus*	–	17.87 ± 0.83	48.20 ± 0.92	18.50 ± 0.97	[139]
Wheat straw	*Fomes fomentarius*	–	35 ± 1	45 ± 1	51 ± 27	[140]
Corn stover	*Auricularia polytricha, Irpex lacteus*	–	17.8 ± 1.0	31.5 ± 0.8	16.8 ± 0.9	[141]

The effect of biological pretreatment of lignocellulose in terms of weight loss, cellulose, hemicellulose, and lignin losses is summarized in Table 1.1.

1.5 Combined Biological Treatment with Other Pretreatment Methods

In view of achieving the effective biological pretreatment, the process can be combined with physical and chemical treatment methods as the main drawback of biological pretreatment is loss of polysaccharide (cellulose/hemicellulose) and the longer pretreatment duration than chemical and physical pretreatment. Combination of biological pretreatment with chemical/physical pretreatment can enhance the fermentable sugar conversion from biomass and can improve the performance of pretreatment as compared to sole pretreatment. It is obvious that chemical/physical pretreatment prior to biological pretreatment allows the substrate more assessable for microbes to degrade lignin. Therefore, optimization is required in order to minimize the overall cost of the pretreatment, time, and energy and maximize the fermentable sugar yield after the enzymatic treatment. This combination can be carried out by two ways (i) chemical/physical treatment prior to biological pretreatment, (ii) chemical/physical treatment after biological pretreatment. The combined biological pretreatment with chemical/physical treatment and pretreatment process conditions are summarized in Tables 1.2 and 1.3, respectively.

Taniguchi et al. [142] treated rice straw with steam explosion prior to biological pretreatment using *P. ostreatu* and found that the pretreatment duration could be reduced from 60 days to 36 days required for obtaining 33 % net glucose yield. Yu et al. [129] reported that the treatment time could be reduced from 60 days to 18 days with considerable sugar yield, when rice straw was pretreated with H_2O_2 (2 %, 48 h).Itoh et al. [143] reported that ethanol yield could be increased by 1.16 times when biological pretreatment was carried out prior to organosolv treatment by using *C. subvermispora* and saved 15 % electrical energy. Indeed, biological treatment can also be used in lignin-based oil production. For example, *Fomitopsis* sp. IMER2 was used in removal of amorphous region of cellulose from corn stover and resulted a significant increase in the oil yield from 32.7 % to 50.8 % in pyrolysis process. Therefore, it can be concluded that biopretreatment favors thermal decomposition of corn stover [134].

1.6 Challenges in Biological Pretreatment

The fermentable sugar loss and relatively long time of the pretreatment compared to physical/chemical pretreatment are major challenges in biological pretreatment process. As discussed earlier, brown-rot fungi are the major consumers of fermentable sugars in the biological pretreatment. Furthermore, biological pretreatment requires

Table 1.2 Combined biological pretreatment of lignocellulose with chemical/physical treatment

Raw materials	Chemical/physical pretreatment	Biological treatment	Achievement	Reference
Rice straw	Steam explosion prior to biological pretreatment	*Pleurotus ostreatus*	Reduction in pretreatment duration from 60 daysto 36 days for obtaining 33 % glucose yield	[142]
Rice straw	Pretreated with H_2O_2 (2 %, 48 h) before biological pretreatment	*Echinodontium taxodii*	Reduction in pretreatment duration from 60 daysto 18 days	[129]
Water hyacinth	After the biological pretreatment, 0.25 % H_2SO_4 acid treatment	*Echinodontium taxodii*, *Eichhorina crassipes*	Sugar yield increased by a factor of 1.13 to 2.11	[47]
Beech wood chips	Biological pretreatment prior to organosolv treatment	*Ceriporiopsis subvermispora*	Ethanol yield increased by 1.16 times and saved 15 % electrical energy	[143]
Pinus radiata	Biological pretreatment carried out prior to ethanolysis	*Gloephyllum trabeum*	Increased solvent accessibility and decreased H factor from 6,000 to 1,156 for obtaining 161 g ethanol/kg of wood	[144]
Corn stover	Thermochemical decomposition after the biological pretreatment	*Fomitopsis* sp. IMER2	Oil yield increased from 32.7 % to 50.8 %	[134]
Corn stalks	Alkaline treatment after the biological pretreatment	*Irpex lacteus*	Lignin loss increased from 75.67 % to 80 %	[130]
Wheat straw	Thermal decomposition after the biological pretreatment	*Phanerochaete chrysosporium*	Significant reduction in the thermal degradation temperature	[131]
Corn straw	Biological pretreatment for 15 days followed by alkali/oxidative pretreatment	*Echinodontium taxodii*	Sugar yield increased by 50.7 %	[129]

