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A Review of natural and engineered enzymes involved in bioethanol production

Ines Cuesta Urena

A REVIEW OF NATURAL AND ENGINEERED ENZYMES INVOLVED IN BIOETHANOL PRODUCTION

By

Inés Cuesta Ureña

Bachelor's of Science in [Biology], University of Barcelona, Barcelona, Spain, 2011

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Approved by:

Sandy Ross, Dean of The Graduate School
Graduate School

Michael Ceballos, Chair
University of Minnesota Morris, Division of Science and Mathematics

Sandy Ross, PhD
Department of Chemistry

Klara Briknarova, PhD
Department of Chemistry

Biswarup Mukhopadhyay, PhD
VirginiaTech, Department of Biochemistry

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Alternative petroleum-derived fuels, such as biofuels, is another form to decrease the dependence of non-renewable energy. The most promising alternative energy is cellulosic ethanol because of the abundance of cellulose and the overall lack of concern for the food-versus-fuel dilemma.

In order to produce ethanol from cellulosic materials, pretreatment is required to "open" the lignocellulosic matrix and make cellulose more susceptible to enzymatic degradation. Enzymatic hydrolysis of lignocellulose is an important area of research due to the absence of negative effects in downstream processes in comparison with acid hydrolysis. Both natural enzymes and engineered enzymes can be used in the process of ethanol production. Natural enzymes are found either individually or as a part of a complex known as cellulosome. Such complexes are the focus of many studies due to the efficiency in the degradation of cellulose. Research in enzymatic engineering is being done in order to mimic these natural systems. Engineered individual enzymes are also used to improve the properties of the enzymes found in nature. Enzymes can be engineered by rational design or directed evolution. Directed evolution is the most efficient technology, since it only requires the knowledge of protein sequences. However, this approach also possesses some limitations. A combination of both methods or a "semi-rational" approach is perhaps the best option to develop higher performance lignocellulolytic enzymes.

Many advances regarding engineering of lignocellulolytic enzymes have been made in the last past years. Further research, however, is required in the development of enzymes systems and enzyme industrial testing to establish cellulosic bioethanol as main substitute for petroleum-derived fuel energy.

Chairperson or Co-Chairperson: Ceballos, Michael

INTRODUCTION

The interest in alternative energies has increased in the past years due to the unsustainable use of natural resources and their growing demand. Biofuels, which can be made from lipid-rich feedstocks (biodiesel) or carbohydrate-rich feedstocks (bioethanol), is one such form energy. Biofuels are considered renewable and, in many cases, sustainable and environmentally-friendly. Although bioethanol possesses numerous advantages, there are still some limitations in commercial production that prevent it from being cost competitive and the liquid fuel of choice. These limitations include: the use of land and water resources to produce corn- and sugarcane-based bioethanol (i.e., first generation biofuels) that competes with food crop production and the inefficient conversion of feedstock molecular substrates to fermentable sugars in cellulosic ethanol (i.e., second generation bioethanol). Current research is focused on overcoming these limitations. Since cellulose is the most abundant polymer on Earth and first generation bioethanol production creates a *food-versus-fuel* dilemma, cellulosic ethanol may be the most promising alternative liquid fuel option if production process bottlenecks can be resolved.

Pretreatment of cellulosic feedstock is typically required to expand or "loosen up" the lignocellulosic matrix. At the molecular level this entails: breaking hydrogen bonds between lignin and holocellulose; disrupting hydrogen bonding within holocellulose; lysing covalent bonds that stabilize lignin; and, destabilizing the crystalline of cellulose to render it more susceptible to degradation by sugar reducing enzymes. Depolymerization of molecular substrates (e.g., cellulose) from pretreated lignocellulosic materials to generate simpler fermentable, sugars (e.g., monosaccharides) is often achieved by one of two different sugar reduction processes: enzymatic hydrolysis; or, acid hydrolysis. Acid hydrolysis is mainly performed via sulfuric acid; however, due to negative effects in downstream processes, the use of this technology is limited. Using hydrolytic enzymes from microorganisms (i.e., bacteria and fungi) as a substitute for chemical approaches is technology continuously in development and an area of intense research. Enzymatic degradation of lignocellulose is performed using a variety of enzymes in specific ratios or sequences so that biomass deconstruction and generation of fermentable sugar may be achieved in an efficient manner. Multi-enzyme systems are designed to optimize synergistic interactions between different classes of enzymes during this process. In general, enzymes used in this process are referred to as "lignocellulolytic enzymes". Most are derived from natural systems and each class of enzymes possesses unique features including structural-functional modularity. Optimization of enzyme-mediated processes is particularly important if bioethanol is to become economically viable. Either enzymes found in nature or engineered enzymes can be utilized in industrial-scale bioethanol production processes. Attempts to mimic the activity of naturally-occurring extracellular macromolecular complexes called cellulosomes have been undertaken,

because of the efficiency by which cellulosomes degrade cellulosic biomass in nature. This has spawned the development of enzyme cocktails and engineered platform systems.

The goal of this article is to provide a review of the enzymes found in nature as well as engineered enzymes that are used in the production of bioethanol with focus on their respective modes of action and a description of the most important multienzyme systems found in nature are discussed, including the cellulosome of C. thermocellum.

NATURAL ENZYMES USED TO CONVERT FEEDSTOCK TO SUBSTRATE

Mode of Action of Primary Lignocellulolytic Enzymes

To use cellulosic material as feedstock for bioethanol production, chipped or ground biomass is typically pretreated to facilitate enzyme access to long chain carbohydrates (e.g., cellulose), which are the macromolecules that are reduced to fermentable sugar for conversion to ethanol. Given the heterogeneous nature of lignocellulose, it is highly recalcitrant even with pretreatment. Numerous methods have been developed for degrading lignocellulose to expose polysaccharides. Likewise, numerous approaches have been developed to reduce these macromolecular substrates. Those approaches that rely on enzymes produced by microorganisms can be highly efficient. Both multi-domain enzymes and enzyme complexes (e.g., mini-cellulosomes) have been applied. One useful feature of many lignocellulolytic enzymes (and their complexes) is innate modularity. In addition to a catalytic core region, many cellulolytic enzymes possess non-catalytic domains. Two notable domains include carbohydrate-binding modules (CBMs) and dockerin domains. CBMs facilitate interactions between enzymes and their respective carbohydrate substrates (Tomme et al., 1988; Tomme et al., 1998; Boraston et al., 1999; Gilbert et al., 2013). Various studies have demonstrated CBMs enhance enzymatic activity against recalcitrant substrates (Black et al., 1996; Bolam et al., 1998; Carrard et al., 2000; Mello and Polikarpov, 2014). Dockerin domains on cellulolytic enzymes from some species of microorganisms mediate cohesin–dockerin interactions, associating the enzymes with larger macromolecular complexes. These complexes, or *cellulosomes*, are found naturally at the cell membrane-cell wall structure of many cellulolytic microorganisms (see Fontes and Gilbert, 2010). Lignocellulolytic enzymes may be generally categorized as: cellulases, hemicellulases, ligninolytic enzymes and pectinases. This section provides a review of primary lignocellulolytic enzymes and their respective functions within natural cellulosomes.

- Cellulases

Cellulases are glycosyl or glycoside hydrolases (GHs) that catalyze cellulolysis, the cleaving of glycosidic bonds in cellulose. The enzyme-mediated cleavage of β-1,4-glycosidic bonds in cellulose occurs via acid hydrolysis, using a proton donor and a nucleophile or base. The products of acid hydrolysis either result in an inversion or retention (via single or double replacement, respectively) of the anomeric configuration of the carbon-1 (C1) at the reducing end (see Koshland, 1953; see Vocadlo and Davies, 2008). In macromolecular complexes, such as in a naturally-occurring cellulosome, cellulolytic enzymes act in a synergistic manner (Wood and McCrae, 1979; Lamed et al., 1983b; Fierobe et al., 2001). Synergism is a phenomenon that results in a mutual increase in the efficiency of action using two or more components in a system. Cellulolytic enzyme synergism can be measured qualitatively and quantitatively; however, predicting synergistic effects of novel combinations of enzymes either free in solution or bound in an artificial cellulosome has proven challenging and is the subject of intense investigation. According to studies on fungi (Selby and Maitland, 1967; Wood and McCrae, 1972; Berghem et al., 1976; Wood and McCrae, 1978; Mandels and Reese, 1999), bioconversion of polysaccharide substrates into simple fermentable sugars requires synergistic interactions of at least three types of enzymes: endoglucanases, cellobiohydrolase, and β-glucosidases. Most of these components are glycoproteins and each presents isoenzymes in natural systems (Wood and McCrae, 1972; Gilkes et al., 1984; Mihoc and Kluepfel, 1990; Jimenéz-Zurdo et al., 1996; Igual et al., 2001; Wei et al., 2005; Begum and Absar, 2009; Khalili et al., 2011). Functionally, cellulases may be categorized into groups based on the type of reaction catalyzed: carbohydrases (including, endoglucanases, exoglucanases and cellobiases); oxidative cellulases (e.g., cellobiose dehydrogenase); and, phosphorylases (i.e., cellobiose phosphorylase and cellodextrin phosphorylase).

- Carbohydrases

Carbohydrases are GHs that hydrolyze the β-1,4-glycosidic bonds of cellulose or cello-oligosaccharides, leading to the formation of short cello-oligosaccharides (cellodextrins) and glucose (Lombard et al., 2014; CAZy, 2015). There are three types of carbohydrases: endoglucanases or endocellulases (EGs); exoglucanases or exocellulases (EXs); and cellobiases, β-glucosidases or β-D-glucoside glucohydrolases (βGs).

EGs are 1,4-β-D-glucan-4-glucanhydrolases that disrupt bonds at random internal sites in the cellulose polysaccharide chain producing oligosaccharides of various lengths. The EGs produce

new chain ends (*see* Figure 10). EGs that do not feature CBMs hydrolyze at amorphous internal sites within the cellulose chain (Rabinovich et al., 1982; Stahlberg et al., 1988; Henriksson et al., 1999; Karlsson et al., 2002). EGs featuring CBMs can also hydrolyze cellulose chains at crystalline internal regions (Tilbeurgh et al., 1986; Gilkes et al., 1988; Tomme et al., 1988; Wang et al., 2012e). Furthermore, EG cellulolysis generates new chain ends for cellobiohydrolase (CBH) activity (Wood and McCrae, 1972; Berghem and Pettersson, 1973; Henrissat et al., 1985). When an enzyme does not readily release a large molecular substrate and catalyzes multiple reactions prior to dissociating from the substrate, it is considered to be "processive". Although processivity is typical of CBHs, some EGs also hydrolyze cellulose processively (Reverbel-Leroy et al., 1997; Irwin et al., 1998; Gilad et al., 2003; Cohen et al., 2005; Zverlov et al., 2005a; Zheng and Ding, 2013). EGs belong to GH families 5, 6, 7, 9, 12, 44, 45, 48, 51, 74 and 124 (Lombard et al., 2014; CAZy, 2015). All well-studied processive EGs are part of the GH-9 family, which includes most plant cellulases, some animal cellulases, and many bacterial cellulases. Surprisingly, very few fungal cellulases are included within the GH-9 family of EGs. Processive EGs from GH-9 family feature CBMs of the family 3c. These EG CBMs are positioned at the C terminus of the enzyme's catalytic domain (Sakon et al., 1997). They are required and responsible for processivity in this class of enzyme (Irwin et al., 1998; Gilad et al., 2003). Additionally, a new kind of processive EG has been reported recently that belongs to the GH-5 family (Cohen et al., 2005; Watson et al., 2009; Zheng and Ding, 2013).

EXs hydrolyze 1,4-β-D-glycosidic linkages in cello-oligosaccharides. Specifically, they cleave from the reducing or nonreducing ends of chains formed by EGs activity. These EXs act in a processive manner due to their shaped tunnel active sites (Rouvinen et al., 1990; Divne et al., 1994; Parsiegla et al., 1998). Several studies indicate that select EXs, such as CBH, are also capable of cleaving internal glycosidic bonds (Stahlberg et al., 1993; Armand et al., 1997; Boisset et al., 2000). There are two main groups of EXs: cellodextrinases and cellobiohydrolases (CBHs). Cellodextrinases, also known as 1,4-β-D-glucan glucanohydrolases and exo-1,4-β-glucosidase, liberate D-glucose from cellodextrins and cellulose (Barras et al., 1969). They belong to the GH families 1, 3, 5, 9 (Lombard et al., 2014; CAZy, 2015). CBHs (1,4-β-D-glucan cellobiohydrolases) liberate D-cellobiose from cellulose chain ends produced by EGs and from crystalline cellulose (Kleman-Leyer et al., 1996; Igarashi et al., 2009; Liu et al., 2011) while CBHII additionally releases D-cellobiose from amorphous cellulose (Koivula et al., 1998) (*see* figure 10). CBHI works processively from the reducing end of cellulose and CBHII works processively from the nonreducing end of cellulose (Fägerstam and Pettersson, 1980; Arai et al., 1989; Barr et al., 1996; Saharay et al., 2010). CBHIIs are grouped into GH families 5, 6, 9, while most of CBHIs belongs to GH families 7 and 48 (Lombard et al., 2014; CAZy, 2015). Due to enzyme processivity and large adsorption ability onto the insoluble cellulose substrates, CBH kinetics deviate from the Michaelis-Menten model of enzyme kinetics and exhibit fractal and "local jamming" effects (Xu and Ding, 2007; Igarashi et al., 2011; Kamat et al., 2013).

βGs hydrolyze the β-1,4-D-glycosidic bonds at the non-reducing ends of soluble cellodextrins and cellobiose to release monomeric β-glucose (Freer, 1993). Unlike other carbohydrases, βGs generally lack distinct CBMs and are therefore not modular in nature. Unlike the majority of biomass degrading enzymes, βGs can be studied using traditional kinetic models primarily due to they act by binding to soluble substrate (Kempton and Withers, 1992; Chauve et al., 2010). βGs serve an important role in the multi-enzyme system synergy by increasing glucose yield and minimizing inhibition of cellulases by cellobiose (Berlin et al., 2005a; Chir et al., 2011). Cellobiose is a strong inhibitor of CBH and EG (Holtzapple et al., 1990; Gusakov and Sinitsyn, 1992; Zhao et al., 2004; Andrić et al., 2010; Teugjas and Valjamae, 2013). Studies show that cellobiose inhibits cellulases 14 times more than glucose (Holtzapple et al., 1984). βGs are classified into GHs families 1, 3, 5, 9, 30, 116 (Lombard et al., 2014; CAZy, 2015).

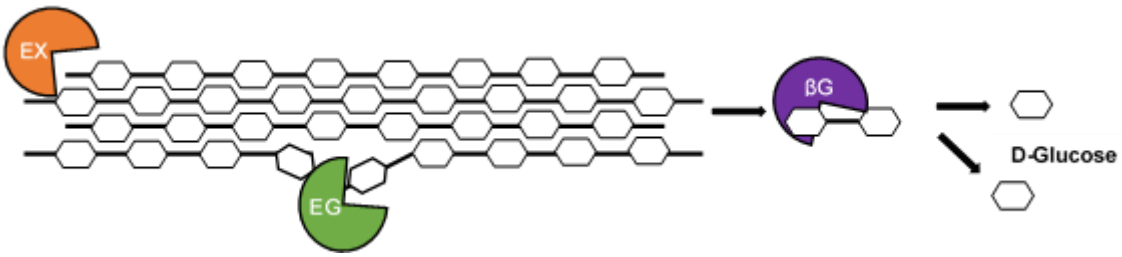

Figure 10. Schematic graphic of cellulose structure and mode of action of cellulolytic enzymes EX, EG, and βG leading to the formation of D-glucose. *Figure adopted from* (van den Brink and de Vries, 2011).

Different EGs possess different mechanisms ("inverting" for GH6, 9, 45, and 48 EGs; "retaining" for GH5, 7, 12 EGs. This EG "plurality" may be in relation with different EGs' side-activities on hemicellulose in deconstructing complex lignocellulose materials (Vlasenko et al., 2010), or synergism between processive and conventional EGs (Tuka et al., 1992; Qi et al., 2007).

- Oxidoreductive Cellulases

For decades, researchers have suggested that there exists an additional non-hydrolytic factor that makes biomass less recalcitrant and more susceptible to enzymatic attack (Reese et al., 1950). This includes a proposed oxidative mechanism for the initiation of cellulose degradation (Eriksson et al., 1974). Subsequently, it was confirmed that cellulose oxidases disrupt cellulose structure via oxidation thus, increasing substrate access to cellulase action (Forsberg et al., 2011; Quinlan et al., 2011). Although these enzymes occur in relatively low concentrations in natural systems, they play a central role in the cellulase systems of both aerobic fungi and bacteria, which degrade

cellulose (Harris et al., 2010; Forsberg et al., 2011). Interestingly, cellulose oxidases are completely absent in anaerobic cellulase complexes.

Oxidoreductive enzyme systems are commonly composed of cellobiose dehydrogenase (CDH), cellobiose quinone oxidoreductase (CBQOR), lactonase, glucose oxidase, and/or polysaccharide monooxygenases (PMOs) (*see* figure 11).

