Shiburaj Sugathan · N.S. Pradeep
Sabu Abdulhameed *Editors*

Bioresources and Bioprocess in Biotechnology

Volume 2 : Exploring Potential Biomolecules

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Dedicated to the memory of
Dr. Christopher Augur (Chris) whose life and scientific contributions in the area of bioresources peaked great on a narrow base.

Dr. Christopher Augur
(1960–2009)

Foreword

I am delighted to introduce Vol. II of "Bioresources and Bioprocess in Biotechnology: *Exploring potential biomolecules",* edited by Dr. Shiburaj Sugathan, Dr. N S Pradeep and Dr. Sabu Abdulhameed. In this regard, I would like to highlight its specific features in the most possible brief form so that the peers may quickly lay their hands on the collection of erudite essays with guiding commentaries and reviews. To be exact, I am happy to say that the editors have done a commendable job in an area of several bio-applications. Technically speaking, there may be other similar edited volumes very coarsely comparable to the present one produced in the past. However, the present compilation makes a better composition, considering its scope and the extent of recent and emerging areas in bioresources and bioprocess technology. The salient features of the outstanding collection of reviews are the much-needed single volume for students, researchers and industrialists in the field of biotechnology, particularly hitherto apparently neglected areas of knowledge with transformational potential. The present volume will be of use to researchers in the fields of antimicrobials particularly toward mycobacterium, plant-based alternative medicines, enzymes, anticancer and anti-inflammatory molecules, medicinal significance of polyphenol-containing fermented products, etc.

The editors must be congratulated for bringing out such an extensive volume beautifully written for universal appeal. The following areas are dealt with utmost care and scholarship. They are chemical alterations of compounds (e.g., a drug) occurring within the body, as by enzymatic activity; plant biosynthetic pathway assemblies for engineering microbial systems to produce targeted chemical compounds; biodiversity of plants ensuring resources for new food crops and medicines; ever nascent ethnopharmacology; etc. No doubt, this volume will be of great use to one and all in the fields of biological resources and biotechnology and

materials research for solving the maladies presently limiting sustainable and comfortable life to humans in a conserved environment with equal rights to all life forms.

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Madathilkovilakath Haridas

Preface

In an era of growing awareness about the threats of biodiversity loss, the society is witnessing an unprecedented interest in novel bioresources, which are increasingly prized for their potential use in many applications. The loss of bioresources is occurring at an alarming rate, a consequence of increasing population pressure, agricultural land degradation, urbanization and above all neglect. Deforestation and forest degradation are large-scale problems in developing countries.

To meet the demands of the society in industrial, healthcare, food and other sectors, the utilization of bioresources is an absolute requirement. At the same time, we need to make sure that the overexploitation should never result in biodiversity loss. To manage this situation, we need to have more knowledge on rarely used or unused resources which are available in bulk and are easy to propagate rapidly. Modern biotechnology is armed with techniques for sustainable utilization of bioresources to meet the increasing demand.

The concept of sustainable development indicates that economic and environmental protection are inseparably linked and that the quality of present and future life fails in meeting basic human needs without destroying the environment on which the life depends. There is a growing recognition worldwide that conservation and sustainable management of bioresources are the need of the hour. The use of biotechnological tools and bioprospecting will open new vistas in many fields viz. agriculture, medicine, horticulture, environment, etc. Since we cannot do without exploiting the available bioresources to our advantage, there has to be a balance between uses of resources and their conservation.

There is an increasing realization that bioresources especially medicinal plants and microbes can provide cheaper means of disease management by analyzing further their functional potential. This interest has led to a better understanding of the role of plant and microbial bioactives in health promotion and disease prevention. Generation of high-throughput data and the study of molecular mechanisms of diseases have all contributed to this effort.

Kerala, India

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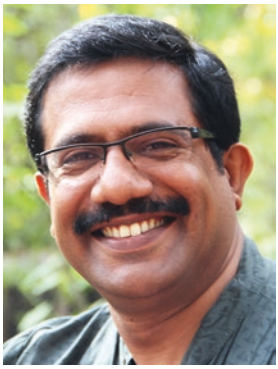

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Part I

Enzymes

Enzymes for Bioenergy

1

Rajeev K. Sukumaran, Amith Abraham, and Anil K. Mathew

Abstract

Lignocellulosic ethanol is emerging as the prominent candidate for renewable liquid transportation fuels, and the conversion of biomass to ethanol requires enzymatic hydrolysis. Enzymes that hydrolyze biomass have been the subject of several studies, since the cost estimations of second-generation ethanol show significant contributions by this single consumable. The chapter introduces biomass-hydrolyzing enzymes in the context of biorefineries and provides an overview on the current knowledge and understanding of these enzymes with respect to their types, mode of action, regulation of gene expression, and synergies. The changing concepts about the role of individual enzymes and the new discoveries on lignocellulose breakdown are presented to highlight the developments in biomass hydrolysis paradigm. It also covers the current strategies employed for commercial production of different lignocellulose-hydrolyzing enzymes and their blending to derive efficient cocktails. Finally, the importance of cost reduction in production and usage of biomass hydrolysis enzymes for a cost-effective bioethanol technology is discussed along with the current approaches in addressing this.

