Chapter 4
Enzymatic Hydrolysis Technologies for the Production of Biofuels

Anahita Eckard

Abstract One of the major challenges in second-generation biofuel production is economical conversion of lignocellulosic biomass to fermentable sugars. The most commercially feasible method for conversion of biomass to fermentable sugars has been the combination of thermochemical and enzymatic hydrolysis treatment of biomass. Nevertheless, even with the most efficient pretreatment method, the use of cellulolytic enzymes accounts for more than half of the sugar production cost. As a result of the high cost of commercial hydrolytic enzyme and the low digestibility of pretreated biomass with minimal enzyme use, sugar production costs are not economical for commercial production of some fermentation products. One of the primary reasons for low digestibility of biomass at minimal enzyme doses is the limited enzyme accessibility to cellulose due to the presence of lignin. Other reasons include (1) the change in reactivity of cellulose during hydrolysis that occurs when amphiphilic substance is depleted and (2) deactivation of enzyme by sugars, sugar degradation products, and both soluble and insoluble lignin. With current pretreatment technologies, commercially relevant methods must be developed that would improve the performance of enzymes and make the application of lower enzyme doses feasible. An attractive solution is to generate on-site enzymes to allow for the utilization of cheap abundant protein activity in-house. This chapter reviews the cellulolytic enzyme system, the mechanism of action, rate-limiting factors of enzymatic hydrolysis, on-site enzyme production, and recovery of enzyme activity by enzyme recycling.

4.1 Introduction

Due to inexpensive prices of petroleum and natural gas in twentieth century, the US petrochemical industry and oil refining was thrived. However, gradually with the increase in price and demand of fossil fuel, many of the petroleum-driven chemical production has been moved to countries with lower production cost and the

A. Eckard (✉)
Cobalt Technologies, Mountain View, USA
e-mail: anahita.eckard@cobalttech.com; anahita.eckard@gmail.com

K. Karimi (ed.), *Lignocellulose-Based Bioproducts*,
Biofuel and Biorefinery Technologies 1, DOI 10.1007/978-3-319-14033-9_4

depletion of fossil fuel in near future started to become of a greater concern (Holtzapple et al. 1990). Clearly, cheaper and abundant resources are necessary to support the production of chemicals and fuel. Among the available alternatives, lignocellulosic biomass such as woody materials and agricultural residues is the most abundant and fast regenerated renewable resources with more than 200 million ton of production annually (Zhang et al. 2008) that can be converted to value-added products.

Lignocellulosic biomass is composed of three major components in which cellulose makes the majority followed by hemicellulose and lignin. Cellulose composed of glucose units linked to each other in linear fashion with β-1, 4 glucosidic bonds. Cellulose has amorphous and crystalline structure. Studies have shown that crystalline cellulose is in flat form, twofold helical conformation; however, minor differences in formation of cellulose chain within the crystalline structure result in 7 crystalline polymorphs identified as Iα, I_{β}, II, III_{I}, III_{II}, IV_{I}, and IV_{II} that are found to vary from each other in solubility, melting point, density or crystal shape, etc. (Kadla and Gilbert 2000). While cellulose is highly insoluble in water, conformational studies shown that other than the bottom and top of cellulose chains that are hydrophobic, the side chains are hydrophilic and able of forming hydrogen bonds. Cellulose and starch polymers are similar to each other as regards that they both are composed of glucose units; however, in opposite of cellulose, the glucose units in starch are connected with α-1, 4 bonds.