CDH, also known as cellobiose oxidoreductase (CBOR) or cellobiose oxidase (CBO), was discovered in 1974 and originally named CBQOR (Westermark and Eriksson, 1974a; Westermark and Eriksson, 1974b). The enzyme is formed by one flavin adenine dinucleotide (FAD) and one heme as prosthetic groups (Ayers et al., 1978; Morpeth, 1985). CBQOR is the term now used for a catalytic active fragment of CDH which lacks the heme group, is produced by posttranslational proteolytic cleavage, and has similar catalytic properties to CDH (Henriksson et al., 1991; Samejima and Eriksson, 1992; Wood and Wood, 1992; Raíces et al., 2002). CDH is the only known example of secreted flavocytochrome and is found in most wood-degrading fungi (see Zamocky et al., 2006). They catalyze the reducing end oxidation of cellobiose, cellodextrins, lactose, and maltodextrins or other oligosaccharides to the corresponding lactones using a wide spectrum of electron acceptors including quinones, phenoxyradicals, Fe^{3+}, Cu^{2+} and triiodide (see Henriksson et al., 2000). These lactones are converted into aldonic acids by spontaneous or enzymatic hydrolysis with lactonase (Brodie and Lipmann, 1955; Beeson et al., 2011). The biological role of CDH is not fully understood yet, but the research done over the last 50 years indicates its participation in the deconstruction of lignocellulose components (cellulose, hemicellulose and lignin) by generating hydroxyl radicals. The enzyme has the ability to reduce Fe^{3+} to Fe^{2+}, which together with hydrogen peroxide (H_2O_2), produces hydroxyl radicals via Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$) (Kremer and Wood, 1992a; Kremer and Wood, 1992b). Also, CDH diminishes the end-product inhibition of cellulases by removal of cellobiose (Ayers et al., 1978; Igarashi et al., 1998). In addition, recent evidence suggests the participation of the enzyme in the transfer of electrons to members of the PMOs to oxidatively breakdown plant-biomass constituents (Phillips et al., 2011; Sygmund et al., 2012; Vu et al., 2014). CDH are classified into auxiliary activity family 3 (Lombard et al., 2014; CAZy, 2015)

Lactonase, also known as a gluconolactonase, aldonolactonase or D-glucono-1,5-lactone lactonohydrolase, catalyzes the hydrolysis of different types of hexose-1,5-lactones to their corresponding aldonic acids (Brodie and Lipmann, 1955; Beeson et al., 2011). It is present in Novozyme 188, a commercial preparation based on enzymes from *Trichoderma reesei* and *Aspergillus niger* (Bruchmann et al., 1987). It can also promote cellulolysis by removing lactones, which are inhibitors of cellulases (Bruchmann et al., 1987; Verma et al., 2011; Rouyi et al., 2014).

Glucose oxidase, also known as notatin, is an oxido-reductase that mainly catalyzes the oxidation of glucose to H_2O_2 and D-glucono-δ-lactone which hydrolyses spontaneously to gluconic acid (Müller, 1928; Bentley and Neuberger, 1949). It is also considered integral components of cellulase, as it relieves inhibition of cellulases by glucose (Holtzapple et al., 1984; Stutzenberger, 1986; Holtzapple et al., 1990; Xiao et al., 2004; see Andrić et al., 2010; Hsieh et al., 2014). Glucose oxidase belongs to auxiliary activity 3 (Lombard et al., 2014; CAZy, 2015).

PMOs were first discovered in 2010 by G. Vaaje-Kolstad (Vaaje-Kolstad et al., 2010). They are copper-dependent metalloenzymes that oxidatively cleave glycosidic bonds at the surface of recalcitrant cellulose structures (Forsberg et al., 2011; Phillips et al., 2011; Quinlan et al., 2011). For efficiency, PMOs require molecular oxygen (O_2) and an electron donor, such as CDH (Phillips et al., 2011; Sygmund et al., 2012; Vu et al., 2014). PMOs introduce O_2 to C−H bonds adjacent to the glycosidic linkage, which leads to the removal of the adjacent carbohydrate moiety (Phillips et al., 2011; Beeson et al., 2012). PMOs can be subdivided into at least three types based on structure and substrate specificity. Type 1 PMOs generate products that are oxidized at C1. Type 2 PMOs generate products oxidized at the non-reducing end of C4. Type 3 PMOs exhibit weaker specificity and release oxidized products from both reducing and non-reducing ends (Beeson et al., 2012; Li et al., 2012). PMOs are classified into auxiliary activity families 9 and 10 (Lombard et al., 2014; CAZy, 2015).

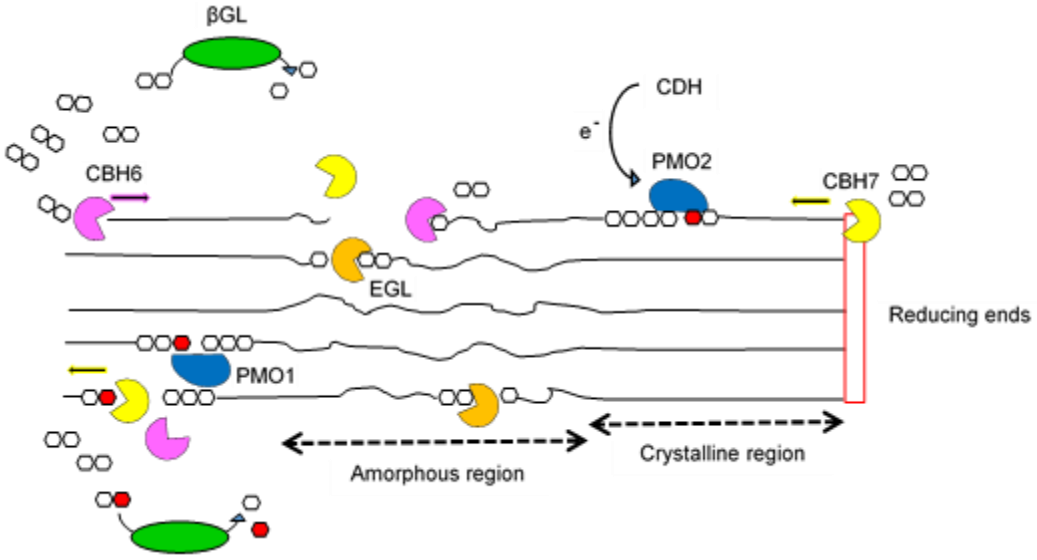

Figure 11. Schematic representation of the enzymatic degradation of cellulose, involving CBH, βG, EG, Type 1 PMOs (PMO1), Type 2 PMOs (PMO2), and CDH. *Figure adopted and modified from* (Dimarogona et al., 2012).

- Phosphorylases

Phosphorylases reduce cellobiose and cellodextrins to glucose using phosphates instead of water (Ayers, 1959; Alexander, 1961; Sheth and Alexander, 1967). Recall that cellobiose and cellodextrin are formed during the enzymatic degradation of cellulose by EGs and EXs. Since phosphorolytic enzymes are located inside cells, organisms transport substrate saccharides from the extracellular matrix across the cell membrane. For example, the thermophile *Clostridium thermocellum* employs ATP-driven transport mechanisms to intake not only glucose, but also cellobiose and cellodextrin (Strobel et al., 1995). Phosphorolysis can then take place on these products of hydrolysis to assist cellulose breakdown. Although phosphorylases are not directly part of the cellulolytic pathway, phosphorolysis may accelerate the rate of overall cellulosic degradation when acting in concert with hydrolytic enzymes by removing inhibitory intermediary products such as cellobiose (Holtzapple et al., 1990; Gusakov and Sinitsyn, 1992; Zhao et al., 2004; see Andrić et al., 2010; Teugjas and Valjamae, 2013). Phosphorylysis is energetically advantageous. Phosphorolysis results in conservation of a portion of the energy from the cleaved glycosyl bond. Glucose-1-phosphate (G1P) leads to the formation of activated glucosyl molecules with the investment of only one additional ATP molecule for the uptake of either cellobiose or cellodextrin. Each glucose molecule produced via hydrolytic cleavage would require two ATPs - one ATP for transport and another for activation (Goldberg, 1975; Strobel et al., 1995). There are two general types of phosphorylases: cellodextrin phosphorylases (CDPs) and cellobiose phosphorylases (CBPs).

CDPs phosphorylate cellodextrins released by EGs to cellodextrin (N-1), where N is the number of glucose units in the chain, and G1P (Sheth and Alexander, 1967). CBPs phosphorylate cellobiose into glucose and G1P during the transport of cellobiose into the cell (Alexander, 1961) (*see* figure 12). Both enzymes are classified into GH family 94 (Lombard et al., 2014; CAZy, 2015)

1) Cellodextrin + Pi ⇌ cellodextrin (N-1) + G-1P
2) Cellobiose + Pi ⇌ glucose + G-1P

Figure 12. Chemical reaction catalyzed by CDP (1) and CBP (2).

- Hemicellulases

Hemicellulases are either GHs or carbohydrate esterases (CEs) that catalyze the hydrolysis and deacetylation of hemicelluloses, respectively (*see* table 7). The mode of action of hemicellulases varies with the type of enzyme (see Vocadlo and Davies, 2008; see Biely, 2012). The heterogeneity and organization of hemicellulose requires the concerted synergistic action of multiple enzymes for complete degradation. The deconstruction of the hemicellulose component in a feedstock exposes cellulose to cellulases and converts hemicellulose into usable saccharides. A principal component of hemicellulose is xylan (Timell, 1967). Therefore, xylanases are common enzymes used to breakdown hemicellulose. Several types of accessory enzymes also play important roles in degrading hemicellulose by acting on side chains of the heteropolymer to facilitate breakdown.

- Main Xylan Degrading Enzymes

Xylan is naturally heterogeneous. Its hydrolysis requires the action of complex enzyme systems. Microbial enzymes act in cooperative manner to convert xylan to its constituent simple sugars. The main enzymes involved are hydrolytic enzymes that hydrolyze β-1,4-xylosidic linkages. These enzymes are grouped into three classes: endo-1,4-β-xylanases (ENs), β-xylosidases (βXs), and exoxylanases (EXYs) (*see* figure 13).

ENs are GHs that hydrolyze β-1,4-xylose linkages in the interior of the heteroxylan backbone and generate xylooligosaccharides. EN action on substrate is not random but instead is determined by the chain length, the degree of branching, or the presence of specific substituents such as arabinofuranosyl groups (Li et al., 2000; von Gal Milanezi et al., 2012). ENs also play a role in lignin removal (Aracri and Vidal, 2011; Valenzuela et al., 2013). They deconstruct the xylan closely associated with lignin, which enhance the accessibility and extractability of lignin (Roncero et al., 2005). ENs are classified into families 5, 8, 10, 11, 30, 43 and 51 of GHs based on amino acid sequence similarities (Lombard et al., 2014; CAZy, 2015). The GH-10 and GH-11 ENs differ in substrate specificity. GH-10 ENs are capable of cleaving glycosidic linkages in the xylan main chain adjacent to substituents (atom or functional group in place of a hydrogen atom on a hydrocarbon), while GH-11 ENs preferentially cleave unsubstituted regions. As a result, GH-10 ENs release products that are shorter than the products of GH-11 EN action (Biely et al., 1997; Ustinov et al., 2008).

βXs release monomeric xylose from the non-reducing ends of xylooligomers and xylobiose produced by EN action on xylan. βXs have molecular weights >100 kDa and typically consist of two or more subunits (Matsuo and Yasui, 1984; Hebraud and Fevre, 1990; Eneyskaya et al., 2003; Eneyskaya et al., 2007). They have catalytic cores of the GH1, 3, 30, 39, 43, 51, 52, 54 and 116, 120 families (Lombard et al., 2014; CAZy, 2015). In general, βX activity on xylooligosaccharides decreases rapidly with increasing chain length (Van Doorslaer et al., 1985; Rasmussen et al., 2006). Many βXs exhibit α-L-arabinofuranosidase activity and some βXs reportedly have βG activity (Rodionova et al., 1983; Uziie et al., 1985; Xiong et al., 2007; Watanabe et al., 2015). Notably, most βXs are susceptible to xylose inhibition, which can significantly affect enzymatic efficiency under process conditions (Dekker, 1983; Poutanen, 1988; Herrmann et al., 1997; Saha, 2003b; Fujii et al., 2011; Kirikyali and Connerton, 2014). By splitting xylobiose, βXs relieve EN end product inhibition (see Sunna and Antranikian, 1997; Williams et al., 2000).

The EXYs are the most recent enzymes to be characterized in xylan degradation. Only a few of these EXYs have been reported and information on their catalytic properties is limited. What is known is that EXYs act on the xylan backbone from the reducing end to release xylose and short xylooligomers (Ganju et al., 1989; Kubata et al., 1994; Kubata et al., 1995; Usui et al., 1999; Honda and Kitaoka, 2004; Fushinobu et al., 2005; Tenkanen et al., 2013; Juturu et al., 2014). EXYs differ from βXs in that the former are inactive on xylobiose (Kubata et al., 1994; Kubata et al., 1995). EXYs can increase the rate of hydrolysis of xylan, since ENs would increase the ends available on the xylan backbone to the EXYs (Gasparic et al., 1995; Juturu et al., 2014). EXYs belongs to GH family 8 (Lombard et al., 2014; CAZy, 2015).

- Accessory Enzymes

Accessory enzymes either degrade the side chains of xylans (debranching enzymes) or act on the backbone chains of different kinds of hemicelluloses (backbone degrading enzymes). They are hydrolases or esterases.

Accessory xylanolytic enzymes

Accessory xylanolytic enzymes include: α-L-arabinofuranosidases (AFs); arabinoxylan arabinofuranohydrolases (AXAHs); endo-1,5-α-arabinanases cumulatively known as arabinases (AR); xylan α-D-glucuronidases or xylan α-1,2-D-glucuronidase (AgluAs); mannan endo-1,4-β-

mannosidase, 4-β-D-mannan mannanohydrolase, endo-1,4-mannanase or, simply, the β-mannanases (MANs); β-mannosidases (MNDs); α-galactosidases (AGL) and β-galactosidases (LACs), or, simply, the galactosidases; βGs; endo-β-1,4-galactanases (EG); xyloglucan-β-1,4-endoglucanases or xyloglucanase, cumulatively known as the xyloglucanendohydrolases (XGHs); α-D-xyloside xylohydrolase or, simpky, α-xylosidases (AXLs); α-fucosidases (AFUs); acetylxylan esterases (AXEs); ferulic acid esterases or feruloyl esterases, also known as, the cinnamoyl esterase hydrolyses (FAEs); *p*-coumaric acid esterases or p-coumaroyl esterase (PAE); glucuronyl esterases (GEs); and, acetyl mannan esterases (AME) (*see* figure 13).

Enzyme	Abbrev	Mode of Action
Hydrolases		
α-L-arabinofuranosidase	AF	non-reducing end of α- 1,2-, α-1,3-, α-1,5-linked arabinofuranosyl groups from arabinans, arabinoxylans, and arabinogalactans
α-fucosidase	AFU	L-fucose residues from xyloglucan branches
α-galactosidase	AGL	non-reducing end of α-linked D-non-reducing end galactose residues from xylan and galactomannans
α-D-glucuronidase	AgluA	non-reducing end of α-1,2-linked 4-O-methyl-D-glucuronic acid residues from glucuronoxylans
endo-1,5-α-arabinanase (arabinase)	AR	α(1→5) glycosidic bonds in arabinan
arabinoxylan arabinofuranohydrolase	AXAH	non-reducing end L-arabinofuranosyl groups from β-1,4-linked arabinoxylans
α-D-xyloside xylohydrolase or α-xylosidase	AXL	α-linked D-xylose residues from the xyloglucan backbone
β-glucosidase	βG	non-reducing end of β-D-glucosyl residues from glucomannan and galactoglucomannan oligosaccharides
β-xylosidases	βX	non-reducing ends of xylooligomers and xylobiose
endo-galactanase	EG	1,4-β-linked galactose residues in arabinogalactans
endo-1,4-β-xylanase	EN	β-1,4-xylose linkages in heteroxylan backbone
exo-β-1,4-xylanase	EXY	reducing end of xylan backbone
β-galactosidase	LAC	non-reducing end of β-linked D-galactose residues from xylan, xyloglucan, and galactoglucomannans
mannan endo-1,4-β-mannosidase, 1,4-β-D-mannan mannanohydrolase or endo-1,4-mannanase (β-mannanase)	MAN	β-1,4-linked bonds in mannan
β-mannosidase	MND	β-1,4-linked mannan oligosaccharides and mannobiose
xyloglucan-β-1,4-endoglucanase or xyloglucanase (xyloglucanendohydrolase)	XGH	1,4-beta-D-glucosidic linkages in xyloglucan
Esterases		
acetyl mannan esterase	AME	acetyl groups from galactoglucomannan
acetylxylan esterase	AXE	acetyl esters in xylan and xylooligomers

ferulic acid esterase or feruloyl esterase (cinnamoyl esterase hydrolases)	FAE	monomeric or dimeric ferulic acid from xylans
glucuronyl esterase	GE	4-O-methyl-D-glucuronic acid residues of glucuronoxylans
p-coumaric acid esterase or *p*-coumaroyl esterase	PAE	monomeric and dimeric p-coumaric acid

Table 7. Summary of lignocellulosic enzyme classes.