Keywords

Cellulase • Hemicellulase • Xylanase • Biomass hydrolysis • Biorefineries • Bioethanol • Bioenergy

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1.1 Introduction

World energy consumption is on the rise, and a significant growth in energy demand of about 48% is projected for the year 2040 from the base value in 2012 (EIA 2016). Economic growth along with accompanying changes can significantly influence energy consumption, and improvement in living standards brings with it a rapidly growing demand for energy. While renewable energy is the world's fastest growing source of energy, fossil fuels continue to provide most of the world's energy, and liquid transportation fuels represent a major share of this (EIA 2016). Even with efforts worldwide on alternative energy resources, it is projected that the demand for fossil fuels will be on the rise at least for the next few decades with serious impacts on the global environment and climate. It is in this context that the renewable liquid transportation fuels including biodiesel and bioethanol gain prime importance as the existing alternatives to petroleum-based transportation fuels. Lignocellulose is inarguably the world's most abundant renewable source of energy and this justifies the enormous efforts put into developing plant biomass-based fuels – primarily bioethanol. While several of the second-generation (2G) ethanol programs claim to have gone commercial, it still is not a reality at consumer level. The major limitation in commercialization of 2G ethanol is the cost of its production. Lignocellulose contains mainly the sugar polymers – cellulose and hemicellulose, and a significant fraction as lignin. Both the sugar polymers can be broken down to their component sugars, which then can be fermented by microbial action to produce bioethanol. The hydrolysis/saccharification of biomass can be achieved by chemical agents (e.g., acids) or through enzymatic hydrolysis. The latter is often much more efficient and requires only ambient conditions, whereas the former needs higher temperature and is plagued by issues like generation of sugar breakdown products, and the need to deal with acidic waste streams (Visser et al. 2015). The seemingly simple enzymatic process is made difficult by the recalcitrance of lignocellulose and the cost of biomass-hydrolyzing enzymes. Recalcitrance of biomass to enzymatic hydrolysis stems from the highly organized structure of lignocellulose, which prevents access of the enzymes to cellulose. Biomass pretreatments are aimed at making the cellulose more accessible to the enzymes and can bring significant improvements in digestibility. The chapter is primarily focused on biomass-hydrolyzing enzymes in the context of bioenergy, and specifically bioethanol. Microorganisms producing cellulase, their regulation at molecular levels, production strategies, enzyme cocktails for biomass hydrolysis, and the emerging strategies for improving production and efficiencies of biomass-hydrolyzing enzymes are discussed.

1.2 Biomass-Hydrolyzing Enzymes and Their Role in Biofuels Production

Plant biomass consists of three major structural biopolymers, namely, cellulose, hemicellulose, and lignin, each having a unique and complex structure. Cellulose is the major component and is a homopolymer of β-1,4-linked glucose units which can have a degree of polymerization (DP) up to 10,000. Often the cellulose chains are

organized into microfibrils and there is cross-linking between adjacent chains through hydrogen bonds leading to crystalline and amorphous domains (Pu et al. 2013). Cellulose can make up to 15–30% of primary cell walls and up to 40% of secondary cell walls (Sticklen 2008). Hemicellulose on the other hand is a diverse group of short chain, branched, substituted polymer of sugars with a DP of ~70–200 (Zhao et al. 2012). The sugar monomers in hemicelluloses can be xylose, mannose, galactose, rhamnose, and arabinose, with xylose being the most abundant one. They can also contain the sugar acids like glucuronic or galacturonic acid and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Gírio et al. 2010). Lignin is a class of complex cross-linked phenyl propane units, primarily comprising the monomeric units—p-coumaryl, coniferyl, and sinapyl alcohols. Typical plant cell wall structure consists of cellulose microfibrils embedded in a matrix of lignin interspersed with hemicellulose fibers forming a very rigid and organized structure, which is rather difficult to break (Fig. 1.1).

Nature's arsenal for breaking plant cell wall structures includes enzymes that can hydrolyze all of these compounds, and these catalysts play a very important role in the recycling of organic carbon on the globe. Different microorganisms are capable of producing enzymes that can degrade cellulose, hemicellulose, or lignin, or a combination of all. Recently, cellulases have taken the center stage in enzyme research, primarily because of their important role in second-generation ethanol (bioethanol) from lignocellulosic biomass. Sugar polymers in the lignocellulosic biomass are linked through β-1,4 glycosidic linkages that can be hydrolytically cleaved to release monomeric sugars. Enzymatic hydrolysis primarily employs cellulases derived from filamentous fungi – especially strains of *Trichoderma, Penicillium*, and *Aspergillus*, mostly in a crude concentrated form. While cellulases have been around for several decades, the enzymes tailored for efficient biomass hydrolysis are a recent development and involve deliberate blending of multiple enzymes from different sources so as to achieve maximum hydrolytic efficiency. Natural cellulases are slow acting and are affected by several parameters from the reaction environment. While recent research has been successful in improving the efficiencies of biomass-hydrolyzing enzymes and their reaction rates, the same cannot be said for their cost of production. It has been realized that the cost of biomass-hydrolyzing enzymes is a major hurdle for developing an economically viable