When it comes to digestibility (breaking the polymer of carbohydrate to sugar monomers) of these two polymers, starch requires 100 times lesser enzyme than cellulose to be converted to glucose. Part of the reason for the low digestibility of cellulose compared to starch was linked to β-bonds in crystalline structure that was more difficult to depolymerize than the α-bonds in amorphous starch. Also, there is a shieling effect imposed by remnant lignin and hemicellulose after pretreatment that limit enzyme accessibility while induce its deactivation and that is why cellulosic biomass requires more severe processing condition such as pretreatment before enzymatic hydrolysis compared to starch feedstocks such as sugarcane or corn. If raw lignocellulosic biomass is exposed to cellulolytic enzymes, the yield of sugar recovery can be found to be minimal. This is because at this point the structures of carbohydrate polymers are not sufficiently accessible for efficient enzyme's catalytic reaction. Therefore, pretreatments, a process during which biomass is exposed to heat and/or chemicals for certain period of time is necessary to enable the enzyme's accessibility to carbohydrate polymers. From a commercial perspective, a desirable pretreatment process should have reasonable capital cost and operating cost, use chemicals that are not highly corrosive and hazardous, and finally would only generate inhibitors in reasonable level that would allow the resulting sugars to be fermented without the need of detoxification. Details of leading pretreatment techniques are presented in Chap. 3. A sample typical process steps in which biomass is converted to value-added product such as alcohols is demonstrated in Fig. 4.1.

Once the cellulose structure has been opened and hemicellulose polymer (mainly C5) was mainly degraded, the solids composed of mainly cellulose and lignin are

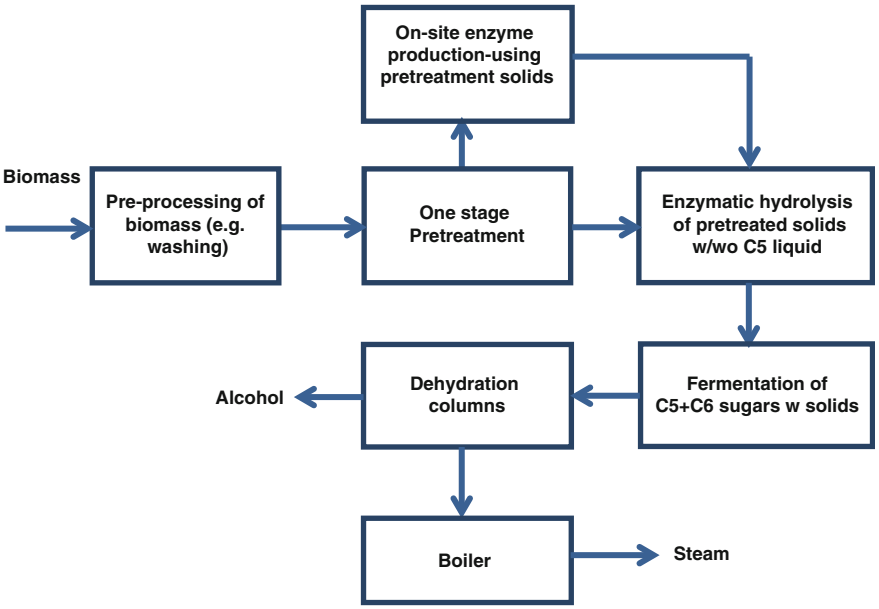

Fig. 4.1 Simplified process flow diagram of lignocellulosic biomass conversion to alcohols

enzymatically hydrolyzed. As a result of the enzyme catalysis on cellobiose or oligomers reacting with water, a single molecule of glucose can be released according to the following reaction. As can be observed, as a result of this reaction, each unit of glucose experiences a mass gain of 11.1 % when released from longer chain, meaning that the mass of glucan with 162 gr converts to 180 gr, when a water molecule is added to glucan:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \tag{4.1}$$

Cellulolytic enzymes act very specific by only catalyzing the addition of water to chain of glucan and as a result in opposite of acid hydrolysis, very minimal by-products are generated. Similar to cellulose, hemicellulose is hydrolyzed by water to individual sugars. Since hemicellulose is composed of both hexoses and pentoses, part of the stoichiometry of hemicellulose (glucan, mannan, galactan) hydrolysis is similar to that of cellulose (Eq. 4.1), and an 11.1 % mass gain is experienced by each molecule of hexose sugars. For xylan and Arabian hydrolysis, a mass gain of 13.6 % is experienced, when molecular weight of the sugar molecule increases from 132 to 150 gr according to the following reaction:

$$(C_5H_8O_4)_n + nH_2O \rightarrow nC_5H_{10}O_5 \tag{4.2}$$

In a typical efficient conversion of biomass, hemicellulose hydrolysis is maximized during pretreatment at temperatures of 100–180 °C, in the absence or

presence of acid as catalyst. By tweaking the process features, a modest inhibitors concentration can be obtained at maximum hemicellulose recovery (~80–90 % conversion); however, at the same time, the outcome of fermentability is very much dependent to temperature profile of pretreatment rather other factors. Then, during enzymatic hydrolysis >95 % of cellulose and <10 % of hemicellulose get exposed to enzymes in the form of polymer or oligomers for conversion to sugar monomers.

After all, the enzymatic hydrolysis has been recognized as the bottleneck of the conversion process since the high price of hydrolytic enzyme has made the sugar production cost from inexpensive and widely available lignocelluloses a challenging task for commercialization. In order to be able to convert the cellulosic fibers to fermentable sugars with high yield that could justify the economics of biomass conversion to alcohols, a large volume of a concentrated enzyme cocktail with cellulase, cellobiose, xylanase, and xylobiose activities is required. At a glance, it may appear that application of up to 5 % (w/w) of concentrated enzyme solution on pretreated biomass is not a large amount; however, cost estimations demonstrate that this amount of enzyme can accounts for more than half of the sugar production cost.

Let's take an example: A typical cellulase loading of 15 FPU/g cellulose would equate to ~30 g of enzyme per liter of ethanol produced. Assuming a liter of ethanol is sold for $0.94; thus, to reduce the cost of enzyme share to <10 % of product cost, it is essential for the enzyme cost to be reduced to less than $2/kg protein, or strategies must be developed to substantially reduce enzyme dosage rates (Himmel et al. 1999; Wingren et al. 2005; Wyman 2007).

One of the strategies for solving the enzyme cost problem is to generate on-site enzyme that uses the existing process streams, and by this way, the cost of enzyme would be reduced significantly. However, the ability to produce a high amount of extracellular enzyme proteins is only the characteristic of certain fungi that have been genetically modified. One of the most studied cellulase generating fungi is *Trichoderma reesei*. From the publically available strain (e.g., RUT C-30), the highest amount of generated extracellular enzyme protein of up to 24 FPU/ml has been reported, while the commercial enzyme cocktails (combination of different activities) contain more than 180 FPU/ml of enzyme activity. For an industrial scale biomass conversion, a high solid loading hydrolysis tank is necessary, otherwise the size of the tank and volume of the water utilized would be larger by several order of magnitude. This setting demands the utilization of an enzyme rich solution that can be produced only from modified commercially owned fungi strains, else increasing the volume of enzyme solution (due to low protein activity) added to biomass could reduce the total solid loading in hydrolysis tank that would consequently increase the capital cost drastically.

In this chapter, cellulolytic enzyme production and enzymatic hydrolysis of pretreated biomass are reviewed.

4.2 Cellulase Classification

Proteins are divided to subclasses based on the nature of reactions they catalyze by International Union of Biochemistry and Molecular Biology (IUBMB). This method uses numbering code system (EC-numbers) to specify their enzymatic activity. In this classification, *T. reesei* cellulases belong to the hydrolases and are found in group EC3 with CBHs (EC 3.2.1.91), EGs (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). However, this system relies on the biochemical description of a protein; therefore, to enable the prediction and classification of new enzymes, a sequence-based categorization was established that assigns sequences to a range of families according to their amino acid similarities. In Cazy database, carbohydrate-active enzymes are categorized into various families such as glycoside hydrolases (GHs) that hydrolyze or re-arrange glycosidic bonds or glycosyltransferase (GTs) that form glycosidic bonds. GHs can be classified as inverting or retaining enzymes. In an inverting enzyme, the result of hydrolysis of β-glucosidic bond is a product with α-configuration, while in retaining enzyme, the product has β-configuration. GHs with retaining mechanism have found commonly to carry out transglycosylation property as well meaning that when they are in high concentrations, they can regenerate oligosaccharides from the hydrolytic reaction products. Cellulases are within several families of GHs, and fascinatingly both CBH and GH are found within the same family.