The AF catalytic domain belongs to the GH2, 3, 43, 51, 54, and 62 families of hydrolases (Lombard et al., 2014; CAZy, 2015). AF acts by cleaving the non-reducing end of α-1,2-, α-1,3- and α-1,5-linked L-arabinofuranosyl groups from hemicellulose, such as arabinoxylans or arabinogalactans (Saha and Bothast, 1998; Verbruggen et al., 1998b; Ahmed et al., 2013). This mode of action is effective in hydrolyzing hemicellulose side chains and disrupting structures anchored by α-glycosidic bonds. AXAHs are essentially AFs from the GH51 family of enzymes (Lombard et al., 2014; CAZy, 2015). However, AXAHs specifically remove the terminal non-reducing arabinofuranosyl residues from the 1,4-β-xylan backbone of arabinoxylans (Kormelink et al., 1991; Ferre et al., 2000; Lee et al., 2001). AFs and AXAHs also facilitate the disruption of lignin–carbohydrate binding at locations where arabinose residues are involved in lignin–hemicellulose ether bonds (Sun et al., 2005). Other AFs that exhibit βX or xylanase activity have also been described (Utt et al., 1991; Matte and Forsberg, 1992; Mai et al., 2000; Lee et al., 2003a). In addition to acting like other AFs, ARs with catalytic domains belonging to the GH43 family can also cleave internal α(1→5) glycosidic bonds in arabinan (Hong et al., 2009; Lombard et al., 2014; Shi et al., 2014; CAZy, 2015). AgluAs, of the GH67 and GH115 family of hydrolases, typically cleave α-1,2-glycosidic bonds of the 4-O-methyl-D-glucuronic/D-glucuronic acid residues from the terminal, non-reducing xyloses of glucuronoxylooligosaccharides or polymeric glucuronoxylan (Tenkanen and Siika-aho, 2000; Nurizzo et al., 2002; Ryabova et al., 2009; Lee et al., 2012; Lombard et al., 2014; Rogowski et al., 2014; CAZy, 2015). MANs cleave β-1,4-linked internal bonds in mannan backbone polymers producing new chain ends and releasing short β-1,4-manno-oligosaccharides (Mandels, 1965; Stålbrand, 1993; Katrolia et al., 2013). MANs belong to the GH5, GH26 and GH113 families (Lombard et al., 2014; CAZy, 2015). MNDs cleave β-1,4-linked manno-oligosaccharides and mannobiose from the degradation products of endomannases (e.g., MANs) producing mannose by acting at non-reducing terminal ends (Gübitz et al., 1996; Andreotti et al., 2005; Zhang et al., 2009). MNDs are GH1, 2 and 5 enzymes (Lombard et al., 2014; CAZy, 2015). Galactosidases are GHs which catalyze the hydrolysis of galactosides into monosaccharides. There are two types of galactosidases. AGLs, which belongs to GH 4, 27, 31, 36, 57, 97 and 110 families, release α-linked D-galactose residues from hemicellulose, eg., xylan or galactomannan by acting at the non-reducing terminal ends (Ademark et al., 2001; Lombard et al., 2014; CAZy, 2015). LACs, belonging to the GH1, 2, 35, 42 and 59 families (Lombard et al., 2014; CAZy, 2015), hydrolyze the non-reducing ends of β-D-galactose residues from

hemicellulose, eg., xylan, xyloglucan or galactoglucomannans (Sims et al., 1997). The βGs are also exo-type enzymes that remove the 1,4-β-D-glucopyranose units from non-reducing ends of oligosaccharides arising from the breakdown of glucomannan and galactoglucomannan by MAN (see Moreira and Filho, 2008). EGs, belonging to the GH53 family, also hydrolyze 1,4-β-linked galactose residues in arabinogalactans (Lombard et al., 2014; CAZy, 2015). XGHs hydrolyze fragmented xyloglucans into oligoxyloglucans and belong to GH 5, 9, 12, 16 and 44 families (Lombard et al., 2014; CAZy, 2015). AXLs release D-xylose residues with α-linkages from the non-reducing terminal of xylogluco-oligosaccharide (Ariza et al., 2011; Larsbrink et al., 2011). They belong to GH 31 family (Lombard et al., 2014; CAZy, 2015). AFUs, belonging to the GH29 and 95 families, release L-fucose residues from xyloglucan branches (Léonard et al., 2008; Lombard et al., 2014; CAZy, 2015). These represent the major hydrolases.

In addition to hydrolases acting on glycosidic linkages in hemicellulose, CEs catalyze the O-de- or N-deacylation of substituted saccharides. This only considers CEs in which sugars play the role of acid, such as in acetylated xylan. CEs can be grouped into different enzyme classes: AXEs, belonging to the CE1, 2, 3, 4, 5, 6, 7, and 12 families (Lombard et al., 2014; CAZy, 2015), hydrolyze acetyl ester bonds at the C-2, C-3 and C-4 positions of xylose in both xylan and xylooligomers (removing O-acetyl groups) (see Biely, 2012). FAEs, belonging to the CE1 family of esterases, hydrolyze hydroxycinnamoyl ester bonds liberating hydroxycinnamic acids, such as monomeric or dimeric ferulic acid (FA) (Lombard et al., 2014; CAZy, 2015). FAE action can target O2 or O5 on α-L-arabinoses's in xylans. Different FAEs exhibit different specificities driven by the nature of cinnamoyl substitution, which may either occur via hydroxylation or methoxylation, and/or the hemicellulose linkages (e.g., arabinose versus galactose ester bonds on xylans or on pectin) (see Benoit et al., 2008). The PAEs hydrolyze ester linkages between arabinose side chain residues of phenolic acids including monomeric and dimeric p-coumaric acid (PA) (Borneman et al., 1991). They are classified into the CE1 family (Lombard et al., 2014; CAZy, 2015). GEs belonging to the CE15 family (Lombard et al., 2014; CAZy, 2015) hydrolyze methyl ester bonds between 4-O-methyl-D-glucuronic acid residues of glucuronoxylans and aromatic alcohols of lignin (Špániková and Biely, 2006; Ďuranová et al., 2009). AMEs release acetyl groups from galactoglucomannan (see Shallom and Shoham, 2003) and belong to family CE16 family (Lombard et al., 2014; CAZy, 2015).

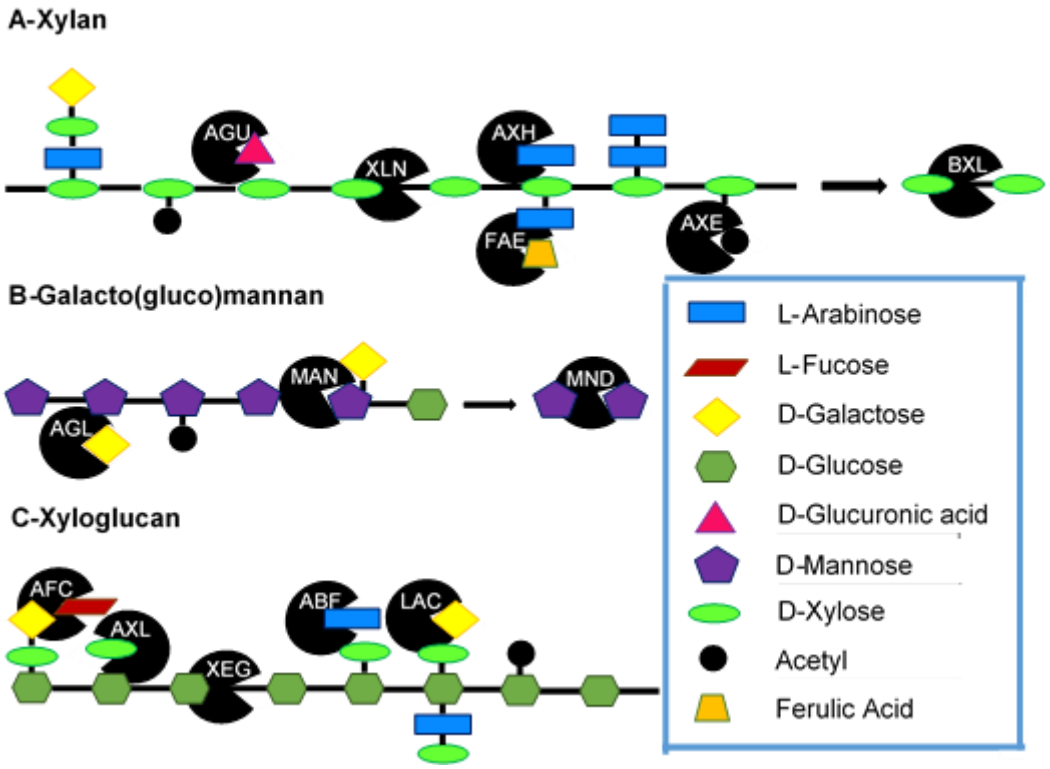

Figure 13. A–C Schematic representation of xylan, galacto(gluco)mannan, and xyloglucan; and mode of action of hemicellulolytic enzymes. *Figure adopted and modified from* (van den Brink and de Vries, 2011).

- Ligninolytic Enzymes

White rot fungi such as *Phlebia spp.* have the ability to effectively degrade lignin to CO_2 and H_2O so that carbohydrate polymers in plant cell walls can be used as carbon and energy sources (Fackler et al., 2006; Arora and Sharma, 2009). Notably, there are multiple studies on the degradation of lignin by white- and brown-rot fungi; however, fewer reports are available on lignin breakdown by soil bacteria (Crawford et al., 1983; Mercer et al., 1996; see Kirby, 2006; see Bugg et al., 2011). Two major families of oxidative enzymes (oxidoreductases) are involved in ligninolysis: peroxidases, including lignin peroxidases, also known as ligninase and diarylpropane oxygenase (LiPs), manganese-dependent peroxidases (MnPs), versatile peroxidases (VPs), and dye decolorizing peroxidase or also known as dyP-type peroxidases (DyPs), and laccases (Lacs). These enzymes catalyze a single-electron oxidation of lignin (transfer of one electron in each step from aromatic lignin components with low reduction potential to the high redox potential active site in the enzyme) that generates highly reactive non-specific free radicals (small agents), such as reactive oxygen species, which initiate lignin depolymerization by various non-enzymatic

reactions (Harvey et al., 1985; Schoemaker et al., 1985; see Hammel et al., 2002). In addition, some accessory enzymes, such as oxidases, are involved in the degradation of lignin by increasing the ligninolytic activity of principal enzymes. Although many enzymes involved in wood lignin degradation cannot penetrate the compact structure of woody tissues (Srebotnik et al., 1988; Flournoy et al., 1993; Blanchette et al., 1997), the enzymes can act at the surface of the cell wall producing low molecular mass agents (see Evans et al., 1994). These low molecular compounds can diffuse through the cell wall and initiate wood decay facilitating the penetration of lignin-degrading enzymes (Galkin et al., 1998).

- Peroxidases

Extracellular heme peroxidases belonging to the auxiliary activity family 2 (Lombard et al., 2014; CAZy, 2015) exhibit high potency in oxidative degradation of lignin and require extracellular H_2O_2 as an electron acceptor. Upon interaction with H_2O_2, these enzymes form highly reactive Fe^{+5}- or Fe^{+4}-oxo species (intermediates in catalytic reactions). These oxoferryl species remove electrons from lignin causing oxidation or radicalization, such in the case of LiPs. Regarding MnPs, an oxoferryl specie oxidazes Mn^{2+} to Mn^{3+}, which mediates lignin oxidation (see Wong, 2009) (*see* figure 14). The two main peroxidases LiP and MnP were discovered in the mid-1980s in *Phanerochate. chrysosporium* and exhibit high redox potential (Tien and Kirk, 1983; Kuwahara et al., 1984; Millis et al., 1989). Common peroxidases found in cellulose degradation systems include LiPs, MnPs, as well as the VPs and DyPs (described below).

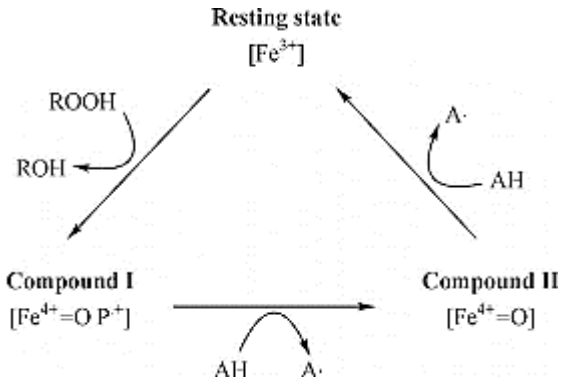

Figure 14. General catalytic cycle of heme-containing peroxidases: First, the resting state (Fe^{3+}) is involved in two-electron oxidation with H_2O_2 to form a Compound I oxo-ferryl intermediate (Fe^{4+}= $OP^{\cdot+}$). Then, Compound I oxidazes electron donor substrates (AH) by one-electron oxidation yielding Compound II (Fe^{4+}=O) and a substrate cation radical (A·). The last step implies another oxidation of substrate by Compound II subtracting one electron and consequently, returning the enzyme to the resting state (see Veitch, 2004). *Figure adopted from (Furukawa et al., 2014)*.

LiP was first discovered in *P. chrysosporium.* LiPs catalyze the H_2O_2-dependent oxidative depolymerization of lignin (Tien and Kirk, 1983; Tien and Kirk, 1984). In most fungi, LiP is present as a series of isoenzymes encoded by different genes (Glumoff et al., 1990; Johansson et al., 1993). LiPs are strong oxidants with higher redox potentials (>1 V) than those found in other types of peroxidases (Ward et al., 2003). This is because the porphyrin ring iron component in LiPs is more electron deficient than in classical peroxidases (Millis et al., 1989). LiPs are considered to be the most effective (and versatile) class of peroxidases. They can oxidize phenolic and non-phenolic compounds (Mester et al., 2001; Ward et al., 2001). LiP-catalyzed reactions include: (1) cleavage of C-C bonds; (2) cleavage of ether (C-O-C) bonds in non-phenolic aromatic substrates; (3) hydroxylation of benzylic methylene groups; (4) oxidation of benzyl alcohols to aldehydes or ketones; (5) phenol oxidation; and, (6) aromatic cleavage of non-phenolic lignin model compounds (Tien and Kirk, 1984; Hammel et al., 1985; Leisola et al., 1985; Renganathan et al., 1985; Renganathan et al., 1986; Umezawa et al., 1986). Note that LiP is too large to enter a plant cell; thus, LiP works on exposed regions of the lumen.

At the molecular level, MnPs are similar to the aforementioned LiPs. MnPs are extracellular heme enzymes that use manganese as a cofactor (Glenn and Gold, 1985; Paszczyński et al., 1986). MnPs were first discovered in *P. chrysosporium* (Kuwahara et al., 1984). The principal function of MnP is to oxidize Mn^{2+} to Mn^{3+} using H_2O_2 (Glenn et al., 1986). Mn^{2+} interacts with MnPs and H_2O_2 leading to the formation of a Mn^{3+}oxalate complexes. However, Mn^{2+} must be first chelated by organic acid chelators to stabilize the Mn^{3+} product. This process produces diffusible oxidizing chelates (Glenn and Gold, 1985; Glenn et al., 1986; Perez and Jeffries, 1992). Although Mn^{3+} is a strong oxidant that can leave the active center and oxidize phenolic compounds, it cannot attack non-phenolic units of lignin (Popp and Kirk, 1991). Resulting phenoxy-radicals undergo a variety of reactions leading to lignin depolymerization (Tuor et al., 1992). MnP can also oxidize non-phenolic lignin model compounds in the presence of Mn^{+2} via peroxidation of unsaturated lipids (Jensen et al., 1996; Kapich et al., 2005; Kapich et al., 2010). It has been suggested that white-rot fungi, which produce MnP and laccase, but not LiP, may produce mediators to enable MnP to cleave non-phenolic lignin substrates (Reddy et al., 2003).

VP from a white fungus (*Pleurotus eryngii*) was reported as a novel peroxidase possessing both MnP and LiP activity. VP oxidizes both phenolic and non-phenolic aromatic compounds, including veratryl alcohol and p-dimethoxybenzene. VP is also able to oxidize Mn2+ like MnPs (Martinez et al., 1996; Martinez et al., 1996; Camarero et al., 1999; Ruiz-Duenas et al., 1999). However, it possesses a high-redox potential for non-phenolic compounds similar to LiP (Camarero et al., 1999). VP is a heme-containing ligninolytic peroxidase with a unique hybrid molecular structure consisting of different active sites that mediate oxidation (Pérez-Boada et al., 2005; Ruiz-Duenas et al., 2009). VP can oxidize hydroquinone without exogenous H_2O_2 if Mn^{+2}

is available for the reaction. Chemical oxidation of hydroquinones promoted by Mn^{+2} may be important for the initial steps of wood biodegradation, since ligninolytic enzymes cannot penetrate unmodified wood cell walls (Gomez-Toribio et al., 2001).

DyPs were first described in fungi (Kim and Shoda, 1999). DyPs are also heme-containing peroxidases, which exhibit no primary sequence or structural similarities to other plant, bacterial, and fungal peroxidases, and perform better than other peroxidases in lower pH (Sugano et al., 1999; Sugano et al., 2007; Sugano, 2009). DyPs possess broad substrate specificity and oxidize all of the typical peroxidase substrates. DyPs can also oxidize high-redox potential synthetic dyes (i.e., anthraquinones), which are not converted by the other peroxidases (Kim et al., 1995; Kim and Shoda, 1999; Sugano et al., 2000; Liers et al., 2010; Santos et al., 2014). Although ligninolytic activity of DyPs has been reported, the actual physiological role of these peroxidases remains unclear. However, evidence suggest that some could be involved in lignin degradation (see Sugano, 2009; Adav et al., 2010; Ahmad et al., 2011; Salvachúa et al., 2013).