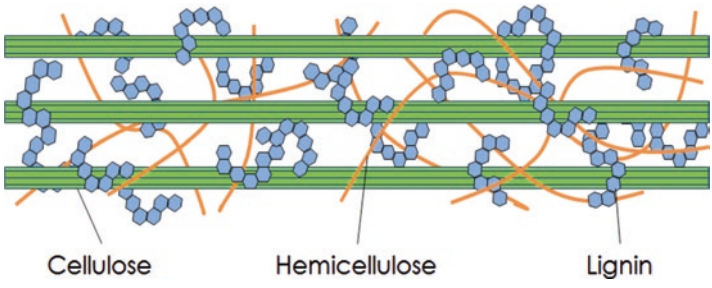

Fig. 1.1 Schematic representation of the lignocellulose structure

cellulosic ethanol industry (Banerjee et al. 2010), and there have been numerous efforts toward bringing down the cost of cellulases at the level of both production strategies and organism engineering. Enzyme majors of the world—Novozymes and Dupont (Genencor)—have been able to bring down the cost of enzyme per unit volume of ethanol produced to levels 10–12 folds lower, but a marketable commercial Lignocellulosic (LC) ethanol remains elusive. This is despite the fact that there are claims from companies on successful running of lignocellulosic ethanol plants. Several research studies have repeatedly highlighted the contribution of enzyme cost to the cost of lignocellulosic biofuels, and have shown that the cost of producing enzymes was much higher than that commonly assumed in the literature (Marcuschamer et al. 2012). A recent report puts the cost of cellulase per liter of ethanol at US$ 0.72 (48% of the production cost) based on the actual purchase price of cellulase in the industrial enzyme market (Liu et al. 2016). The above discussions highlight the importance of cellulase cost reduction in bioethanol production and active research efforts are now directed toward this cause worldwide. The strategies range from using cost-effective carbon sources and onsite production of enzymes (Johnson 2016) to developing genetically modified source organisms (Seiboth et al. 2012a; Fuji et al. 2013). However, reducing the production cost of cellulase alone is not the solution, and the ways to reduce the cost of cellulase for bioethanol production involve a range of possible solutions including development of efficient pretreatment regimes that allow better access of enzymes to the biomass, preventing lignin redeposition on biomass after pretreatment, use of surface active agents to aid hydrolysis, etc. A better understanding of these strategies would require knowledge on the enzymes and their mechanism of action.

1.2.1 Enzymes for Biomass Hydrolysis – Types and Mode of Action

While there are a multitude of proteins aiding in biomass hydrolysis, the major enzymes involved in biomass hydrolysis can be grouped as cellulases, hemicellulases, and lignin-degrading enzymes. Also there are a large number of accessory enzymes and proteins that are involved in helping the deconstruction, which cannot be grouped into any general categories. The following discussions will introduce the biomass-hydrolyzing enzymes and the major emphasis will be on cellulases since these are the major enzymes which are directly involved in breaking down the carbohydrate polymers to fermentable sugars and therefore important in biofuel production. Hemicellulases and lignin-degrading enzymes shall also be introduced.

1.2.1.1 Cellulases

Cellulases are enzymes, which hydrolyze the β-1,4-D-glucan linkages in cellulose and produce as primary products glucose, cellobiose, and cello-oligosaccharides. Cellulases are produced by a number of microorganisms and comprise several different enzyme classifications. Three major types of cellulase enzymes are involved

in the hydrolysis of native cellulose, namely, endoglucanases (EG), exoglucanases/cellobiohydrolases (CBH), and β-glucosidase (BGL) (Schulein 1998). In cellulase-producing organisms, there are multiple enzymes under each of these classifications, which act synergistically to break down cellulose. The classical model for cellulase hydrolysis emerged from the work done on *Trichoderma reesei* in the late 1990s by several groups as reviewed in Payne et al. (2015). In this model, EGs (Cel7B in *Trichoderma reesei*) attack the amorphous regions on the surface of cellulose microfibril revealing new reducing and nonreducing ends in the cellulose chain, which then serve as sites for attack by exoglucanases. The exoglucanases can also attack the available free ends of the cellulose chains. In *T. reesei*, the exoglucanase that attacks the reducing end of the cellulose chain is cellobiohydrolase I (CBHI/Cel7A) and that which attacks the non-reducing end is cellobiohydrolase II (CBHII/Cel6A). The cellobiose released by exoglucanases is cleaved to glucose units by the final enzyme in the cascade – beta glucosidase. The role of each cellulase and its synergism is described below.

1.2.1.2 Endoglucanase

Endoglucanases or endo 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.4) are enzymes which randomly act on the cellulose polymer producing nicks in the amorphous regions of cellulose (endo-initiating) to expose the reducing and nonreducing ends by cleaving the endo β-1,4 linkages between adjacent glucose units. They are generally measured by detecting the reducing groups released from the soluble carboxymethylcellulose substrate (Sheehan and Himmel 1999). Endoglucanases are classically considered as the initiators of cellulose hydrolysis by the cellulase complex, since their action is essential for exposing the reducing and nonreducing ends in the cellulose polymer, essential for the action of exoglucanases/cellobiohydrolases. Recent view on endoglucanases also proposes its role to help cellobiohydrolases to overcome blockage at amorphous regions of cellulose (Payne et al. 2015). Endoglucanases are represented in several glycosyl hydrolase (GH) families, and in the model organism *T. reesei* there are six endoglucanases represented in families GH5, GH7, GH12, GH45, and GH74 (Kubicek 2012).

1.2.1.3 Exoglucanases

Exoglucanases or exocellulases are of two types, namely, cellulose 1,4-β-D-cellobiosidase (reducing end) EC 3.2.1.176 (cellobiohydrolase I/CBHI) and cellulose 1,4-β-D-cellobiosidase (nonreducing end) EC 3.2.1.91 (cellobiohydrolase II/CBHII). These enzymes attack the available reducing or nonreducing free ends or the ends generated by the action of EGs to liberate cellobiose units. While CBHI attacks the reducing ends of the chain, CBHII attacks the nonreducing ends (Cantarel et al. 2009). The current view on exoglucanases does not consider them as having exclusive exoglucanase action, but as exoglucanases with endo-initiating action (Kurasin and Valjamae 2011). In the model organism *T. reesei*, cellobiohydrolases are represented in glycosyl hydrolases families GH6 and GH7.