It is interesting to note that the three-dimensional structure of many members of GHs studied so far shown to share similar overall protein folding and reaction mechanism, e.g., inverting or retaining (Seiboth et al. 1997).

4.3 Cellulase System and Mechanism of Hydrolysis of Cellulose and Hemicellulose

Cellulase system can be partitioned into two categories of secreted or cell-bound cellulases. They are categorized based on their mode of action and structural properties. In opposite of some bacteria, the cellulase multienzymes of fungi are not gathered in large cellulosome complex, but the different fungal enzymes are generated independently and their combined impact on the cellulose causes the decomposition of this polymer.

The fungal cellulolytic system can be divided into three major enzyme classes composed of **endo-glucanases (EGs) (1-4, β-D glycanohydrolases; E.C.3.2.14)** that either cleaves the insoluble cellulose fibrils internally at amorphous sites in random fashion and creates new ends or works on soluble 1,4-β-glucan substrates. The activity of this enzyme was commonly measured by detecting the reducing groups released from carboxymethylcellulose (CMC) or reduction in viscosity (Whitney et al. 1999). Some of the EGs have two domain structures.

Exo-glucanases include (1-4, β-D glycanohydrolases; cellobiohydrolases (CBH); E.C.3.2.1.74) that liberate D-glucose from 1, 4-β-D-glucans while hydrolyze D-cellobiose slowly, and 1, 4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) that generates D-cellobiose from 1, 4-β-glucans. Despite this overall rough model, some CBHs have been found even to cleave the cellulose internally (Seiboth et al. 2011). *T. reesei,* the main cellulase producing fungi has two cellobiohydrolases, CEL6A and CEL7A, which are composed of two separate domains. A catalytic domain and a cellulose-binding domain that are linked via a flexible linker (Seiboth et al. 2011). Cellulose-binding domain belongs to carbohydrate-binding modules (CBMs) and is responsible for making a stable anchor for the attachment of enzyme to cellulose. X-ray crystallography has shown a wedge-shaped structure for CBMs with one face being hydrophilic and another one more hydrophobic. Three tyrosines form a regular flat surface in the CBM of CEL6A that play a role in binding to cellulose (Rouvinen et al. 1990; Divne et al. 1994) (Fig. 4.2).