- Laccases

Lacs are glycosylated multi-copper phenoloxidases of the auxiliary activity family 1 found in plants, fungi, and bacteria (see Dwivedi et al., 2011; Lombard et al., 2014; CAZy, 2015). Lacs are widely distributed across the wood-degrading fungi (see Baldrian, 2006). Lacs do not require manganese or H_2O_2. They catalyze the single-electron oxidation of substrates through a concomitant four electron reduction of O_2 to H_2O (see Solomon et al., 1996; Messerschmidt, 1997; see Solomon et al., 2001). Lacs can directly oxidize phenolic components of lignin. Direct oxidation of phenolic lignin units generate phenoxy-free-radical products, which ultimately lead to polymer cleavage (Kawai et al., 1988). Substrates with high redox potential are not susceptible to Lac activity, since the enzyme possesses a relatively low redox potential ($\leq$0.8 V) (Reinhammar, 1972; Schneider et al., 1999; Johnson et al., 2003; Uzan et al., 2010). For example, non-phenonlics, which have a redox potential above 1.3V (Zweig et al., 1964), cannot be directly oxidized by Lacs. To degrade non-phenolic components, Lacs require the assistance of suitable mediators. Upon oxidation by Lacs, these low molecular weight compounds are converted to radicals and act as redox mediators that, in turn, oxidize other compounds that are not directly Lac substrates (Bourbonnais and Paice, 1990; Barreca et al., 2003; Cho et al., 2008). In bioethanol production, if Lacs are used directly as a biomass pretreatment, the addition of exogenous mediators, such as 2,29-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Bourbonnais and Paice, 1990), may not be necessary, since natural mediators will likely be generated. For example, initial Lac activity on phenolic lignin units can result in the release of phenoxy radicals, which are natural mediators that oxidize more recalcitrant non-phenolic lignin moieties (d'Acunzo et al.,

2006; Nousiainen et al., 2009). It has been reported that this enzyme also possesses demethylating activity in lignin subunits (Trojanowski et al., 1966; Harkin and Obst, 1974; Ishihara and Miyaxaki, 1976; Leonowicz et al., 1979; Ander et al., 1983; Malarczyk et al., 2009) and lignin preparations (Ishihara and Miyaxaki, 1974; Ander and Eriksson, 1985; Crestini and Argyropoulos, 1998; Ibrahim et al., 2011). During demethylation, laccases act on aryl-O-alkyl C-O bonds.

- Accessory Enzymes and Mediators

There are some accessory enzymes and non-enzymatic components known as mediators that increase ligninolytic activity of principal enzymes. Some of these enzymes are involved in the production of H_2O_2 required by peroxidases. However, other enzymes catalyze the reduction of phenolic products derived from lignin degradation avoiding their posterior repolymerization. These enzymes include oxidases and reductases. Oxidases are grouped into: Glyoxal oxidase (GLOX; a copper radical enzyme) described in *P. chrysosporium* (Kersten and Kirk, 1987; Kersten, 1990; Takano et al., 2010), aryl alcohol oxidase (AAO) (or veratryl acohol oxidase, VAO) described in *P. eryngii* (Guillén et al., 1990; see Hernandez-Ortega et al., 2012), pyranose 2-oxidase or glucose 2-oxidase (Ruelius et al., 1968; Janssen and Ruelius, 1968a; Volc et al., 1978; Daniel et al., 1994), glucose oxidase (or glucose 1-oxidase) (Müller, 1928; Muller, 1936; Franke and Lorenz, 1937; Franke and Deffner, 1939; Kelley and Reddy, 1986), and alcohol oxidase (AOX) or methanol oxidase (Janssen et al., 1965; Janssen and Ruelius, 1968b; Suye, 1997). In addition, fungi produce reductases, such as aryl-alcohol dehydrogenases (AAD) (Muheim et al., 1991; Gutierrez et al., 1994), quinone reductases (QR) (Guillen et al., 1997; Bazzi, 2001) and CDH (or CBOR), CBO or CBQOR (Westermark and Eriksson, 1974a; Westermark and Eriksson, 1974b; Temp and Eggert, 1999).

- Pectinolytic Enzymes (Pectinases)

Pectinases are enzymes that catalyze the cleavage of pectic substances, such as pectin. Depending on the cleavage sites utilized, pectinases are categorized into one of three groups: esterases, lyases, and hydrolases (see Sharma et al., 2013b).

- Main Pectinases

The most studied pectinolytic enzymes are homogalacturonan-degrading enzymes: polygalacturonases (PG) or pectin depolymerase; polymethylgalacturonases (PMG); lyases or transeliminases and pectinesterases (PE), which is also known as pectin methylesterases (PME).

PGs catalyze the hydrolytic cleavage of α-1,4-glycosidic linkages in polygalacturonic acid chains by introducing water across the oxygen bridge to form D-galacturonate. They are classified into GH family 28 (Markovic and Janecek, 2001; Lombard et al., 2014; CAZy, 2015). They are divided into: endo-polygalacturonases (PGA) and exo-polygalacturonases (XPG). PGA randomly attacks the pectic acid (polygalacturonic acid) to produce a number of Gal A oligosaccharides. The enzyme cleaves internal α-1,4-D-glycosidic linkage between two low methyl esterified or non-methylated acid residues in pectic acid, because such enzyme can only act on glycosidic linkages adjacent to galacturonic acid residues with free carboxyl groups (Yuan and Boa, 1979; Mohamed et al., 2006). XPG catalyzes the hydrolysis of α-1,4-glycosidic linkages of homogalacturonan (HG) chains from the non-reducing end releasing monogalacturonate. The enzyme needs a non-esterified GalpA unit at subsites -2, -1 and +1 (Kester et al., 1999a). In addition, it is tolerant of xylose substitution by removal of the Gal A-Xyl dimer. As a consequence, XGA is also an XPG substrate (Beldman et al., 1996a; Kester et al., 1999b) (*see* figure 15a).

PMG performs the hydrolysis of α-1,4-glycosidic bonds of the pectin backbone and specially catalyzes highly esterified pectin, forming 6-methyl-D-galacturonate bonds (Seegmiller and Jansen, 1952) (*see* figure 15a).

Lyase performs the trans-eliminative reaction of the α-1,4 glycosidic bond of polygalacturonic acid polymer to form an Δ-4,5 unsaturated C-C bond at the non-reducing end of the cleaved pectin polysaccharide (Albersheim et al., 1960; Moran et al., 1968). They are classified as pectate lyase (pectate transeliminases or PGL) and pectin lyase, (pectin transeliminase or PL). PGL cleaves glycosidic linkages on pectin and produce unsaturated oligogalacturonates or digalacturonates. PGLs are usually specific for non-esterified pectin (pectate) and depend on Ca^{2+} (Starr and Moran, 1962; Pickersgill et al., 1994; Mayans et al., 1997; Seyedarabi et al., 2010). The enzyme is grouped into five of the polysaccharide lyase (POL) families 1, 2, 3, 9, and 10 (Lombard et al., 2014; CAZy, 2015). It exists two types of PGLs: endo-PGL, which acts on substrates at random internal sites within the chain, and exo-PGL, which catalyzes the substrate cleavage from the reducing end. PL catalyzes the random cleavage of high esterified pectin and produces unsaturated

methyloligogalacturonates. PL does not depend on Ca^{2+} (Albersheim et al., 1960; Edstrom and Phaff, 1963; Delgado et al., 1992; Vitali et al., 1998) (*see* figure 15c). PL belongs to POL family 1 (Lombard et al., 2014; CAZy, 2015).

PE or PME catalyzes the de-esterification of methyl ester linkages by removing the methoxyl group at O6 through catalyzing the hydrolysis of the ester linkage into pectate and methanol. The enzyme preferentially targets methyl ester groups in galacturonate units that are next to non-esterified galacturonate units (Solms and Deuel, 1955; Fries et al., 2007). The enzyme is active prior to PG and PGL enzymes, which require non-esterified substrates. After the action of PE, PGs and lyases, then PE catalyze the resulting pectin. PE is a part of CE family 8 (Lombard et al., 2014; CAZy, 2015) (*see* figure 15b).

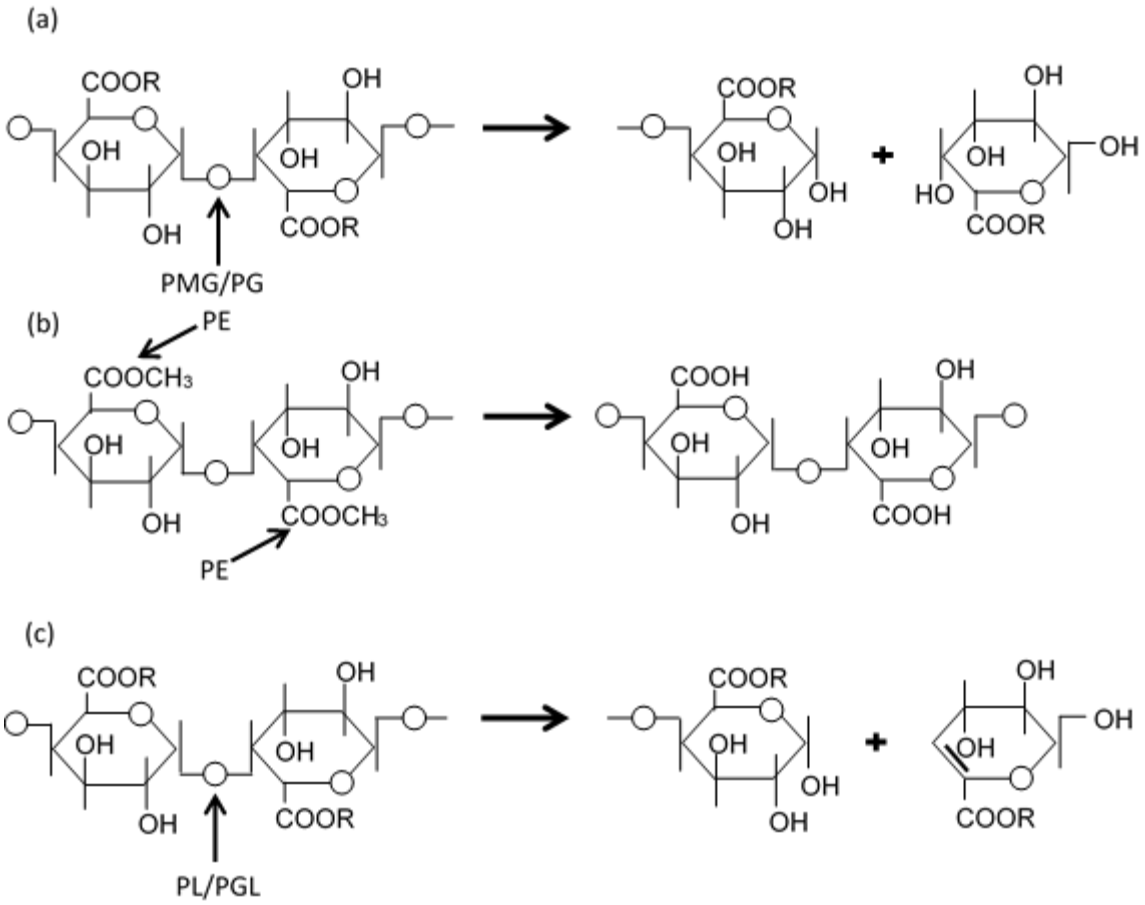

Figure 15. Mode of action of pectinases: (a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH3 for PL. The place where the pectinolytic enzymes react with pectin are showed by the arrows. *Figure adopted from* (Pedrolli et al., 2009).

- Other Pectinases

Pectinases that have not been as extensively studied are: pectin acetyl esterases (PAE); rhammnogalacturonase (RGase), or also known as rhamnogalacturonan hydrolases (RGH); rhamnogalacturonan rhamnohydrolases (RGRH); rhamnogalacturonan galacturonohydrolases (RGGH); rhamnogalacturonan endolyases (RGL); rhamnogalacturonan acetylesterases (RGA); xylogalacturonan hydrolase (XGH); and accessory enzymes (*see* figure 16). They are either backbone degrading enzymes or debranching enzymes.

PAE hydrolyzes the acetyl ester group of HG and rhamnogalacturonan I (RG-I) forming pectic acid and acetate (Williamson et al., 1990; Williamson, 1991; Shevchik and Hugouvieux-Cotte-Pattat, 1997; Bolvig et al., 2003; Bonnin et al., 2008). PAE is classified into CE families 12 and 13 (Lombard et al., 2014; CAZy, 2015). RGase or RGH is an endo acting enzyme able to randomly hydrolyze the α-D-1,4-GalpA-α-L-1,2-Rhap linkage in the RG-I backbone producing oligogalacturonates. The enzyme is intolerant toward acetyl esterification of the RG-I backbone (Schols et al., 1990; Kofod et al., 1994). RGase is grouped into GH family 28 (Lombard et al., 2014; CAZy, 2015). RGRH is an exo-acting pectinase that catalyzes the hydrolytic cleavage of the rhamnogalacturonan chain of RG-I at the non-reducing end, which produces rhamnose (Mutter et al., 1994). This enzyme belongs to GH family 28 (Lombard et al., 2014; CAZy, 2015). RGGH is an exo-acting pectinase that catalyzes the hydrolytic cleavage of the rhamnogalacturonan chain of RG-I at the non-reducing end, which produces monogalacturonate (GalA moety) (Mutter et al., 1998a). RGGH is classified into the GH family 28 (Lombard et al., 2014; CAZy, 2015). RGL catalyzes the random transelimination (β-elimination) of the RG-I α-L-1,2-Rhap-α-D-1,4-GalpA backbone leaving an unsaturated galacturonate at the non-reducing end and a rhamnose at the reducing end (Kofod et al., 1994; Mutter et al., 1996). The RGL activity is hindered by the presence of the acetyl groups in the RG-I backbone (Kofod et al., 1994; Mutter et al., 1998b). These enzymes are classified as polysaccharide-lyase families 4 and 11 (Lombard et al., 2014; CAZy, 2015). RGA catalyzes the hydrolytic cleavage of acetyl groups from the rhamnogalacturonan chain in RG-I (Searle-van Leeuwen et al., 1992). RGA is classified into CE family 12 (Lombard et al., 2014; CAZy, 2015). XGH hydrolyzes the α-1,4-D linkages of xylose substituted galacturonan moieties in XGA producing xylose galacturonate dimers (van der Vlugt-Bergmans et al., 2000; Zandleven et al., 2005). XGH is grouped into GH family 28 (Lombard et al., 2014; CAZy, 2015). Accessory enzymes acting on the lateral chains of RG-I and rhamnogalacturonan II (RG-II) include endogalactanases, exogalactanases, AGLs and LACs, AFs, AR, exoarabinases and FAE (see de Vries and Visser, 2001).

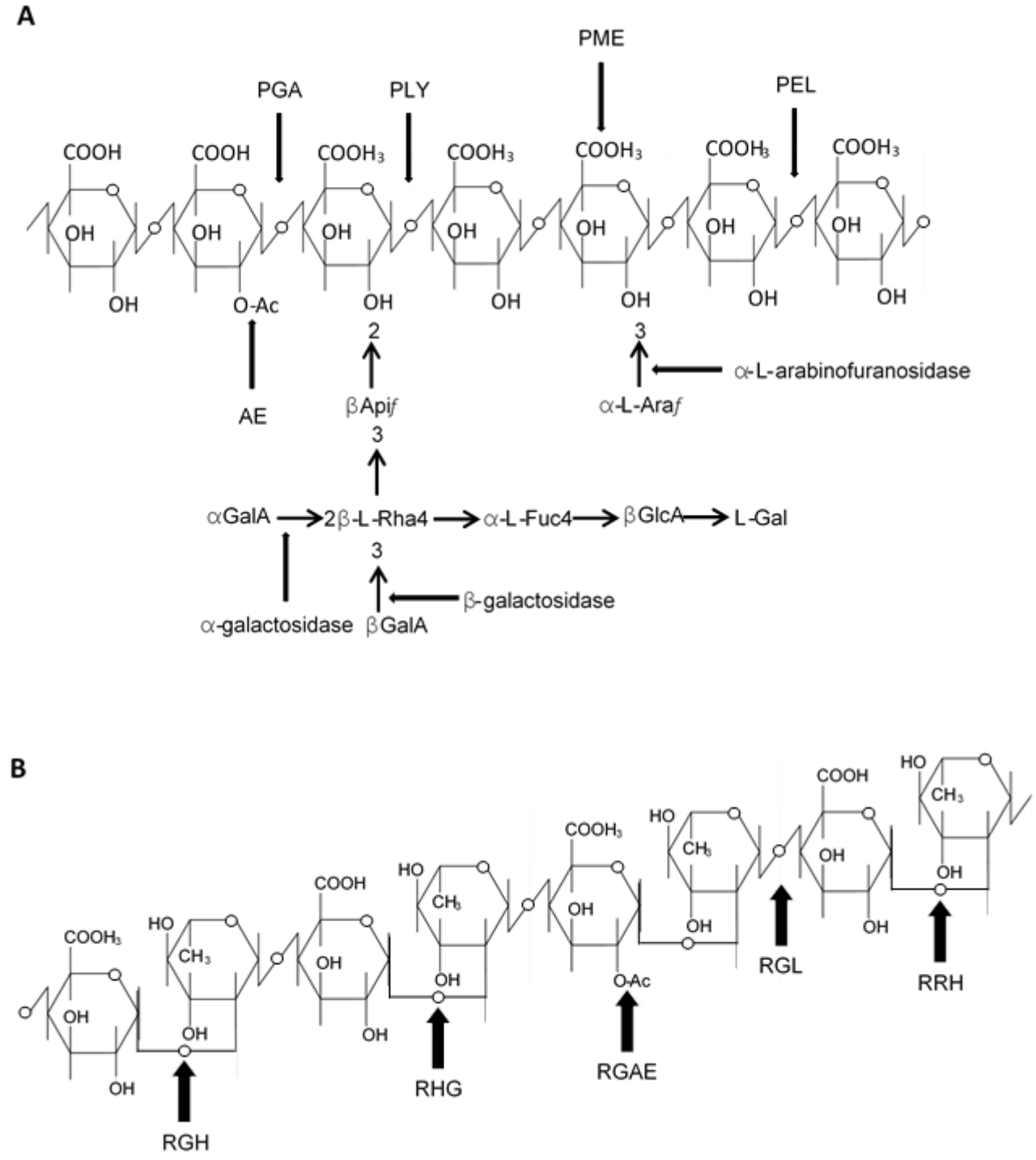

Figure 16. A-B schematic representation of HG and lateral chains of RG-II, and mode of action of pectinolytic enzymes involved in their degradation. *Figure adopted and modified from* (Lara-Márquez, Zavala-Páramo et al., 2011).