1.2.1.4 Beta Glucosidases

Beta glucosidases or cellobiases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of terminal, nonreducing beta-D-glucosyl residues with release of beta-D-glucose (Leah et al. 1995). Beta glucosidases (BGLs) catalyze the final reaction in cellulose hydrolysis, namely, the hydrolysis of cellobiose to two molecules of glucose, and are responsible for the regulation of the cellulolytic cascade through their own feedback inhibition by their reaction product glucose. Most of the microbial BGLs employed in biomass hydrolysis belong to GH family 3, while they can be found in families 1, 3, 9, 30, and 116 (Teugjas and Väljamäe 2013). BGL action is considered as a critical step in cellulose hydrolysis since the substrate of BGL – cellobiose – is a strong inhibitor of CBHs and its hydrolysis is essential to overcome product inhibition of the exoglucanases. Since glucose accumulation can lead to BGL inhibition which in turn leads to CBH inhibition through accumulation of cellobiose, the regulation of cellulase production in response to the hydrolysis of cellulose is of critical importance in most of the organisms producing these enzymes. In several cases the BGLs are also inhibited by their substrate, believed to be caused by the transglycosylation reaction capable of being performed by these enzymes (Bohlin et al. 2013).

1.2.1.5 Other Cellulolytic Enzymes and Accessory Proteins

It has long been recognized that the hydrolysis of the dense crystalline lattices of cellulose has to be mechanically disrupted for access of the hydrolytic enzymes and the role of a "swelling factor" which was nonhydrolytic and was proposed as early as 1950 (Reese et al. 1950). "Swollenin", a protein with sequence similarity to plant expansions, was described in *T. reesei* by Saloheimo et al. (Saloheimo et al. 2002). It was believed that swollenin and similar nonhydrolytic swollenin-like proteins act like a zipper opening up the cross-linking of cellulose microfibrils just like plant expansins (Arantes and Saddler 2010). It was also proposed originally that these proteins lack hydrolytic activity since only negligible quantities of sugar release were observed with their independent action, while they enhanced hydrolysis of cellulosic substrates (Gourlay et al. 2012). The mechanism of promoting cellulose breakdown was speculated to be through a nonhydrolytic weakening of hydrogen bonding (Jäger et al. 2011, Gourlay et al. 2012). However, the most recent works have indicated that the protein does have hydrolytic activity and shows a unique mode of action with similarities to the action of both endoglucanases and exoglucanases (Andberg et al. 2015). Apart from swollenin, the "disrupting" or "amorphogenesis inducing" class of biomass-degrading proteins includes expansins, bacterial expansin-like proteins, fungal expansin-like proteins, loosenin, etc. (Arantes and Saddler 2010; Gourlay et al. 2013).

Revolutionary changes in the conventional cellulose deconstruction paradigm have emerged with the discovery of a class of enzymes that share conserved structural features binding a metal ion and following a hitherto undescribed oxidative mechanism (Vaaje-Kolstad et al. 2010). These types of enzymes which are now considered ubiquitous have been termed as Lytic Polysaccharide Mono Oxygenases (LPMOs). The most important feature of these enzymes is their ability to attack the

highly crystalline regions of cellulose where EGs are unable to bind productively. Thus they are able to synergize with glycosyl hydrolases, likely as endo-acting enzymes that act directly on the surface of crystalline cellulose. It is now known that LPMOs require a reducing agent and molecular oxygen and a copper ion in the active site (Payne et al. 2015). The electron donor can also be a co-secreted enzyme like cellobiose dehydrogenase (CDH), the only known example of a secreted flavocytochrome (Dimarogona et al. 2012).

1.2.1.6 Mechanism of Cellulose Hydrolysis by Cellulases

With more and more studies on cellulase action being undertaken, it is now becoming clearer that our understanding of cellulose hydrolysis is probably not complete, and there are paradigms not yet characterized. However, decades of research in this field have given insights into a generally appreciated mechanism of action, and recent discoveries like that of the LPMOs have improved that understanding. The following mode of hydrolysis is a summary of what is currently accepted as the mechanism of cellulose breakdown by cellulases. Cellulose structure is complex with crystalline array of cellulose microfibrils with glucan chains interlinked through hydrogen bonds. There are regions of disorder in the arrangement of glucan chains along the cellulose microfibrils, which are called the amorphous regions. The biomass-degrading enzymes work at the solid liquid interfaces, which implies that a high concentration of catalytic units is required at the surface for efficient hydrolysis of the polymer. This is achieved by the unique adaptation of several of the endo- and exoglucanases in having a three-domain structure with a carbohydrate binding module (CBM) which attaches to the cellulose surface, a catalytic module which does the actual hydrolysis, and a linker which serves mobility and also aids the enhanced binding of the enzyme to the cellulose surface. A detailed description of the structural features of cellulose-hydrolyzing enzymes is beyond the scope of this chapter and the readers are directed to Payne et al. (2015) for a comprehensive discussion on this topic. The typical three-domain structure aids in the processivity of the exoglucanases and endoglucanases that possess this structure. Processive enzymes are those which catalyze consecutive reactions without release of their substrate, and in the cases of cellulases with this modular structure, they help to keep the catalytic domain near the substrate (Teeri et al. 1998).

On recognition of a free chain end, the cellulase threads the chain into the tunnel (exoglucanase) in the catalytic domain of the enzyme to form a catalytically active complex (CAC). Hydrolysis occurs following a retaining or an inverting mechanism (Davies and Henrissat 1995) depending on the type of enzyme and the product is expelled. The processive cycle is continued with multiple events of hydrolysis before finally dissociating from the chain and reinitiating the processive cycle at a new site (Payne et al. 2015). The processive mechanism for exocellulase-mediated cellulose hydrolysis is represented in Fig. 1.2.