more space and longer time; hence the probability of risk of contamination increases. Consequently, these factors increase the process cost. In order to overcome the above problems and making the process more cost effective and beneficial, a dedicative microorganism must be used in the process, where it could decrease the lignocelluloses recalcitrance with a minimum loss of sugar and a short time for incubation. The effective biological pretreatment process is influenced by many factors, such as (i) strain selection: The strain must have a high affinity to lignin rather than the other part of

Table 1.3 Biological pretreatment conditions for various applications

Strain	Raw material	Pretreatment condition	Result(s) achieved	Reference
Trametes hirsuta	Paddy straw	Solid state fermentation at 30 °C for 10 days	Enhanced carbohydrate content by 11.1 %	[146]
Irpex lacteus	Corn stover	In 250 ml Erlenmeyer flasks at 28 °C for 25 days	Highest saccharification ratio reached 66.4 %	[114]
Stereum hirsutum	Japanese red pine chips	cultivated at 30 °C for 8 weeks in cultivation bottle	Sugar yield increased up to 21.01 %	[5]
Phanerochaete chrysosporium	Wheat straw	Solid state fermentation at 37 °C for one week supplemented with Tween 80	Highest lignin loss (25 %) and approx. 250 % higher efficiency for the total sugar release	[128]
Epitrimerus taxodii	Bamboo culms	Cultures maintained at 25 °C for 120 days in 250 ml Erlenmeyer flasks	Sugar yield increased 8.7 fold and caused high lignin loss (>20 %)	[135]
P. chrysosporium	Wheat straw	solid substrate fermentation at 30 °C in 500 ml Erlenmeyer flasks for 3 weeks	30 % loss of total lignin	[131]
Ceriporiopsis subvermispora	Japanese cedar wood	300 ml Erlenmeyer flask at 28 °C with 70 % relative humidity for 4–8 weeks supplemented with wheat bran	74–76 % of β-O-4 aryl ether linkages in the lignin and methane yield reached 35 %	[147]
C. subvermispora	Corn stover	solid-state fermentation at 28 °C for 42 days	57–67 % overall glucose yield increased	[137]
C. subvermispora	Corn stover	pretreated at 28 °C with 75 % moisture content for 35 days	Lignin degradation up to 31.59 % and glucose yields of 66.61 %	[138]

lignocelluloses; (ii) high degradation rate of lignin; (iii) simple nitrogen source requirement; (iv) simple micronutrient requirements. These factors have already been optimized and implemented by many researchers in their biological pretreatment process for various applications.

In view of reducing the capital cost, incubation time and effective biological pretreatment with minimum fermentable sugar loss, the following approaches can be implemented in near future:

1. Combined biological and chemical/physical treatment may be effective for treatment of lignocelluloses.

2. Using some advance tools like bioinformatic tools, metagenomic tools, and high throughput screening, the process can be implemented effectively. For example, as discussed earlier, altering the pathway of lignolytic enzyme or removing cellulase/hemicellulase enzymes may provide the alternative solution.
3. Novel strains or novel enzymes can be isolated with the help of metagenomic tools for the better degradation or conversion of lignocelluloses.
4. To inhibit the action of cellulolytic enzyme or to increase the lignolytic enzyme action during the process, a specific enzyme inhibitor or mediator can be used.

Acknowledgments We thank Dr. A.K. Jain, Director, SSS-NIRE, Kapurthala, for his valuable suggestions and encouragement to write this chapter. One of the authors (Nandhagopal N.) gratefully acknowledges the Senior Research Fellowship under 'Bio-energy Promotion Fellowship' awarded by the SSS-NIRE, Kapurthala and MNRE, Govt. of India, New Delhi.

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