Natural Cellulosomes

Since bacteria and fungi are unable to engulf particles, these organisms need to secrete cellulases in order to degrade plant cell walls. The plant cell wall degrading apparatus of aerobic and anaerobic microorganisms differ considerably in their macromolecular organization. Cellulases and hemicellulases synthesized by anaerobes frequently assemble into a large multienzyme complex (molecular weight >2 MDa) called cellulosome (see Bégum and Lemaire, 1996; see Bayer et al., 1998; see Shoham et al., 1999; see Bayer et al., 2004; see Gilbert, 2007; see Smith and Bayer, 2013). Cellulases and hemicellulases produced by most aerobic microorganisms are free enzymes that are secreted at high concentrations and contain a CBM (Wilson, 2008).

Cellulosomes are supramolecular assemblies that are usually bound to the outer surface of the microorganism (see Smith and Bayer, 2013) and exploit the synergistic interactions of their enzyme components to efficiently degrade recalcitrant crystalline lignocellulosic substrates (Fierobe et al., 2002; Fierobe et al., 2005). A cellulosome from the anaerobic thermophilic bacterium *Clostridium thermocellum* was the first identified and characterized during the early 1980s (Bayer et al., 1983; Lamed et al., 1983a; Lamed et al., 1983b). The complex contains not only cellulases, but also a large array of hemicellulases (Morag et al., 1990; Kosugi et al., 2002) and pectinases (Tamaru and Doi, 2001). Enzyme activities include POLs, CEs, and GHs. Experimental evidence also demonstrates the presence of cellulosomes in anaerobic fungi that correspond to the genera Neocallimastix, Piromyces, and Orpinomyces (Wilson and Wood, 1992; Ali et al., 1995; Fanutti et al., 1995; Li et al., 1997; Fillingham et al., 1999; Steenbakkers et al., 2001; Steenbakkers et al., 2003; Nagy et al., 2007; Haitjema et al., 2013; Wang et al., 2014). In addition to the cellulosome, some anaerobic bacteria also produce free cellulases, but their function in cellulose degradation is still unknown (Gilad et al., 2003; Berger et al., 2007).

- Cellulosome Structure

The cellulosome is an extracellular protein complex on bacterial and fungal cell surfaces that adheres to plant materials and deconstructs plant cell wall lignocellulose (Lamed et al., 1983b). The cellulosome comprises several subunits, each of which displays a modular architecture. Some cellulosomes are structural and others are catalytic. The core structural components are known as scaffoldins, which attach all other subunits (Tokatlidis et al., 1991). The cellulosomal catalytic components contain non-catalytic modules called dockerins (Hall et al., 1988), which bind to the cohesin modules located in the scaffoldins (Tokatlidis et al., 1991; Schaeffer et al., 2002; Carvalho et al., 2003). The high affinity protein–protein interactions established between dockerins and

cohesins allow integration of the enzymes into the complex (Carvalho et al., 2003). In addition, scaffoldins usually contain a non-catalytic module called CBM that anchors the entire complex onto the plant cell wall (*see* figure 17).

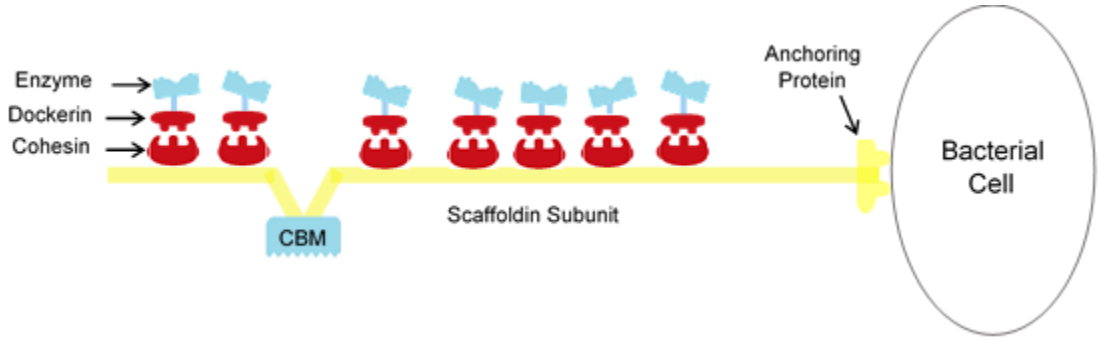

Figure 17. Basic schematic representation of the *C. thermocellum* cellulosome. *Figure adopted from* (Stahl et al., 2012).

Scaffoldins are large non-catalytic modular cohesin-containing proteins that are critical for cellulosome assembly and substrate binding through the CBM (Tokatlidis et al., 1991; Salamitou et al., 1992). Scaffoldins have been identified in most cellulosome-producing bacteria, such as *Acetivibrio cellulolyticus*, *Clostridium cellulolyticum*, *Clostridium cellulovorans* and *Clostridium josui* (Shoseyov et al., 1992; Kakiuchi et al., 1998; Pagès et al., 1999; Dassa et al., 2012). Dockerin contains a highly conserved duplicated segments of approximately 22 amino acids each connected by a peptide containing 8 to 17 amino acid residues and are usually present in a single copy at the C-terminus of cellulosomal enzymes (Grépinet and Béguin, 1986; Yagüe et al., 1990). The reaction between dockerins and cohesins requires Ca^{2+} in *C. thermocellum* (Yaron et al., 1995; Choi and Ljungdahl, 1996) and *C. cellulolyticum* (Pagès et al., 1997). The first 12 residues of each duplicated segment of dockerins resemble the calcium-binding loop in the EF-hand motif (Pagès et al., 1997). Ca^{2+} has shown to be essential for dockerin stability, function and compression into its tertiary structure (Choi and Ljungdahl, 1996; Lytle et al., 2000). These discoveries explain why Ca^{2+} is essential for the cohesin-dockerin interaction and hence the structural stability of the cellulosomes (Lytle et al., 1999; Lytle et al., 2000). The cohesin–dockerin interaction is among the highest affinity protein–protein interaction known. For example, the type I cohesin-dockerin interaction is characterized by a dissociation constant of the order of 10^{-10} M (Fierobe et al., 1999). The dockerin modules are believed to bind to cohesins in two different configurations. The dual binding mode would confer plasticity in dockerin-cohesin interactions and consequently, in cellulosome assembly, allowing the flexible incorporation of enzyme activities into the cellulosome. Furthermore, the plasticity in dockerin-cohesin recognition would also provide alternative modes of interaction between the enzymes and substrates (Carvalho et al., 2007). Cohesins are approximately 150-residue modules and are usually present as tandem repeats in

scaffoldins (Fujino et al., 1992; Shoseyov et al., 1992; Kakiuchi et al., 1998; Ding et al., 1999). There is a notable degree of amino acid homology between cohesins from different species. This similarity is also reflected in their structure; however, the cohesin-dockerin interactions appear to be species-specific (Pagès et al., 1997). The structure of the complex further revealed that protein-protein recognition is mainly mediated by hydrophobic interactions of the beta-sheet cohesin domain and one of the helices of the dockerin protein (Spinelli et al., 2000; Lytle et al., 2001; Miras et al., 2002; Schaeffer et al., 2002; Carvalho et al., 2003; Carvalho et al., 2007). In some cellulosome systems, including that of clostridial, the primary scaffoldin anchors the whole cellulosome onto the cell surface through interaction with a another type of cohesin from an anchoring protein (Leibovitz and Béguin, 1996). Most of the catalytic components of the cellulosome are devoid of the CBMs, and they depend on the CBM present on the scaffoldin protein for attachment to the polysaccharide substrates. Although, there are several reports that propose that CBMs disrupt crystalline structure (Knowles et al., 1987; Din et al., 1994; Wang et al., 2008), CBMs more likely act through targeting and proximity effect. They bring enzymes into intimate contact with plant cell walls enhancing proximity of the enzymes with it and thus, increasing the concentration of enzymes onto the polysaccharide substrates, which in turn enhances the catalytic efficiency of the associated enzymes (Black et al., 1996; Bolam et al., 1998; Hervé et al., 2010). They contain from 30 to approximately 200 amino acids and are located within the parental protein at either the C-terminal or N-terminal (Juge et al., 2002; Abe et al., 2004; Lunetta and Pappagianis, 2014; Peng et al., 2014). Originally, these domains were named, cellulose binding modules (CBD), because the first protein domains discovered bound primarily crystalline cellulose (Tilbeurgh et al., 1986; Gilkes et al., 1988; Tomme et al., 1988). Later, this name was replaced by CBM in order to reflect the diverse ligand specificity of these modules (Boraston et al., 1999). CBMs have been grouped into 67 families based on their amino acid sequences according to the CAZy database (Lombard et al., 2014; CAZy, 2015). Nevertheless, CBMs have also been classified into three types by Boraston based on their binding specificity (see Boraston et al., 2004). Type A CBMs interact with flat surfaces of insoluble polysaccharides, including crystalline cellulose; type B CBMs bind to internal regions of single polysaccharides (glycan chains); and type C CBMs recognize small saccharides such as mono-, di-, or trisaccharide. The orientation and positioning of the aromatic residues in the binding sites of CBMs are the primary drivers of specificity and affinity in these proteins (Simpson et al., 2000), but other interactions, including direct hydrogen bonds (Notenboom et al., 2001; Xie et al., 2001; Pell et al., 2003) and calcium-mediated coordination (Bolam et al., 2004; Jamal-Talabani et al., 2004), also play an important role in CBM ligand recognition. Some CBMs have become primordial to the substrate specificity and mode of action in cognate enzymes. For instance, family 3c CBMs may play a role in the processivity displayed by GH9 family 'endo-processive' cellulases (Sakon et al., 1997; Irwin et al., 1998; Li et al., 2007a; Burstein et al., 2009; Oliveira et al., 2009). Also, CBM 22 was proved to change the specificity of a GH10 family xylanase such that it displayed primarily β-1,4-β-1,3-glucanase activity (Araki et al., 2004).

- Biological Functions of Cellulosomes

It has been suggested that cellulosomes are more efficient at deconstructing plant structural polysaccharides than the corresponding "free" enzyme systems produced by aerobic bacteria and fungi. As an example, *C. thermocellum* requires much less protein in order to solubilize crystalline cellulose substrate than *T. reesei* (Johnson et al., 1982). Indeed, *C. thermocellum* holds one of the highest rates of cellulose hydrolysis (see Lynd et al., 2002). The *C. thermocellum* cellulosome displays a specific activity against crystalline cellulose, which is 50-fold higher than the corresponding *Tricoderma*-free cellulolytic system (see Demain et al., 2005). It has been reported that holding plant cell wall–degrading enzymes onto a macromolecular complex leads to a spatial enzyme proximity that maximizes the potential for synergy between different cellulosomal enzymes against recalcitrant substrates. These enzymes are further augmented by enzyme-substrate targeting scaffoldin-borne CBM (Fierobe et al., 2002; Fierobe et al., 2005). Several studies have shown the importance of recruiting lignocellulolytic enzymes into the cellulosome to mediate efficient hydrolysis of the crystalline substrate. This organization prevents non-productive adsorption of break down products by optimizing component spacing. Sets of enzymes with strong binding domains bind to a single site on the substrate, which prevents competition for a limited number of binding sites on the substrate. The presence of other enzymes with different specificities prevents hydrolytic cessation of one structural type of cellulose. Furthermore, it takes advantage of the synergistic interaction among the enzymes by the correct ratio between the components.

- Cellulosome of *Clostridium thermocellum*

C. thermocellum is a thermophilic and strictly anaerobic spore-forming bacterium, which hydrolyzes a wide range of polysaccharides from lignocellulosic biomass. More than thirty years ago, the *C. thermocellum* cellulosome was discovered. It was the first cellulosome discovered in a microorganism and is now one of the most widely studied nanomachines (Bayer et al., 1983; Lamed et al., 1983a; Lamed et al., 1983b; see Gilbert, 2007; see Bayer et al., 2008; see Fontes and Gilbert, 2010; see Kothari et al., 2011; see Akinosho et al., 2014). Crystalline cellulose is most efficiently degraded by this cellulosome (see Lynd et al., 2002). The extracellular enzyme complex has been shown to be > 2 MDa (Coughlan et al., 1985). In some strains, the cellulosomes aggregate with larger supercomplexes called polycellulosomes. Polycellulosomes have a molecular mass up to 100 MDa (Mayer et al., 1987). The complex composition varies with the carbon source (Bhat et al., 1993). Although *C. thermocellum* exclusively breaks down cellulose, the literature indicates that it has the potential to degrade a number of other polysaccharides (Spinnler et al., 1986; Zverlov et al., 2002b; Zverlov et al., 2005b).

- Structure

Several cellulosome-producing microbes express more than one type of scaffoldin. The primary scaffoldin of *C. thermocellum* is known as CipA (Lamed et al., 1983a; Gerngross et al., 1993; Kruus et al., 1995). CipA contains, nine type I cohesins that recognize type I dockerins in catalytic subunits, and a CBMIIIa, which binds crystalline cellulose and exhibits broad binding specificity for different sites on cell wall (Tokatlidis et al., 1991; Gerngross et al., 1993; Tormo et al., 1996; Blake et al., 2006; Yaniv et al., 2013). In addition, it contains a C-terminal type II dockerin, which is linked to a hydrophilic X-domain and does not recognize CipA cohesins. Instead, it recognizes type II cohesins located at the amino-terminal ends of cell-surface proteins, also known as anchoring proteins (SdbA, Orf2P, and OlpB), leading to the anchorage of cellulosomes or free enzymes to the cell (Lemaire et al., 1995; Leibovitz and Béguin, 1996; Leibovitz et al., 1997; Adams et al., 2006; Xu and Smith, 2010) (*see* figure 18).

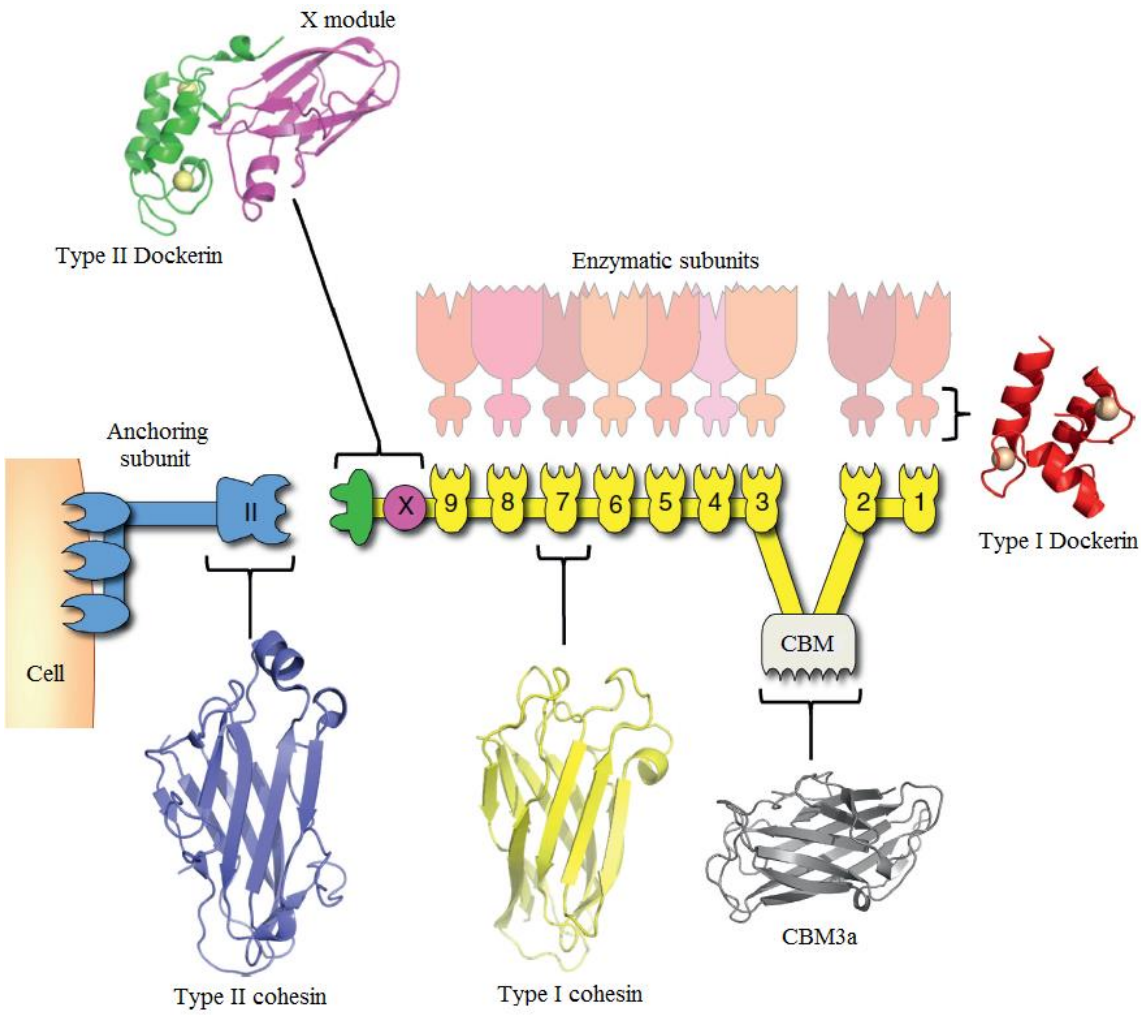

Figure 18. The schematic representation of the cellulosome from *Clostridium thermocellum* with the X-ray crystal structures of the individual cellulosomal components. *Figure adopted from* (Smith and Bayer, 2013).