In the case of endoglucanases, the processive cycle is different in that the chain threading and product expulsion are omitted. The binding site of endoglucanase has a cleft instead of a tunnel, which allows chain acquisition without threading. It is now known that cellulose hydrolysis by the exoglucanases proceeds by movement

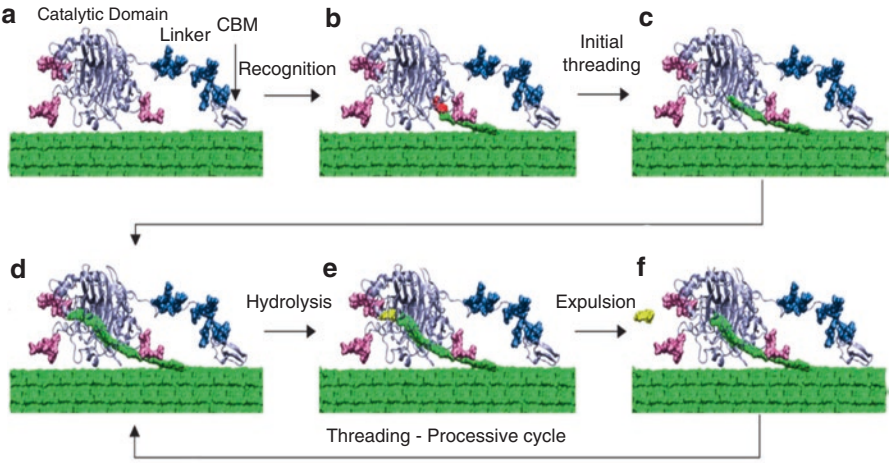

Fig. 1.2 Mechanism of processive cellulose hydrolysis by *T. reesei* cellobiohydrolase (Cel7A). (**a**) Enzyme binding to cellulose (**b**) recognition of the reducing end of a glucan chain (**c**) initial threading of the glucan chain into the catalytic tunnel (**d**) formation of CAC by threading (**e**) processive hydrolytic cycle showing product formation (cellobiose shown in *yellow*) (**f**) product expulsion (Reproduced from Beckham et al. (2011), with permission from Elsevier)

of the enzyme through the cellulose surface while the glucan chain is threaded to the active site tunnel, and this movement requires that the cellulose chain is threaded and is being hydrolyzed (Igarashi et al. 2009). It is considered that the rate-limiting step in processive CBH action is the dissociation from cellulose chains. Processively acting CBH molecules can get stalled at amorphous regions of cellulose and this leads to a diminished hydrolysis rate (Praestgaard et al. 2011). The renewed concept on the role of EGs is that they are acting not only to generate reducing and nonreducing ends and thus helping CBH to attach, but also to help CBH dissociate from the cellulose chain when they encounter amorphous regions during their processive action (Jalak et al. 2012).

In light of the above findings, the roles of different cellulases may have to be redefined. EGs and LPMOs do the endo-initiation in the amorphous and crystalline regions of cellulose respectively by breaking down the glycosidic bonds. Endo-initiation is also aided by the exoglucanases. The liberated reducing and nonreducing ends are attacked respectively by cellobiohydrolase I and II, which act in a processive fashion to liberate cellobiose units, and the cellobiose units are eventually cleaved to glucose by the beta glucosidases (Fig. 1.3).

1.2.1.7 Hemicellulases

Hemicellulose is a hetero-polysaccharide made up of various carbohydrate monomers having different linkages and substitutions, and its structure and composition changes with the plant source and geographical origin (Juturu and Wu 2012). The different types of hemicelluloses recognized include xyloglucans, xylans, ferulate

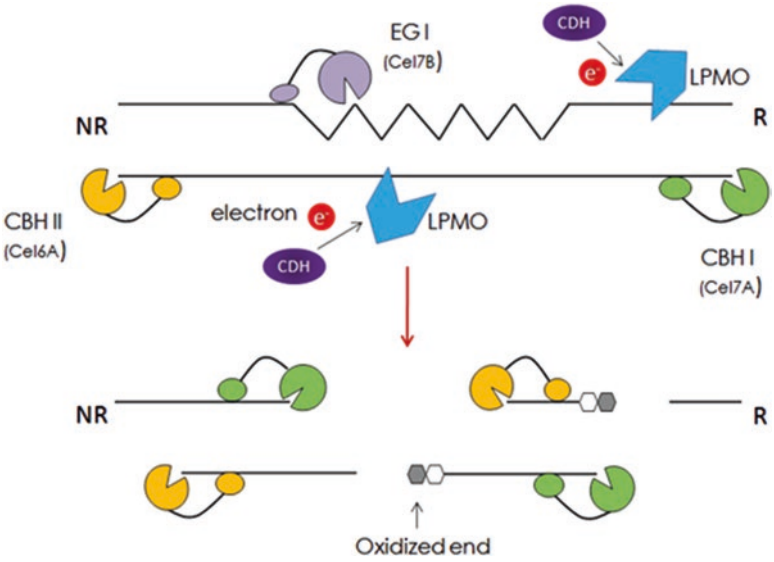

Fig. 1.3 Mechanism of cellulose hydrolysis. Present concept on the hydrolysis of cellulose by filamentous fungi incorporating hydrolytic and oxidative breakdown. Action of LPMO requires an electron donor which in this case is the cellobiose dehydrogenase (CDH) enzyme. LPMO action liberates a new chain end which is oxidized. *R* reducing end, *NR* Nonreducing end