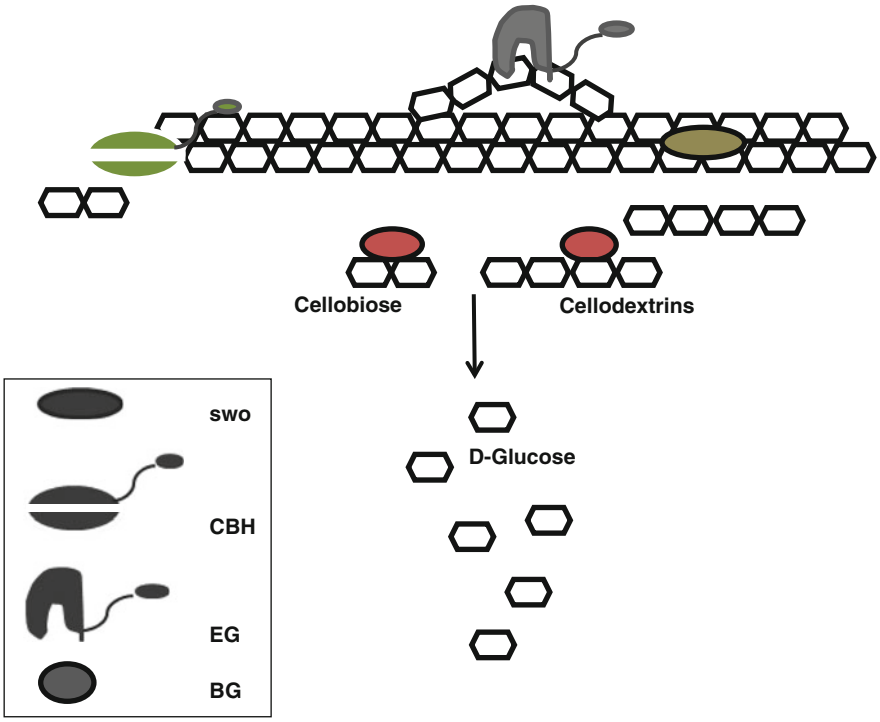

Fig. 4.2 Digestion of cellulose to monomeric sugars is the result of the action of several enzyme mono-components, in which CBHs and some of the EGs are composed of two parts which includes a large catalytic domain and a carbohydrate-binding module (CBM). CBHs, EGs, and BGLs break the cellulose synergistically to glucose. The non-enzymatic protein of swollenin (SWO) was found to aid in the degradation of cellulose by disrupting the crystalline structure and thus improving the accessibility of cellulose for enzymatic proteins (Adopted from Seiboth et al. 2011)

Overall, the CBMs of cellulases are specific for binding to the surface of crystalline cellulose but not on soluble substrates (Bayer et al. 1998). But are CBMs necessary for the catalytic activity of CBHs? It was found that the average velocity (ca. 3.5 nm/s) of CEL7A along the cellulose remained un-changed in CBM-deleted enzyme compared to the intact one, suggesting that the CBMs are not necessary for the movement of CEL7A, and the catalytic domain seems to be enough for the sliding of this enzyme on the substrate (Igarashi et al. 2009).

β-glucosidases (BG) (β-glucoside glucohydrolysase, EC 3.2.1.21) hydrolyzes the soluble cellodextrin and cellobiose to glucose monomers which is the digestible form for majority of fermenting microorganisms.

It is important to note that the majority of hydrolysis process happens simultaneously (Beguin and Aubert 1994; Tomme et al. 1995), and there is a synergism between different GHs family. It was first demonstrated by Gilligan and Reese (1954) that the amount of the reducing sugars generated from cellulose with the filtrate of combined fractions of fungal culture was larger than the sum of the sugar amounts generated by the individual fractions of these enzymes. Also, repeatedly the cross-synergism between endo- and exo-acting from different fungi has been shown (Selby 1969; Coughlan et al. 1987; Baker et al. 1994).

The action of cellulases from glycosyl hydrolase families 6, 7, and 9 is in possessive manner on cellulose, meaning that these enzymes do not become disengaged from single cellodextrin substrate until that cellulose chain is completely hydrolyzed or the enzyme becomes denatured (Wyman et al. 2005). For instance, it was found by small angle X-ray scattering (Receveur et al. 2002) that the movement of cellulase of *T. reesei* CEL7A on cellulose is caterpillar like. This is due to the fact that this enzyme has two domains connected to each other by an amino acid linker peptide (made of 30 amino acid) leading to creation of a maximum extension between the two mass of binding and catalytic modules that cover four glucose units. Despite that the binding of enzyme to cellulose is the first step in hydrolysis and crucial, cellulases that carry a fused cellulose-binding module (CBM) or an attached one by a linker peptide appear to be highly susceptible to loss on cellulose (Linder et al. 1995). This is because the highly small and reactive binding module of the cellulases may result in nonproductive adsorption to cellulose and other sites.

4.4 Impact of Supplementary Enzymes on Hydrolysis of Biomass

Similar to what is explained for the cellulase system, the conversion of biomass is dependent on the complex of few enzyme's mono-components. In addition to endo- and exo-glucanase, the action of beta-glucosidase is necessary for the conversion of cellobiose to monomeric sugars. Furthermore, both xylan and pectin can prevent from cellulase accessibility to cellulose which as a result, supplementation of xylanase to cellulase seems helpful for improvement of the overall digestibility of

biomass. It was found that with xylanase supplementation, the hydrolysis of glucan and xylan was improved by 42.5 and 43.6 % for acid treated hybrid poplar. This improvement was even higher for ammonia recycles percolation (ARP)-treated corn stover for which the digestibility of glucan and xylan was increased by 4.79 and 10.74 %, respectively (Qing and Wyman 2011). In addition to xylanase, pectin causes steric hindrance for the contact of cellulase to cellulose and hemicellulose and therefore reduces the rate of cellulose and hemicellulose hydrolysis. According to Zheng et al. (2009), addition of pectinase improved the hydrolysis of both glucose and xylose by 7.5 and 29.3 %, respectively.