As it was mentioned earlier, cohesin-dockerin interactions appear to be species-specific (Pagès et al., 1997). However, there are exceptions, and thus, the Xyn11A dockerin of *C. thermocellum* binds to various cohesins of *C. josui* with high affinities (KD of 10^{-8} M) (Jindou et al., 2004). Cohesin I domains are connected to the scaffoldin by O-glycosylated linker segments of about 20 residues containing a majority of proline, serine, and threonine (Gerwig et al., 1989; Gerwig et al., 1991; Gerwig et al., 1992; Gerwig et al., 1993). Inter-cohesin linkers appear intrinsically disordered and display a high degree of conformational flexibility (Hammel et al., 2004; Hammel et al., 2005; Bomble et al., 2011; Garcia-Alvarez et al., 2011; Currie et al., 2012; Currie et al., 2013). The interaction of *C. thermocellum* type-I cohesion-dockerin complex structure is achieved via a binding interface with an extensive hydrogen-bonding network and supporting hydrophobic interactions. These interactions primarily involve only one of the two main helices present in the

symmetric dockerin structure and a face of the cohesin module formed by strands 8, 3, 6, and 5 (Carvalho et al., 2003). Structural and mutagenesis data reveal that type I dockerins contain two almost identical cohesin binding interfaces. Residues participating in cohesin recognition at the two binding interfaces (specifically a serine-threonine pair at positions 11 and 12 and a lysine-arginine pair at positions 18 and 19) are highly conserved in the two segments of the majority of *C. thermocellum* dockerins. This suggests that both binding interfaces display similar protein specificities (Carvalho et al., 2003; Karpol et al., 2008; Garcia-Alvarez et al., 2011). The plasticity of the type-I cohesion-dockerin interaction may reduce steric constraints, which allows the enzymes to assume an alternative conformation for substrate degradation (Carvalho et al., 2007). It also facilitates dockerin alternation through recognition of unbound cohesins, which leads to a continuous reorganization of the cellulosome. This provides the structural flexibility necessary to enhance substrate targeting and improves the synergistic interactions between additional enzymes, including exo- and endo-acting cellulases (Carvalho et al., 2003; Carvalho et al., 2007). Most significantly, type I and type II cohesin/dockerin partners do not interact. This ensures that there is a clear distinction between the mechanism for cellulosome assembly and cell-surface attachment (Leibovitz and Béguin, 1996). The CBMIIIa from CipA corresponds to family 3 of CBM. The crystal structure of CBM3 from *C. thermocellum* displays a nine-stranded β-sandwich fold and one β-sheet presenting a planar topology, which interacts with crystalline cellulose. It is an internal domain that consists of approximately 155 residues (Tormo et al., 1996; Yaniv et al., 2013). In comparison to other type A CBMs, CBM3s bind more extensively to cellulose (Blake et al., 2006). The absorption of the CBM to cellulose depends on the structure arrangement of the cellulosic substrate. The CBM from *Clostridium thermocellum* possesses higher binding capacity for amorphous cellulose than for crystalline cellulose. The capacity for amorphous cellulose is 20 fold higher (Morag et al., 1995). The X module is involved in the dockerin stability and cohesion recognition (Adams et al., 2006). The CipA dockerin II module binds only to the type II cohesins of one cell wall binding protein or anchoring scaffolding SdbA, Orf2P, or OlpB (which carry 1, 2 and 4 type II cohesin(s), respectively) in a highly specific and ultra-tight manner (Leibovitz et al., 1997; Adams et al., 2006). In addition, two type-II cohesin-containing CipA anchoring proteins (Cthe_0735 and Cthe_0736) have been found. The latter comprises 7 type-II cohesin modules, which increases the potential to form polycellulosome structures (Raman et al., 2009; Raman et al., 2011). Two type-I cohesin-containing cell-surface anchoring proteins (OlpA and OlpC) have also been identified, which bind individual cellulosome components (Salamitou et al., 1994; Pinheiro et al., 2009) (see figure19).

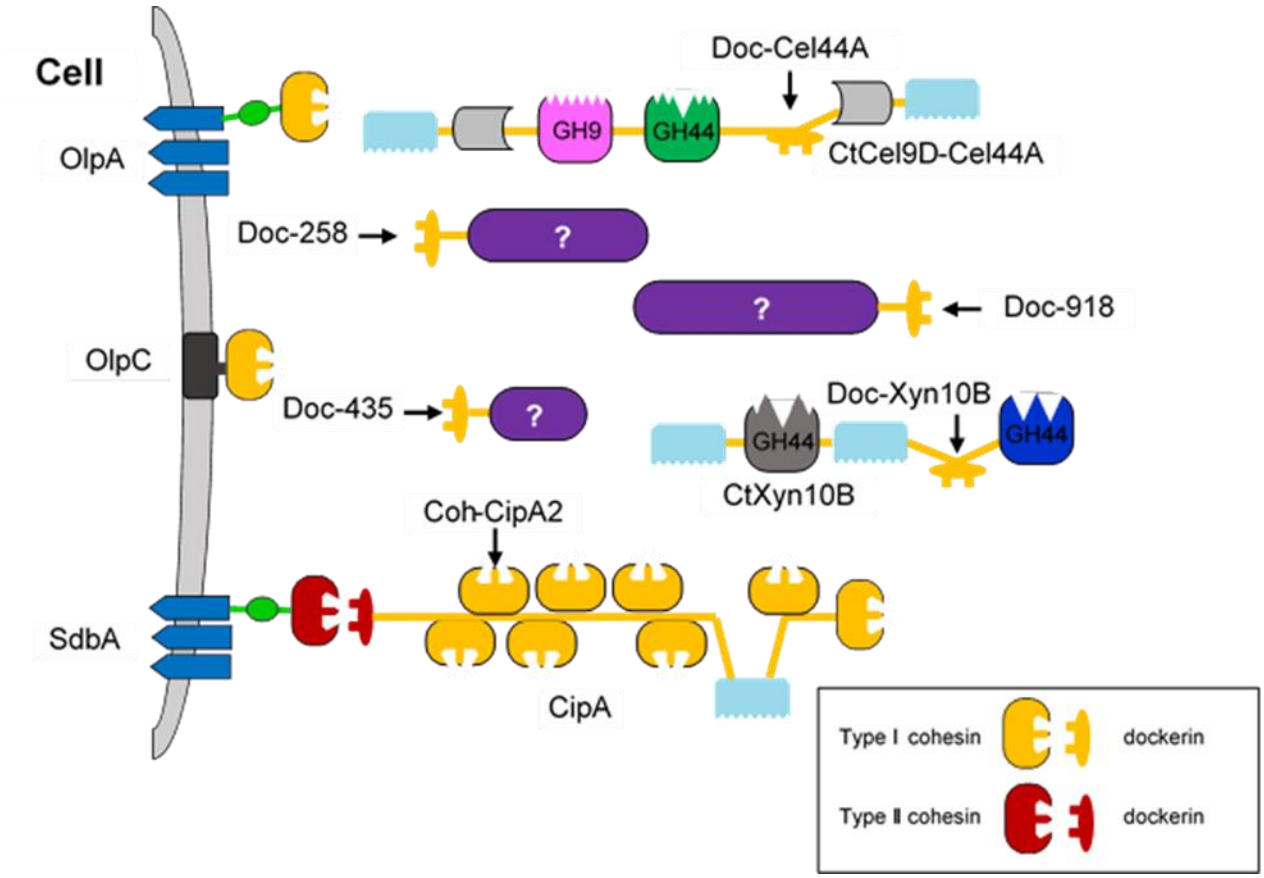

Figure 19. Simplified representation of *C. thermocellum cellulosome* with cell-anchored proteins, OlpA and OlpC and SdbA. *Figure adopted from* (Pinheiro et al., 2009).

While SdbA, OlpB, and Orf2 in *C. thermocellum* are bound to the peptidoglycan layer of the cell envelope, OlpA and OlpC interact with secondary cell wall polymers in the S-layer of the cell envelope (Lemaire et al., 1995; Zhao et al., 2006a; Zhao et al., 2006b; Pinheiro et al., 2009). However, the biological significance of these identified differences in cell-wall specificity remains unclear.

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- Catalytic Subunits

Several cellulosomal enzymes have been revealed in *Clostridium thermocellum*. More specifically, the complex is comprised of numerous EGs (Shinmyo et al., 1979; Ait et al., 1979a; Garcia-Martinez et al., 1980; Ng and Zeikus, 1981; Petre et al., 1981; Beguin et al., 1983; Cornet et al., 1983; Béguin et al., 1985; Pétré et al., 1986; Schwarz et al., 1986; Joliff et al., 1986a; Joliff et al., 1986b; Soutschek-Bauer and Staudenbauer, 1987; Schwarz et al., 1988; Mel'nik et al., 1989;

Hazlewood et al., 1990; Fauth et al., 1991; Jung et al., 1992; Romaniec et al., 1992; Kobayashi et al., 1993; Mosolova et al., 1993; Singh and Akimenko, 1994; Bhat et al., 2001; Zverlov et al., 2003; Zverlov et al., 2005a); four EXs or CBHs, which include CbhA (formerly Cbh3; component S3) (Tuka et al., 1990; Mel'nik et al., 1991; Singh and Akimenko, 1993; Zverlov et al., 1998) CelS (S8) (Wang et al., 1993; Wang and Wu, 1993), CelK (S5) (Kataeva et al., 1999), and CelO (Zverlov et al., 2002a); a CBP (Sih and McBee, 1955; Alexander, 1968); a CDP (Sheth and Alexander, 1967; Sheth and Alexander, 1969); two βGs (bglA and bglB) (Aït et al., 1979b; Gräbnitz et al., 1989; Gräbnitz et al., 1991); at least six ENs (XynA, XynB, XynC, XynY, XynZ and XynD) (Grépinet et al., 1988; Morag et al., 1990; Fontes et al., 1995; Hayashi et al., 1997; Hayashi et al., 1999; Zverlov et al., 2005b), which comprise xylan esterase modules in XynY and XynZ to remove feruloyl residues from native xylan (Blum et al., 2000); one XGH (XghA) (Zverlov et al., 2005b); two lichenases (1,3-1,4-β-glucanases) (Schwarz et al., 1985; Schimming et al., 1991); two laminarinases (1,3-β-glucanases) (Tuka et al., 1990); and minor activities of βX, LAC, and MND (Kohring et al., 1990). *C. thermocellum* has been shown to degrade pectin and probably produces pectin lyase, polygalacturonate hydrolase, pectin methylesterase (Spinnler et al., 1986), one chitinase (Chi18A) (Zverlov et al., 2002b), and one MAN (Halstead et al., 1999).

- Lignocellulolytic System of *Tricorderma reesei*

Most aerobic microorganisms, including fungi (e.g. *Trichoderma reesei*), the bacterium *Thermobifida fusca*, and other aerobic bacteria do not possess a cellulosome for the degradation of lignocellulolytic material. Instead, they produce single enzyme components at high concentrations, which are connected to synergistic binding modules (Wilson, 2008). These enzymes also play an important role in degrading the polysaccharide component of biomass, and are mostly from fungi that belong to the genus *Trichoderma* (Gosh and Gosh, 1992) in particular, *Trichoderma reesei.* *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is a filamentous mesophilic soft-rot ascomycete fungus that is widely used in industry as a source for cellulases and hemicellulases in the hydrolysis of plant cell wall polysaccharides (Kuhls et al., 1996; Merino and Cherry, 2007). The exceptional capacity for extracellular protein production and the ability to grow on a wide range of substrates make filamentous fungi the source of choice for industrial enzymes (Schaffner and Toledo, 1991). *T. reesei* is particularly efficient at producing extracellular enzymes. Certain industrial strains produce over 100 grams of extracellular proteins per liter (see Schuster and Schmoll, 2010). *T. reesei* serves as the primary industrial source for cellulases and hemicellulases (Merino and Cherry, 2007), which are useful in pulp and paper industries (Buchert et al., 1998), textile industries (Galante et al., 1998), food and feed industries (Hjortkjaer et al., 1986; Roldán et al., 2009), and biofuel production (Kataria and Ghosh, 2011). The production of the primary *T. reesei* enzymes that are related to lignocellulosic biomass degradation is transcriptionally regulated, and the relative proportions of expressed proteins may vary widely

depending on the growth medium used, carbon source, and cultivation conditions (Allen and Roche, 1989; Foreman et al., 2003; Juhasz et al., 2005; Stricker et al., 2008; Sipos et al., 2010; Maurya et al., 2012; Coffman et al., 2014). For example, the production of ENs and βX by *T. reesei* is induced very specifically by lactose and xylose, respectively (Kristufek et al., 1995; Xiong et al., 2004). The genome sequence of *T. reesei* has helped researchers identify the diversity of hydrolytic enzymes secreted by this fungus (Martinez et al., 2008). Unexpectedly, previous studies have indicated that *T. reesei* produces a relatively smaller number of cellulases, hemicellulases, and pectinases compared to other plant cell wall degrading fungi (Martinez et al., 2008). Enzyme expression by *T. ressei* depends on the carbon sources (Jun et al., 2013b). Protein engineering have been used in these enzymes to improve properties, such as alkali-tolerance, stability or activity (Wang et al., 2005a; Nakazawa et al., 2009).

- Catalytic Subunits

T. reesei has the smallest repertoire of genes for cellulases, hemicellulases, and pectinases (Martinez et al., 2008). As a result, several enzyme families involved in polysaccharide degradation are reduced or absent in *T. reesei.*

Carbohydrate Active Enzymes (CAZymes) are enzymes that degrade, modify, or create glycosidic bonds, which include GHs (enzymes that hydrolyze or rearrange glycosidic bonds), glycosyltransferases (GTs; enzymes that form glycosidic bonds), POLs (enzymes with a non-hydrolytic cleavage mechanism for glycosidic bonds) and CEs (enzymes that hydrolyse carbohydrate esters). Many of the *T. reesei* genes encoding CAZymes involved in polysaccharide degradation are not distributed randomly in the genome; instead, they are located in clusters. In general, the *T. reesei* genome encodes a number of CAZymes that are slightly below the average found among Sordariomycetes (Martinez et al., 2008). Unexpectedly, the number of genes encoding GHs (201) is below average for the number of GHs found in Sordariomycetes (Martinez et al., 2008; Hakkinen et al., 2012). The *T. reesei* genome also has the smallest number of CBM-containing proteins (36) among the Sordariomycetes (Martinez et al., 2008). In addition, *T. reesei* contains 22 CEs and 5 POL genes (Hakkinen et al., 2012). With respect to the content of GTs (99), however, *T. reesei* is close to average within the same lineage (Martinez et al., 2008; Hakkinen et al., 2012). *T. reesei* lacks several protein families that are important for lignocellulosic degradation. These enzymes are included in PGL, pectin esterase, tannase, and FAE families. Of all possible CAZyme genes involved in pectin degradation, T. reesei possesses the smallest amount of pectinolytic enzymes (family GH28) among the plant cell wall–degrading fungi. The enzyme invertase is also absent (family GH32) (Martinez et al., 2008).