esters, mannans, glucomannans, and β-1,3 and β-1,4 glucans (Scheller and Ulvskov 2010). While xylans are the major components of hemicellulose in hardwood and herbaceous plants, mannans form the major component in the hemicellulose of softwoods. All of the xylans of the higher plants are based on a β-1,4-linked xylopyranose backbone which is substituted with acetyl groups and other sugar residues (Fig. 1.4) (Dodd and Cann 2009). The heterogeneous nature of hemicellulose necessitates the requirement of multiple enzymes that act synergistically and/or sequentially. Different microorganisms employ different strategies for degradation of hemicellulose. Several of the biomass-degrading filamentous fungi secrete an entire cocktail of hemicellulases together, and these act synergistically on the hemicellulose to break down the polymer into its monomers. On the other hand, aerobic bacteria accomplish this in two stages, where the first step is the secretion of enzymes that break the hemicellulose backbone and release oligomers; the second one is its further cleavage to monomeric sugars by cell-wall-bound or intracellular enzymes. In yet another strategy, anaerobic bacteria uses cellulosome-like structures to hydrolyze hemicellulose (Shallom and Shoham 2003).

The major hemicellulose-degrading enzymes are the enzymes which break down the xylan backbone (endo- and exoxylanases and β-xylosidases) and the side chains (arabinofuranosidases, glucuronidases, acetyl xylan esterases, ferulic acid esterases, and alpha galactosidases). A total degradation of xylan requires the synergistic action of mainly endoxylanases, which cleaves the β-1,4 xylose linkages of xylan backbone; exoxylanases, which hydrolyzes β-1,4 linkages of xylan from the

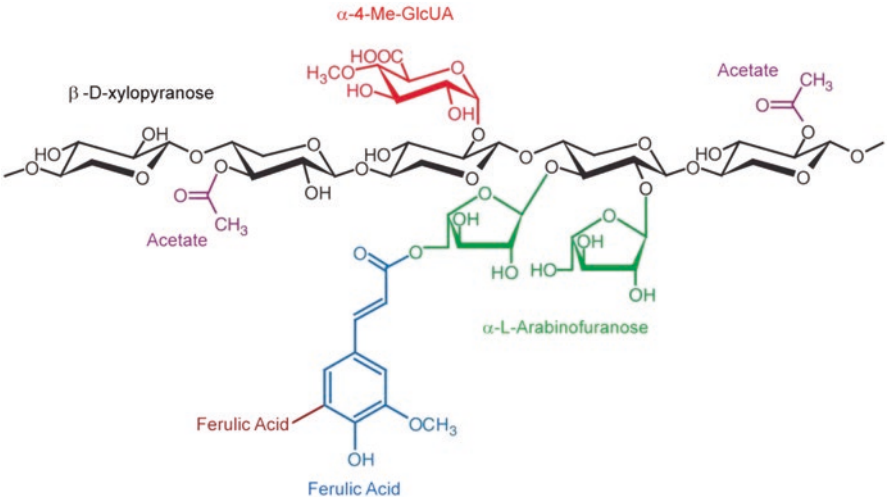

Fig. 1.4 Xylan structure (Reproduced from Dodd and Cann 2009, with permission from John Wiley & Sons)

reducing ends releasing xylooligosaccharides; and β-xylosidases, which cleaves the xylobiose and xylooligosaccharides to release xylose. In addition, the enzymes α-arabinofuranosidases and glucuronidases remove arabinose and 4-*O*-methyl glucuronic acid substituents from the xylose backbone, and the esterases – acetylxylan esterase, ferulic acid esterase, and ρ-coumaric acid esterase – hydrolyze the ester-bonded substituents – acetic acid, ferulic acid, and ρ-coumaric acid – from the xylan (Sukumaran 2009). An overview of the functions of various hemicellulases as provided by Juturu and Wu (2013) is given in Table 1.1.

Endoxylanase (EX, EC 3.2.1.8) hydrolyzes the xylan backbone and has catalytic cores belonging to GH families 8,10,11,30, and 43 with the most common ones being GH 10 and 11. These differ in their substrate specificities and the GH10 is more active on substituted xylan. Similar to cellulases, they may also contain CBMs (Sweeney and Xu 2012). Endoxylanases randomly cleave the xylan backbone from inside releasing long chain xylooligomers on which the β-xylosidases act. β-Xylosidase or xylan-1,4-β-xylosidase (BX, EC 3.2.1.37) acts on the xylo oligosaccharides and xylobiose released by BX to form xylose. These enzymes have catalytic cores belonging to the GH3, 30, 39, 43, 52, and 54 families. These two enzymes are often collectively called xylanases. A third class of enzyme which acts on the xylan backbone is also recognized and is called the exoxylanase that hydrolyzes short chain xylo oligomers acting from the reducing end. Unlike the BX, these are inactive on xylobiose and are also inert on pure polymeric xylan (Juturu and Wu 2014). Hemicellulases are frequently blended to cellulases in commercial biomass-hydrolyzing enzymes due to their ability to synergize with cellulases. However, the need for hemicellulase addition depends on the type of pretreatment employed as well, since some pretreatments (e.g., acid) remove the hemicellulose portion