4.5 Impact of Pretreatment on Efficacy of Enzymatic Digestibility of Biomass

There are few key factors that are known to affect the digestibility of biomass during enzymatic hydrolysis. Optimum solubilization of hemicelluloses during pretreatment process results in an increase of accessible pore volume and the specific surface area of cellulose and eliminates the barrier of enzyme accessibility to cellulose (Stone and Scallan 1969; Saddler et al. 1982; Brownell and Saddler 1987; Puls and Schuseil 1993; Mooney et al. 1998). This is in agreement with Chang and Holtzapple (2000) that showed the maximum acetyl removal of biomass can significantly improve the digestibility of biomass regardless of moderate lignin and high crystalline content of the biomass (Chang and Holtzapple 2000). It was found that only a limited swelling can take place when there is close association between lignin and cellulose during enzymatic hydrolysis (Mooney et al. 1998). According to Chang and Holtzapple (2000), lignin and crystallinity index of biomass is the greatest driver for enzymatic digestibility of biomass such that high digestibilities were obtained when only one of these two factors was low (Fig. 4.3). A prediction model developed by analysis of different pretreated samples suggested

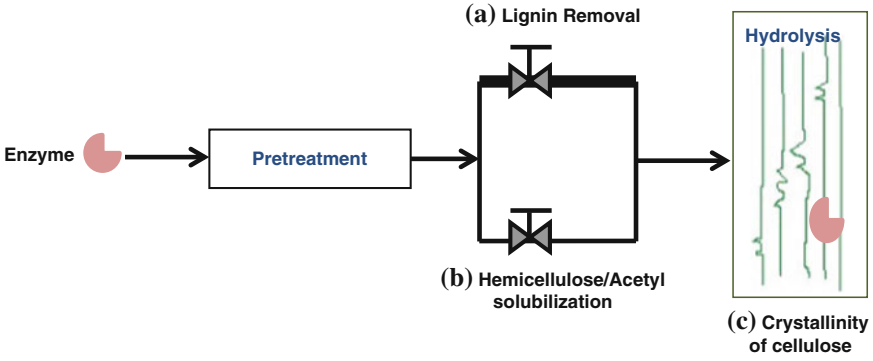

Fig. 4.3 Simplified schematic of key factors impacting the enzymatic digestibility of biomass

that if the crystallinity and lignin content of the biomass is reduced to <30 and 15 %, respectively, the biomass becomes hypothetically nearly 100 % digestible meaning that minimum amount of enzyme (e.g., >5 FPU) can be sufficient for hydrolysis (Chang and Holtzapple 2000). Lignin is well known to decrease the efficacy of enzymatic hydrolysis for numerous reasons. (i) Lignin forms a shield for cellulose against of the chemical, physical, or microbial degradation and limits the significant swelling of the cell wall, thus restricting the accessibility of cellulose to the enzyme (Sutcliffe and Saddler 1986); (ii) Lignin also irreversibly attracts a large fraction of the cellulase, making it unavailable for enzymatic hydrolysis of cellulose (Lu et al. 2002; Uribe and Sanpedro 2003; Eckard et al. 2013b). A sample schematic diagram of key factors affecting digestibility of biomass is shown in Fig. 4.3. In this figure, if assuming the lignin and hemicellulose or acetyl groups as two critical valves on the path of pipes that transfer the enzymes to cellulose, then it is important to open at least the valve on the larger pipe (A-lignin) to allow the enzymes flow to biomass. However, opening the thinner pipe (B-hemicellulose solubilization) also helps in improving the enzyme flow (accessibility) to biomass, especially when lignin removal is minimal. These two factors are highly dependent to severity and type of pretreatment method. A simple example of a scenario with two closed valves is the enzymatic hydrolysis of raw biomass without pretreatment. The third key factor is reduction in biomass crystallinity that was shown to be the second most important factor in biomass digestibility after lignin removal (Chang and Holtzapple 2000). Consideration of these three factors can help to reduce the enzyme dose significantly.