The components of the *T. reesei* cellulolytic system include: two CBHs (CBHI/CEL6A and CBHII/CEL7A) (Shoemaker et al., 1983; Teeri et al., 1983; Teeri et al., 1987); ten EGs, which include five characterized enzymes (EGI/CEL7B, EGII/CEL5A, EGIII/CEL12A, EGIV/CEL61A and EGV/CEL45A) (Penttilä et al., 1986; Saloheimo et al., 1988; Saloheimo et al., 1994; Saloheimo et al., 1997; Okada et al., 1998) and five putative enzymes (CEL5B, CEL61B, CEL74A, gene ID 53731, gene ID 77284) (Foreman et al., 2003; Martinez et al., 2008; The Regents of the University of California, 2015); and eleven βGs, which comprise two characterized enzymes (BGLI/CEL3A, BGLII/CEL1A) (Barnett et al., 1991; Fowler and Brown, 1992; Takashima et al., 1999; Saloheimo et al., 2002) and nine candidate enzymes (CEL3B, CEL3D, CEL1B, CEL3C, CEL3E, bgl3i, gene ID 66832, bgl3j and bgl3f) (Foreman et al., 2003; Ouyang et al., 2006; Martinez et al., 2008). Both CBHs have been shown to act processively. Whereas CEL6A cleaves the cellobiose dimers from the non-reducing end of the cellulose chain, CEL7A acts from the reducing end (Barr et al., 1996). The GH5 cellulase CEL5B has a putative GPI (Glycophosphatidylinositol)-anchor at the C-terminus, which binds this protein to the plasma membrane and fungal cell wall. CEL74A was later characterized as a putative XGH (Grishutin et al., 2004). Enzymes from the GH family 61 have been shown to enhance lignocellulosic degradation by an oxidative mechanism (Langston et al., 2011). In addition, several novel candidate cellulolytic enzymes have been identified from the genome of *T. reesei* (Foreman et al., 2003).

There are several hemicellulases produced by *T. reesei*: seven ENs, that include four characterized ENs from the families GH10 (XYNIII), GH11 (XYNI, XYNII) and GH30 (XYNIV) (Tenkanen et al., 1992; Torronen et al., 1992; Xu et al., 1998), and three candidates enzymes (gene ID 112392 or XYNV, 41248 and 69276) (Metz et al., 2011; The Regents of the University of California, 2015); one MAN (MANI) (Stålbrand et al., 1995); one candidate AXL (gene ID: 69944); one candidate β-1,3-mannanase (or gen ID 71554); six candidate MNDs (gene ID: 5836, 69245, 59689, 57857, 62166 and 71554) (The Regents of the University of California, 2015); four AXEs, which comprise one characterized enzymes (AXEI) and three predicted enzymes (AXEII and gene ID: 70021 and 54219) (Margolles-Clark et al., 1996a; Foreman et al., 2003; Herpoel-Gimbert et al., 2008; The Regents of the University of California, 2015); a candidate cutinase (gene ID60489) (The Regents of the University of California, 2015); a putative XGH (CEL74A) (Grishutin et al., 2004); two AgluAs, one characterized from the family GH67 (GLRI) (Margolles-Clark et al., 1996b) and a candidate from the family GH115 (gene ID 79606) (Hakkinen et al., 2012); five AFs, that include one characterized AF (ABFI) (Margolles-Clark et al., 1996c) and four candidate AFs (ABFII,ABFIII and gene ID 3739 and 68064) (Foreman et al., 2003; Herpoel-Gimbert et al., 2008; The Regents of the University of California, 2015); nine AGLs, that consist of three characterized enzymes (AGLI, AGLII, AGLIII) (Zeilinger et al., 1993; Margolles-Clark et al., 1996d) and six candidate enzymes (gen ID: 27219, 27259, 59391, 75015, 55999 and 65986) (Metz et al., 2011; The Regents of the University of California, 2015); two LACs, which comprise one characterized

enzymes (bga1) and a candidate enzyme (gene ID: 76852) (Seiboth et al., 2005; The Regents of the University of California, 2015); five βXs, that consist of one characterized enzyme (BXLI) (Margolles-Clark et al., 1996c) and four candidates (xyl3b and gen IDs: 73102, 3739 and 68064) (Ouyang et al., 2006; The Regents of the University of California, 2015); two AXEs, one characterized (AESI) (Li et al., 2008b) and one candidate (gene ID: 103825) (Hakkinen et al., 2012); one characterized GE (CIPII) (Foreman et al., 2003; Li et al., 2007b; Pokkuluri et al., 2011); five candidate β-glucuronidases (gene ID: 76852, 71394, 106575, and 73005) (The Regents of the University of California, 2015); two α-glucuronidases, one characterized (GLRI) (Margolles-Clark et al., 1996b) and one candidate enzyme (gen ID: 79606) (Hakkinen et al., 2012); and five candidate AFUs (gene ID: 69944, 72488, 5807, 111138 and 58802) (The Regents of the University of California, 2015).

Engineered Enzymes

Natural lignocellulolytic enzymes have limitations when used in industrial processes. When compared to chemical catalysis, biocatalysis provides enormous advantages, including high efficiency, high degree of selectivity (regio-, chemo-, and enantio-), and ‘‘green’’ reaction conditions (see Hudlicky and Reed, 2009; see Reetz, 2009). Thus, it is not strange that industrial catalysis becomes more and more dependent on enzymes. However, most naturally occurring enzymes are not optimized for industrial applications. Multiple traits need to be satisfied to create the ideal industrial enzyme catalyst (see Burton et al., 2002). The search for superior enzymes has been the interest of many researchers over the past decade. In order to overcome the limitations of naturally occurring enzymes, researchers have developed different methods to obtain biocatalysts with better traits. Over the years these methods have changed. For many years, the identification of better biocatalysts depended only on labor-intensive screening of microbial cultures to achieve the desired activities. The basis for this process was that isolated cultures permit extended and reproducible growth, which in turn allow phenotypic and genotypic characterization (Ferrés et al., 2015). But only a small number of microbes can successfully be cultivated in the laboratory. Thus, this approach will miss the majority of the biodiversity found in nature. Another strategy is the metagenome approach, which allows the sequence of entire genomes from environmental samples by the extraction of genomic DNA, fragmentation, and clonation to yield the corresponding metagenome libraries (see Handelsman et al., 1998; Srivastava et al., 2013). This process allows faster access to catalytic activities from organisms that cannot be cultured, but screening larger libraries of DNA or microbes may not be the fastest or most efficient route to obtain a good catalyst. One of the latest and most promising alternatives to address this problem is protein engineering, which uses molecular biological methods and/or computational techniques to adapt enzyme functions for applied ends. There are two general

approaches for protein engineering: rational design and directed evolution. Although there is a third approach for protein improvement based on statistical analysis, it is used less often.

In the case of lignocellulosic biofuels, the high cost of enzymes is a major obstacle for economic and industrial production of cellulosic biofuels. The high cost is attributable to the large amounts of cellulases that are required to breakdown cellulose into fermentable sugars (Merino and Cherry, 2007; Klein-Marcuschamer et al., 2012). A cost effective enzyme technology used to degrade polysaccharides into fermentable sugars is crucial for economically viable biofuels. In order to address these issues, four primary strategies can be carried out: (1) metagenome screening (see Handelsman et al., 1998; Srivastava et al., 2013); (2) genome mining in sequenced microbial genomes (see Ahmed, 2009; see Davidsen et al., 2010); (3) exploring the diversity of extremophiles (see Schiraldi and De Rosa, 2002; see Kumar et al., 2011b); and (4) developing superior enzymes, such as cellulases with improved characteristics, including higher catalytic efficiencies, increased stability at elevated temperatures, and higher tolerance to end product inhibition, using advanced biotechnology like enzyme engineering, which plays an important role in developing superior enzymes including cellulases. Currently, protein engineering is a well-established technology for modifying the properties of enzymes; general strategies, as well as numerous successful examples, have been published (Peters et al., 2003; seeKazlauskas and Bornscheuer, 2009; see Turner, 2009; see Bornscheuer et al., 2012; see Davids et al., 2013). Two enzymatic engineering strategies have been proposed for lignocellulolytic enzymes: (1) improving properties of individual cellulases; (2) synergy engineering by optimizing the enzyme cocktails for maximized synergy or by the creation of a multi-enzyme cellulolytic complex called cellulosome (Zhou et al., 2009; see Mohanram et al., 2013; Ji et al., 2014; Hu et al., 2015). So far, the most remarkable results in protein engineering of cellulases are improvements in thermostability and thus, diverse thermostable cellulases have been constructed (Heinzelman et al., 2009a; Heinzelman et al., 2009b; Heinzelman et al., 2010; Komor et al., 2012; Smith et al., 2012; Wu and Arnold, 2013; Trudeau et al., 2014). On the other hand, commercial development of hemicellulases for enzymatic hydrolysis of lignocellulosic material is not as advanced as cellulases, since current commercial enzymes mixtures have mostly been developed for the hydrolysis of biomass pretreated with acid, which removes the majority of hemicellulose (Pedersen et al., 2011).

- Engineered Individual Enzymes

Engineering for single enzymes is achieved via rational design, which is primarily based on the enzymes structure knowledge and the catalytic mechanism (Johnsson et al., 1993; Pleiss, 2012); or directed evolution, in which the improved enzymes or ones with new properties, are selected or

screened after random mutagenesis, molecular recombination or focused mutagenesis (see Packer and Liu, 2015) (see figure 20).

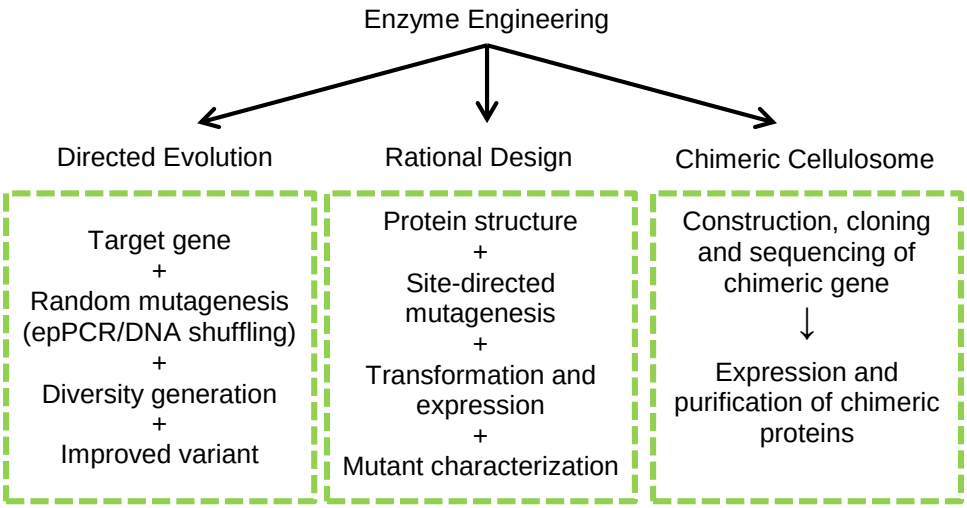

Figure 20. Routes to advancement in cellulase enzyme technology. *Figure adopted and modified from* (Mohanram et al., 2013).

- Rational Design

Ratinal design is the oldest technique in protein engineering introduced after the invention of recombinant DNA methods and site-directed mutagenesis. Rational design uses biochemical data, protein structures and molecular modeling data to propose mutations that would be introduced by site-specific mutagenesis. This approach is dependent on enzyme structural information, function and mechanism. The process of rational design involves: (1) choice of a suitable enzyme, (2) identification of the amino acid sites to be changed, typically based on a high resolution crystallographic structure, and (3) characterization of the mutants via sequencing and purification of the mutant enzymes following each round of mutagenesis (Johnsson et al., 1993; Pleiss, 2012). Assuming that structural information on the target enzyme is available, rational design may be the easiest and fastest approach to enzyme engineering. Computational models (or in silico studies) are typically used to predict which amino acid(s) should be altered in the protein (see Tiwari et al., 2012). Then, targeted mutagenesis is used to change the corresponding gene. Rational design requires the knowledge of the enzymatic structure of interest and/or its sequence in several related species. As a result, crystallography and spectroscopic analysis has been a powerful tool for computer modeling. Advances in modeling, specifically calculations of free energy perturbation and molecular dynamics, help predict mutations. Techniques that help identify mutations include

algorithms (see Desjarlais and Clarke, 1998). Thermostability improvement using rational design via molecular dynamic simulations is applied without reducing enzyme activity. This occurs by taking into account protein-surface properties instead of protein-core characteristics, such as core packing and cavity filling (Joo et al., 2010; Joo et al., 2011). Similarly, the integration of different structural prediction techniques can be applied in the rational design of enzymes. This is also the case for molecular docking, fragment molecular orbital (FMO) calculation, and three-dimensional quantitative structure-activity relationship with comparative molecular field analysis (3D-QSAR CoMFA) modeling (Zhang et al., 2008). Moreover, the creation of structural databases helps researchers understand the functional role of individual amino acids within the enzymatic structure. Among the huge number of data analysis software, two new internet-based computational tools are particularly useful: (1) The HotSpot Wizard server and (2) the commercial 3DM database. The HotSpot Wizard server combines information from extensive sequence and structural database searches with functional data to create a mutability map for a target protein (Pavelka et al., 2009). Likewise, the commercial 3DM database integrates protein sequence and structural data from GenBank and the PDB to create comprehensive alignments of protein super families (Kuipers et al., 2010). A new strategy involves the use of nuclear magnetic resonance relaxation (NMR) dispersion experiments coupled with mutagenesis studies for enzyme catalysis. NMR methods provide a powerful tool to help characterize the effects of controlling long range networks of flexible residues that affect enzymatic function (see Doucet, 2011). Another new approach involves the combination of site direct mutagenesis with immobilization. This approach offers support to improve the activity, stability, and selectivity of the immobilization of biocatalysts (see Mateo et al., 2007; see Hernandez and Fernandez-Lafuente, 2011). However, the application of pure rational design is still limited by some factors, such as the lack of understanding of structural properties and their contributions to function, or the limited knowledge of protein dynamics (Ruscio et al., 2009).

Site-directed mutagenesis on cellulases was first reported using the *T. reesei* exo I gene (Chen et al., 1987). The goal of this experiment was to provide information on the role of individual residues during catalysis. Recently, thermostability of βGs from *Trichoderma reesei* and *Penicillium piceum* H16 have been improved by rational design (Lee et al., 2012; Zong et al., 2015). Also, Arnold and coworkers used a computational approach and site-directed mutagenesis to produce a thermostable fungal CBHI I (Cel7A) and achieved a 10°C increase in optimal active temperature (Komor et al., 2012). Another study reported the shifting of the pH optimum of an EG (PvEGIII) from *Penicillium verruculosum* using a rational design approach (Tishkov et al., 2013). Escovar-Kousan and his team reported an increase of 40% in the activity of the *T. fusca* EG/EX Cel9A on amorphous cellulose or soluble cellulose using the same method (Escovar-Kousen et al., 2004). In addition, Rignall and coworkers changed significantly the mixtures of products released from phosphoric acid swollen cellulose by a single mutation in the active site cleft of the EG-I from *Acidothermus cellulolyticus* (Rignall et al., 2002). On the other hand, to

date only a few cellulases modified by site directed mutagenesis have been reported to possess significantly higher activity on insoluble substrates. One significant example used a modified EG Cel5A from Acidothermus cellulolyticus to report a 20% improvement in its activity against microcrystalline cellulose by decreasing product inhibition (Baker et al., 2005). In some cases, mutant enzymes with higher activity do not increase the activity of a synergistic mixture containing several cellulases (Zhang et al., 2000). Although the research on cellulase mechanisms started by 1950, the mechanism by which cellulases catalyze the hydrolysis of crystalline cellulose is not entirely understood. There is insufficient data regarding the mechanism by which a cellulase binds a segment of a cellulose chain from a microfibril into its active site. Also, there is an incomplete understanding of the way in which certain free CBM stimulate cellulase hydrolysis (Moser et al., 2008; Wang et al., 2008). Finally, although the mechanism for cellulase synergism has been studied and documented (see Woodward, 1991; see Kostylev and Wilson, 2012), there is still much more to understand about this essential process: in particular, how mixtures of cellulases hydrolyze both crystalline and amorphous regions of bacterial cellulose, while most individual enzymes only seem to degrade amorphous regions (Chen et al., 2007). Therefore, rational design is limited, since it requires a detailed understanding of structure–function relationships for cellulase crystalline cellulose activity, which is still lacking. Other enzymes that have also been engineered by rational design are hemicellulases. In this case, rational design has been used in order to shift the pH optimal (Pokhrel et al., 2013; Xu et al., 2013a; Xu et al., 2013b) or improve the thermostability (Fonseca-Maldonado et al., 2013; Satyanarayana, 2013) or the catalytic performance (Huang et al., 2014; Cheng et al., 2015). On the other hand, although some studies can be found regarding the application of rational design on ligninolytic enzymes and pectinases in other to improve their properties, much less research has been published (Xiao et al., 2008; Fang et al., 2014). Rational design has also been used to engineer methabolic pathways (Eriksen et al., 2014).

- Directed Evolution

Directed evolution is another choice for engineering individual enzymes. It has become the most important tool for improving critical traits of biocatalysts, including the improvement of thermal (Koksharov and Ugarova, 2011; Steffler et al., 2013; Zhou et al., 2015), oxidative or activity stability (Stemmer, 1994a; Oh et al., 2002; Akbulut et al., 2013; Wang et al., 2015), enantio-selectivity (Reetz et al., 1997; May et al., 2000; Kim et al., 2015b), pH range (Ness et al., 1999; Wang et al., 2005a; Melzer et al., 2015), substrate specificity (Glieder et al., 2002; Gupta and Farinas, 2010; Ng et al., 2015) and tolerance or stability towards organic solvents (Moore and Arnold, 1996; Reetz et al., 2010a; Yamada et al., 2015). As rational design, directed evolution can also be applied to engineer metabolic pathways and even whole organisms (see Eriksen et al., 2014; see Guenther et al., 2014). Furthermore, directed evolution can be applied to the generation of individual novel enzyme functions (Raillard et al., 2001; Chen and Zhao, 2005). Applying this

approach to the genomes of whole organisms provides the potential to evolve whole-cell biocatalysts within a whole sequence process (Patnaik et al., 2002; Snoek et al., 2015). Directed evolution tends to be more successful than the rational approach (see Gerlt and Babbitt, 2009). With directed evolution, which requires only knowledge of the protein sequence, the amino acid sequence of an enzyme is iteratively altered until the enzyme functions in the desired manner. The process involves iterative cycles of producing mutants and finding the mutant with the desired properties via screening or selection (see Arnold, 1998) (*see* figure 21).