Table 1.1 Hemicellulose-degrading enzymes and their native functions

Enzyme type	Native function	Action sites
(I) Glycosyl hydrolases		
Endoxylanase	cleaves β-1,4 bond of xylan backbone releasing xylooligomers	β-1,4 xylan backbone
β-Xylosidase	cleaves exo β-1,4 bond of xylooligomers releasing xylose	β-1,4 xylooligomers
Endo-1,4-mannanase	cleaves β-1,4 bond of mannan releasing mannan oligomers	β-1,4 mannan
β-Mannosidase	cleaves exo β-1,4 bond of mannan oligomers releasing mannose	β-1,4 mannan oligomers
α-L-Arabinofuranosidase	cleaves arabinan at O-2 and O-3 positions on xylan backbone	α-L-arabinofuranosyl oligomers
α-L-Arabinanase	cleaves xylooligomers generating arabinose	α-1,5-arabinan
α-D-Glucuronidase	cleaves α-1, 2 bond between glucuronic acid side chain substitutions releasing glucuronic acid	4-*O*-methyl-α-glucuronic acid
(II) Carbohydrate esterases		
Acetyl xylan esterase	cleaves acetyl side chain substitutions releasing acetic acid	2- or 3-*O*-acetyl xylan
Feruloyl xylan esterase	cleaves ferulic acid side chain substitutions releasing ferulic acid	Ferulic acid substitutions

Table reproduced from Juturu and Wu (2013), with permission from John Wiley & Sons

completely, thereby reducing the requirement of hemicellulases. Nevertheless, hemicellulases are a major component of the biomass-hydrolyzing enzymes, since the pretreatment methods are seldom capable of complete removal of hemicellulose, and there are multitudes of pretreatment regimes that result in intact or near intact hemicellulose component. Also the understanding of hemicellulases cannot be regarded as complete and we still have hemicellulose-active enzymes whose role and mode of action is ambiguous (Tenkanen et al. 2013).

1.2.1.8 Lignin-Degrading Enzymes

Most of the cellulolytic organisms produce oxidoreductases as part of the lignocellulolytic machinery and the main function of these enzymes is considered to be degradation of lignin (Sweeney and Xu 2012). Lignin degradation is important for access of cellulases and hemicellulases to the carbohydrate polymers and also in diminishing the inactivation of these enzymes through nonproductive binding on lignin. In biofuel production, the major applications of lignin-degrading enzymes are considered to be delignification and detoxification. Delignification applies to the pretreatment of biomass to remove lignin, whereas the detoxification is relevant in the context of post-hydrolysis processing of biomass to remove potential inhibitors of fermentation (Placido and Capareda 2015). While there are different

microorganisms that produce lignin-degrading enzymes, white rot fungi are considered to be the most efficient producers. Most of the current understanding on lignin-degrading enzymes has emerged from studies on the enzymes of white rot fungi. In these organisms the ligninolytic enzyme system consists of three major classes, namely, laccases, manganese peroxidases, and lignin peroxidases.

Laccases (EC 1.10.3.2) or benzene diol oxygen oxidoreductases are oxidoreductases which enjoy wide distribution among microorganisms. These are enzymes having copper in their active site and are generally classified as multicopper oxidases or blue multicopper oxidases (Rodríguez Couto and Toca Herrera 2006). They employ oxygen as an oxidizing agent and cofactor. Laccases have low substrate specificity and therefore can degrade several compounds having phenolic structure (Placido and Capareda 2015). Other major enzymes that act on lignin – lignin peroxidase (LiP) and manganese peroxidase (MnP) – are heme peroxidases having protoporphyrin IX as the prosthetic group. Lignin peroxidases (EC1.11.1.14) are capable of oxidizing sites of very high redox potential including moderately activated aromatic rings of nonphenolic model lignin compounds. Manganese peroxidase (EC 1.11.1.13) on the other hand cannot oxidize nonphenolic lignin model compounds and depend on the generation of Mn3+ as a diffusible charge transfer mediator. Yet another peroxidase is the versatile peroxidase described in the fungus *Phanerochaete chrysosporium* and capable of both LiP and MnP activities (Fischer and Fong 2014).

Direct use of lignin-degrading enzymes in biomass hydrolysis is not practiced and often the applications of these enzymes are in the delignification of biomass as a pretreatment step. Here again, the pretreatment is more often accomplished by whole microorganisms elaborating ligninases rather than use of their enzymes in isolation. Nevertheless, there are several successful reports on the use of enzymes in isolation for delignification (Gutiérrez et al. 2012; Wang et al. 2013). Mostly laccases are employed for such applications, though MnP, LiP, or combinations of these enzymes may be employed. Another major application of ligninases is the detoxification of the biomass hydrolysates. Several of the conventional pretreatment processes generate toxic compounds classified as furan derivatives, sugar degradation products, weak acids, and phenolic compounds from lignin. These compounds can affect the growth and ethanol production by yeasts or other microbes used for bioethanol production and are sometimes removed prior to fermentation so that the ethanol yields are improved. Ligninolytic enzymes are an efficient means of degrading these inhibitors and offer the advantages of reduced or no sugar loss, ambient conditions of operation. Mostly phenolic compounds are removed, while lesser known ligninolytic enzymes like aryl-alcohol oxidases (AAOs) are being investigated for removal of furan derivatives (Carro et al. 2015). Detailed reviews on the ligninolytic enzymes and their applications for biofuels may be found in Placido and Capareda (2015) and Fisher and Fong (2014).