4.6 Rate-Limiting Factors in Enzymatic Hydrolysis Reaction

Despite of a generally known concept that the hydrolysis of beta bonds in a crystalline structure is far more difficult to depolymerize than the alpha bonds in amorphous starch (Wyman et al. 2005), still the hydrolysis of cellulose is rate limited due to many other reasons. It has been clearly shown that the rate of the enzymatic hydrolysis per adsorbed enzyme (specific rate) was decreased significantly as the hydrolysis proceeded (Wyman et al. 2005). In spite of many hypotheses, still the clear-cut reason for the decline in the rate of hydrolysis is not well understood (Ooshima et al. 1991; Kurakake et al. 1995; Mansfield et al. 1999; Zhang et al. 1999).

Declined hydrolysis rate was primarily postulated to be related to the depletion of the amorphous cellulose (the reactive substance), as a result, reduced reactivity of cellulose particles with the progress of cellulosic substrates conversion is one of the key reasons to reduced hydrolysis rate (Nutor et al. 1991; South et al. 1995). It was found that when enzyme concentrations were increased, the specific hydrolysis rate remained unchanged; however, when the substrate concentrations were increased,

the specific hydrolysis rate was further reduced, both observations suggesting that the reduced hydrolysis rate is highly impacted by substrate conversion to a non-reactive form over time in addition to enzyme deactivations (Wyman et al. 2005).

Other studies suggest that the falloff is also related to enzyme inactivation with several other factors that are further explained below. The nature of this deactivation can be the deformation of major enzyme's substructure and/or unavailability of enzyme in solution for catalytic activity due to irreversible adsorption (Castanon and Wilke 1981; Parke et al. 1992). Several factors such as thermal effects, shearing force, high surface tension, and interfacial or air–liquid contacts have been shown to adversely impact the enzyme activity (Kim et al. 1982; Aymard and Belarbi 2000). This irreversible adsorption was assessed in several studies, using direct measurement of adsorbed enzyme on biomass at 4 °C incubation (no hydrolysis) with nitrogen combustion analyzer (Yang and Wyman 2006; Kumar and Wyman 2008). It was found that the irreversible adsorption of enzyme was not only limited to insoluble lignin, but also to pure cellulose (Eckard et al. 2011).

High concentration of several molecules showed inhibitory effects to enzymatic hydrolysis. This include glucose and cellobiose (Holtzapple et al. 1990), monomer sugars of hemicellulose (i.e., xylose, mannose, galactose) (Xiao et al. 2004; Qing et al. 2010), xylo-oligomers (Qing et al. 2010), soluble lignin, and lignin degradation products, polymeric phenol tannic acids, and to less extent the monomeric phenolic compounds (Ximenes et al. 2011).

In aqueous pretreatment liquors, a release of 2–5 g/l of lignin in solution is expected as phenols (Ximenes et al. 2011). It was found that the digestibility of pure cellulose (Avicel) that was hydrolyzed with 15 FPU of cellulose was dropped by 10–23 % by addition of 20 % lignin (w/w). This seemed to vary strongly based on the source of the lignin (Cantarella et al. 2014). According to this result, phenolic compounds (at 10 mM) demonstrated 1–5 % more inhibition than non-phenolic ones due to impact of hydroxyl groups on enzyme activity. This result also suggests that the phenolic's hydroxyl groups may play more severe role than physical barrier and non-specific adsorption in enzymatic hydrolysis of cellulose.