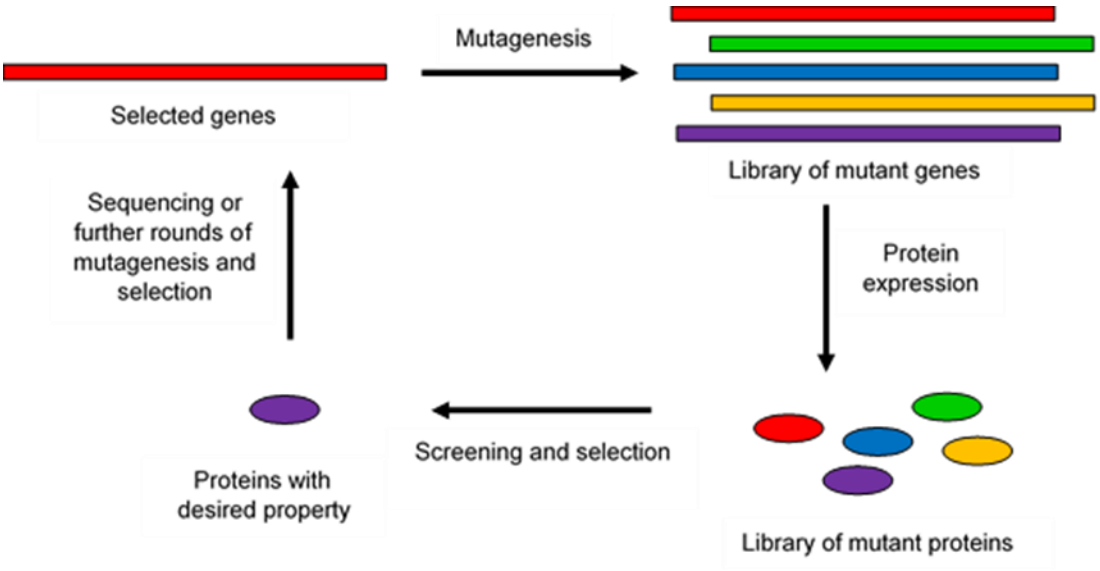

Figure 21. General steps of directed enzyme evolution. The gene encoding the protein of interest is mutated to generate a library of mutant genes. Expression of the mutant genes provides the library of mutant proteins. The proteins are screened or selected based on a desired property, and the variants with modified activity are sequenced or used for further rounds of mutagenesis and selection. *Figure adopted from* (Tao and Cornish, 2002).

The idea of directed evolution for biomolecules in vitro and on a molecular level was first introduced by the pioneering work of Spiegleman et al. (Mills et al., 1967). Regarding nucleic acids, Eigen and Kauffman (Kauffman, 1993) proposed a theory for molecular evolution. Arnold's group was among the first to apply the principles of molecular evolution for the creation of improved enzymatic biocatalysts. They improved the activity of the protease subtilisin E in organic co-solvents (Chen and Arnold, 1993). The greatest advantage of directed evolution compared to rational design is that it is independent of the knowledge of enzyme structure and the interactions between enzyme and substrate. This allows scientists to engineer enzymes whose functions are not yet fully understood (*see* figure 22).

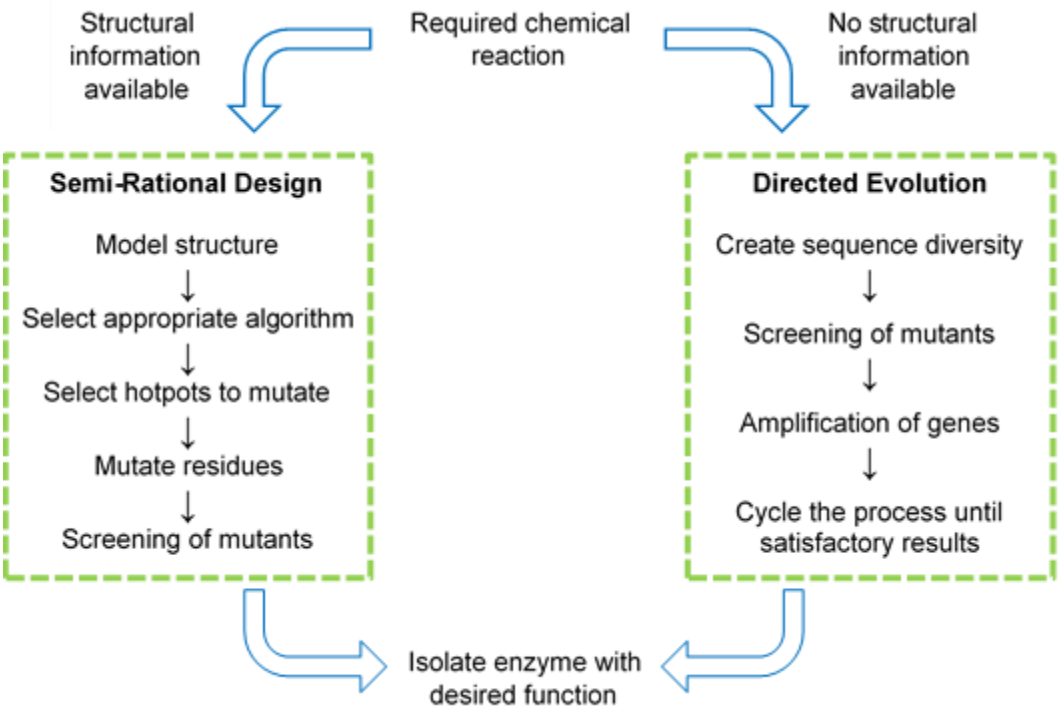

Figure 22. Summary of the different processes required by semirational design and directed evolution. *Figure adopted from* (Quin and Schmidt-Dannert, 2011).

In general, mutations can be introduced throughout the gene via three strategies: (1) random mutagenesis, (2) random recombination or (3) focused mutagenesis (see Packer and Liu, 2015). A wide variety of methods have been developed to create a mutant library. The most commonly employed techniques are: error-prone PCR (epPCR), which was first described by Goeddel and coworkers (Leung et al., 1989), and DNA shuffling , which was first recognized by Pim Stemmer (Stemmer, 1994a; Stemmer, 1994b). The epPCR inserts random point mutations across genes due to the low fidelity the Taq polymerase under certain conditions, such as the increase of magnesium concentrations, supplementation with manganese, or the use of mutagenic dNTP analogues (Zaccolo et al., 1996). The DNA shuffling method is based on mixing and subsequent joining of different, but related small DNA fragments, forming a complete new gene. It is typically achieved by creating hybrid gene libraries via the homologous recombination of related parent genes (Crameri et al., 1998). Non-homologous recombination can also be employed (Sieber et al., 2001). New directed evolutionary methods have been used, including Look-Through Mutagenesis (LTM), which was developed as a method for rapid screening of amino acid mutations in protein sequence selected positions that introduce favorable properties, and Combinatorial Beneficial Mutagenesis (CBM), which is used to identify the best ensemble of individual mutations (Hokanson et al., 2011). The success of a directed evolutionary experiment depends highly on the method that is used to find the best mutant enzyme among a large number of mutants in the library

(You and Arnold, 1996). Identifying interesting variants within large combinatorial libraries (generated by most directed evolution experiments) can be accomplished either by assaying all the members individually (screening) or applying conditions that allow only variants of interest to appear (selection) (see Packer and Liu, 2015). In general, selective methods are preferred over screening methods, because of the higher performance (see Olsen et al., 2000; Griffiths et al., 2004; see Otten and Quax, 2005; Seelig, 2011). The advantage of screening is that the difference between substrate and product of an enzymatic reaction can be determined directly or indirectly in almost every case. The disadvantage of screening is that all individual mutants have to be tested in order for the desired enzymatic reaction to occur. This even includes mutants that might not be active, accurately folded, or when there is a possibility of a high percentage of mutants. Furthermore, the development of a high-throughput screening method is not available for all enzyme stabilities and not all screening methods are easy to implement at the required scale. Screening can be performed via facilitated screening, which distinguishes mutants on the basis of distinct phenotypes, including chromospheres released and halos formed. If facilitated screening is not available, then random screening is utilized, which randomly chooses mutants (see Taylor et al., 2001). The last screening method is often implemented using microtiter plates along with fluorescent substances (Yang et al., 2010; Despotovic et al., 2012; Yu et al., 2014; Zeng et al., 2015). It can also be achieved utilizing chromatography, mass spectrometry, capillary electrophoresis, or IR-thermography (see Wahler and Reymond, 2001). Selection mimics the survival of the fittest strategy. The primary advantage of selection over screening is that many more library members can be analyzed simultaneously, because uninteresting variants are not observed. Consequently, library surveying is much faster and can be carried out with higher output. In the best screening protocols available, the maximum number of library members that can be assayed is approximately 10^8. In contrast, up to 10^{13} clones can be assessed with selection methods (see Packer and Liu, 2015). Selection is based on the advantage that mutants with the desired enzyme function provide to the host cell over bacteria bearing wild type enzymes. Although, many enzymatic activities are not essential to the bacterium, by coupling the activity of interest to an essential feature of the bacterium, this can be changed. Selection approach is often carried out based on the principles of resistance to cytotoxic agents (e.g., antibiotics) (Stemmer, 1994a; Stemmer, 1994b; Siau et al., 2015) or complementation with an auxotroph (Smiley and Benkovic, 1994; Jürgens et al., 2000; Griffiths et al., 2004; De Groeve et al., 2009). Despite the fact that directed evolution is a powerful method to overcome some of the limitations of biocatalysts, it possesses some limitations. Evolutionary analysis of enzymatic families suggest that drastic changes in enzyme function might require significant changes in polypeptide backbones (Matsumura, 2000). However, with in vitro evolution, this cannot be achieved since enzymes are improved only by point mutations, which have a significant bias for transitions over transversions. This limits access to a broader spectrum of substitutions. Another limitation is the considerable time required to implement, because the number of all possible protein variants (including the inactive ones) in a directed evolution experiment is too large. Thus, more recently, the emphasis has been on producing smaller libraries (although also including diversity) and higher

quality libraries. In order to reduce the large number of mutants in the library, one approach is to employ a more efficient screening. Modifications in substrate specificity may be monitored with high-output methods, such as fluorescence-activated cell sorting (Bernath et al., 2004; Becker et al., 2008; Fernandez-Alvaro et al., 2011), which can screen tens of millions of variants in a short amount of time. Directed evolution is also sometimes employed in conjunction with rational design to produce 'smarter libraries' (semi-rational approach) (Ba et al., 2013; Teze et al., 2015; Zhang et al., 2015). The prior knowledge of either the sequence or the three-dimensional crystal structure of the enzyme leads to the design of a more specific set of mutations, which allows creation of a much smaller library with a higher proportion of mutants displaying beneficial traits. This approach takes advantage of rational and random protein design to produce smaller smarter libraries and make the directed evolution faster and more efficient (Reetz et al., 2010b). Another strategy to reduce the number of mutants in libraries involves limiting the location of changes in the active site. The type of changes to those known from sequence comparisons often occur at these sites and mutations closer to these regions seem to be more beneficial (Morley and Kazlauskas, 2005; Jochens and Bornscheuer, 2010; Liebgott et al., 2010). In addition, an important advance that allows multiple mutations is the recognition that mutations often destabilize proteins (Guo et al., 2004; Drummond et al., 2005; see Tokuriki and Tawfik, 2009; Worth et al., 2011). Starting with a very stable protein allows it to tolerate a greater number and range of changes (Bloom et al., 2007; Gupta and Tawfik, 2008). Two more approaches involve the assumption that beneficial mutations are mostly additive (Wells, 1990) and that synergistic effects are rare with the exception of nearby changes. Subsequently, the number of useful changes made during the improvement of a protein has increased considerably in the past decade and the size of the libraries has decreased. The number of mutations has increased from 1–5 mutations in the early 2000s, to 30–40 amino-acid substitutions by 2010 (Fox et al., 2007; Savile et al., 2010).

Some properties of several enzymes involved in lignocellulosic degradation have been changed using directed evolutionary techniques. They are mostly EGLs (Kim et al., 2000; Murashima et al., 2002b; Catcheside et al., 2003; Wang et al., 2005a; Nakazawa et al., 2009; Liu et al., 2010; Liang et al., 2011; Vu and Kim, 2012; Liu et al., 2013a; Lehmann et al., 2014) and βGs (Arrizubieta and Polaina, 2000; González-Blasco et al., 2000; Lebbink et al., 2000; McCarthy et al., 2004; Hardiman et al., 2010; Pei et al., 2011; Drevland et al., 2014) whose activities were principally assayed in a high throughput manner with the help of artificial substrates, either soluble or chromogenic. Only a few examples of directed evolution on EXs existed due to a lack of reliable screening methods (Wang et al., 2012f; Wu and Arnold, 2013). Directed evolution has only achieved moderate success on improving individual cellulases. This is primarily due to the difficulties in developing high throughput screening methods on activities towards the insoluble cellulosic substrates (see Zhang et al., 2006b). Most of the cellulases that have been obtained by directed evolution were screened on artificial substrates and the enzymes have not shown considerable improvement in the hydrolysis of natural substrates (Lin et al., 2009; Nakazawa et

al., 2009; Hardiman et al., 2010). As a result, high throughput screening on natural substrates is needed, although it is difficult to carry out (see Zhang et al., 2006b; Liu et al., 2010). Recently, researchers tried to address these challenges during the development of various automated microplate platforms, which can evaluate the enzymatic hydrolysis of lignocellulose in a high throughput manner (Chundawat et al., 2008; Navarro et al., 2010; Song et al., 2010; Bharadwaj et al., 2011). These automated high throughput systems would greatly facilitate future protein engineering on biomass degrading enzymes or enzyme cocktails. Directed evolution has also been used to change properties of hemicellulases, such as pH optimum (Ruller et al., 2014), thermostability, (Singh et al., 2014; Zheng et al., 2014) or activity (Wang et al., 2013; Du et al., 2014). In addition, directed evolution has been used for the improvement of ligninolytic enzymes and pectinases however, not as much work has been published about it (Solbak et al., 2005; Garcia-Ruiz et al., 2012; Liu et al., 2013b; Viña-Gonzalez et al., 2015; Zhou et al., 2015).

CONCLUSION

The aim of this paper has been to review both seminal and current research and technologies regarding the use and development of enzyme systems for deconstructing lignocellulosic materials into simpler sugars, which can then be fermented to generate bioethanol products. Descriptions of natural enzymes and explanations of the modes and action of engineered systems illustrate the complexity and challenges in efficiently reducing cellulose such that bioethanol may be produced in cost-competitive manner.

The composition and structural organization of cellulolytic enzyme systems from two classes of microorganisms indicate that lignocellulose degradation is efficiently achieved in nature. However, attempts to artificially mimic natural systems in whole or in part have proven difficult. Indeed, the literature demonstrates large variations in efficiency and particularities in the modes of action of different plausible enzyme-based solutions to lignocellulose deconstruction. Nonetheless, advances in the selection of synergistic enzyme complements, the engineering of highly catalytic cellulolytic enzymes, and the development of enzyme sequestration platforms provide promising technological avenues to overcome current challenges.

Future breakthroughs in engineered enzyme systems may soon boost process efficiency so that cost-competitive commercial-scale bioethanol may be produced. Such breakthroughs may include: engineering enzymes based on the improvement of select structural-functional features; synergy engineering for enzyme cocktails; and/or the development of enzyme sequestration

platforms that increase enzymatic efficiency. Enzymes can be engineered by rational design, based on knowledge of the enzyme's structure and catalytic mechanism or by directed evolution. Given that the structure and catalytic mechanisms for many enzymes are not available, only a few studies using rational design have been successful in making significant improvements in overall sugar reduction efficiency. Directed evolution, which possesses the advantage of only requiring the knowledge of the protein sequence, is a powerful emerging technology that may result in novel solutions to the lignocellulose deconstruction problem. However, two limitations must be addressed in directed evolution. First, only point mutations can currently be performed by this method, which limits the magnitude of change that can be induced with respect to enzyme function. If significant change in an enzyme's sequence is required before a high performing enzyme evolves, then the single point mutation approach may be prohibitively time consuming. Furthermore, due to the large number of possible protein sequence variants, a method to narrow down libraries will be required. Perhaps, a hybrid approach that combines both rational design and directed evolution or a "semi-rational" approach will ultimately prove to be the best method for generating high performance cellulolytic enzymes. This can lead to the development of a more specific set of variants. Indeed, recent work on EGs and βGs support this possibility. However, advanced high throughput screening methods for assessing enzymatic activity will need to be developed before semi-rational design is touted as the best option for development of cellulolytic enzymes.

In conclusion, the development of processes for enhancing the production of ethanol from natural feedstocks has substantially progressed over the past few decades; however, further development of enzyme systems and industrial scale testing of technologies is required before an environment-friendly hazardous materials-free solution is achieved.

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