1.3 Microbial Production of Cellulases and the Systems for Cellulose Hydrolysis

While cellulases, hemicellulases, ligninolytic enzymes, and a myriad of different accessory enzymes and proteins are involved in the hydrolysis of lignocellulosic biomass in nature, not all of them are used in the preparations for commercial hydrolysis of biomass for biorefinery applications. In commercial preparations of biomass-hydrolyzing enzymes, enzymes are only used in their crude form, the only processing steps employed being concentration, stabilization, and formulation. Major attention is often only given to cellulases, though it is also implied that other enzymes are present in the preparations, since there are no elaborate purification steps involved. Often cellulase preparations contain hemicellulases, LPMOs, ligninolytic enzymes, etc. depending on the source organisms and techniques employed for production. It may be noted that the most commonly employed microorganism for cellulase production is *Trichoderma reesei*, which is often genetically modified for enhanced cellulase expression and is derepressed for carbon catabolite repression. *T. reesei* is limited in its ability for synthesis of beta glucosidase, and, often in biomass hydrolyzing blends BGL and xylanase from heterogeneous sources are added to make the enzyme more effective. Since cellulases are the major determinants of the efficacy of the biomass-hydrolyzing enzyme cocktails, the current discussion is limited to cellulases. Detailed discussion on hemicellulases and other enzymes may be found in Shalom and Shoham (2003), Juturu and Wu (2013), and Placido and Capareda (2015).

Ability to degrade cellulose is not a common trait among microorganisms and only a few specialized microorganisms – mostly bacteria and filamentous fungi – are capable of cellulose depolymerization (Quiroz-Castañeda and Folch-Mallol 2013). The machinery for cellulose degradation is radically different in the anaerobic bacteria and the rest of the organisms, and these involve cell wall-bound complex structures known as cellulosomes. Aerobic bacteria and filamentous fungi normally secrete a complex array of free enzymes that act synergistically to convert cellulose like the *T. reesei* cellulases. In yet another mechanism recently hypothesized, certain bacteria (e.g., *Fibrobacter succinogenes*) found in the rumen of herbivores use a mechanism involving both cell wall-bound and free enzymes for cellulose hydrolysis (Burnet et al. 2015). The following discussion will describe the free and bound systems of cellulose hydrolysis in the context of microbial degradation of cellulose, and the third mechanism shall be introduced separately.

1.3.1 Cellulose Hydrolysis Through Cell Wall-Bound Enzyme Complexes

Cell wall-bound cellulase-degrading enzyme complexes called cellulosomes are employed by several anaerobic bacteria for breaking down cellulose in nature. These include several Clostridia including the typical strain *Clostridium thermocellum*. The other common anaerobic bacteria include *C. cellulovorans, C.*

cellulolyticum, C. acetobutylicum, Acetivibrio celluloyticus, Bacteriodes cellulosolvans, Ruminococcus albus, R. flavifaciens, etc. (Fontes and Gilbert 2010). There are also anaerobic fungi like *Neocalimastix, Pyromices*, and *Orpinomyces* which employ the cellulosomes for degradation of celluloses (Haitjema et al. 2014). These systems employed by the anaerobic microorganisms are called "complexed systems" as the cellulosomes are multiprotein complexes anchored to the microbial cell wall. Cellulosomes are the largest extracellular enzyme complexes found in nature, and there are polycellulosomes as large as 100 MDa (Doi and Kosugi 2004). Cellulosome contain high-molecular weight noncatalytic proteins called scaffoldin onto which the enzymes are attached. The modular cellulases and hemicellulases produced by anaerobic microbes contain a dockerin appended to the catalytic module (the enzyme) and a noncatalytic carbohydrate binding module (CBM) (Fontes and Gilbert 2010). Dockerins are proteins of ~70 aminoacids usually present in single copy at the C terminal end of cellulolytic enzymes. They serve the purpose of anchoring the enzyme to the large scaffoldin protein which bears modules called cohesins that directly bind the dockerins. Cohesins are modules that are ~150 residues in length and are present as internal repeats in the scaffoldin. Typically about 1–11 cohesin modules are found in a scaffoldin, and it is recognized that the interaction of cohesions with dockerins may not be highly specific allowing different dockerins (bearing different enzymes) to be assembled on the cellulosome complex. Also the scaffoldin molecules contain a cellulose-specific family 3 CBM and a C terminal divergent dockerin which serve respectively the functions of targeting the cellulosome to the cellulose and to the bacterial cell wall (Fontes and Gilbert 2010). A typical cellulosome assembly is represented in Fig. 1.5.

The co-localization of different enzymes and CBMs on the cellulosome allows them to act in close proximity on the cellulose surface, which in turn is proposed to enhance the hydrolytic ability (Resch et al. 2013).

In addition to the anaerobic cellulolytic bacteria inhabiting the rumen or gut microbiomes and aquatic environments, there are anaerobic fungi that are capable of efficient cellulose degradation. It is now known that an early branch of fungi belonging to the order *Neocallimastigomycota* inhabit the digestive tracts of mammalian and reptilian herbivores that consume highly fibrous diets (Haitjema et al. 2014). They are suggested to be responsible for 40–70% of plant biomass digestion in the ruminant and nonruminant herbivores (Akin et al. 1990). These fungi possess both the complexed and the free enzymes and are believed to act by developing a highly branched rhizoidal network of rhizomycelia that penetrates the substrate and exposes it for attack by the secreted cellulases (Haitjema et al. 2014). While the studies on anaerobic fungi have confirmed that the enzymes of these fungi can form large complexes and they encode fungal dockerin domains, more is yet to be known about the cohesins or scaffoldins in them. While most of the studies have identified fungal dockerin domains in the cellulolytic enzymes elaborated by the anaerobic fungi, the type and structure of scaffoldins have largely remained elusive. Recently, scaffoldins have been described in *Neocallimastix* (Wang et al. 2014). It is also known that the dockerins displayed by one enzyme can bind another cellulase from the same organism (Nagy et al. 2007), implying that the mechanism of cellulose