According to the study of Cantarella et al. (2014), the stability of endo-glucanase was impacted by all the phenolics (1–2 g/l) through reduction in half-life of the enzyme and the reaction rate. Out of the phenolic compounds tested, the most inactivating ones were vanillin, hydroxybenzaldehyde, and protocatechuic acid (at 1 and 2 g/l). This inactivation was irreversible meaning that after the removal of phenolics, the inactivation still lasted. Similarly, the exposure to syringaldehyde (at 1 and 2 g/l) reduced the enzyme activity, and after syringaldehyde removal, the activity of enzyme was not recovered. Other phenolics such as p-coumaric acid when increased from 1 to 2 g/l, the enzyme reaction rate was reduced by fourfold, but the inactivation was ceased upon removal of the compound. Similarly, when hydroxybenzoic acid was tested, the inactivation was increased by 5 times upon the increase of concentration from 1 to 2 g/l; however, the enzyme activity was recovered when hydroxybenzoic acid was removed from contact with enzyme (Cantarella et al. 2014).

4.7 Cellulase Generating Microbes

When it comes to the industrial cellulase producing fungus, the commercially used strains were mainly derived from an isolate which was collected on the Solomon Islands during World War II (Reese and Mendels 1984; El-Gogary et al. 1990) and have been mutated to an enhanced cellulase producing form from *T. reesei*, (the anamorph or asexual reproductive stage of the *Hypocrea jecorina*). Cellulolytic enzyme secretion from *T. reesei* includes eight EGs (CEL5A, CEL5B, CEL7B, CEL12A, CEL45A, CEL61A, CEL61B, and CEL74A), two CBHs (CEL6A and CEL7A), and seven β-glucosidases (CEL1A, CEL1B, CEL3A, CEL3B, CEL3C, CEL3D, and CEL3E). The low pectin degrading content of *T. reesei* shows that the enzymes from this microbe are the most suitable ones for degrading the dead plant rather than attacking living intact plants.

Montenecourt and Eveleigh developed two lines of mutants that resulted in RUT C-30 (the hypercellulolytic strains) and RL-P37 (El-Gogary et al. 1990) (Fig. 4.4). Today, *H. jecorina* RUT C-30 is the most frequently used strain for cellulase production in laboratory research (Domingues et al. 2000; Shin et al. 2000; Bailey and Tahtiharju 2003; Collen et al. 2005; Levasseur et al. 2006; Zhang et al. 2007).

It is important to understand that the genetic modifications of *T. reesei* RUT C-30 is a truncation in the cre1 gene, the carbon catabolite repressor, and β-glucosidase II encoding gene (Geysens et al. 2005). As a result, in the presence of sugar monomers, still enzyme secretion can be observed at *T. reesei* RUT C-30, as far as the inducer is present, but the titer of enzyme production is minimal and does not exceed, e.g., 24 FPU/ml of fermentation broth (Seiboth et al. 2011). However, as a result of strain improvement, the capacity of cellulolytic enzyme protein secretion in the industrial strains has reached to 100 g/l (El-Gogary et al. 1990) with up to 60 % of the cellulase been composed of CEL7A (CBHI) and 20 % of CEL6A (CBHII) (Seiboth et al. 2011).

Trichoderma spp. have great skill in dealing with a vast variety of environments such as tropical forest as well as the dark and sterile environment of a biotechnological reactors and flask. When it comes to identification, other than pigmentation,

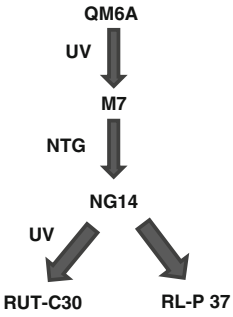

Fig. 4.4 Pedigree of strain *T. ressei* RUT C30 and its relationship to the wild-type isolate QM6A. Classical mutations were introduced by UV light (UV) and nitrosoguanidine (NTG) (Adopted from Seidl et al. 2008)

species identification within the genus is difficult because of the narrow range of variation of the simplified morphology in *Trichoderma* (Gams and Bissett 1998). *T. reesei* generates a wide range of pigments from bright greenish-yellow to reddish, with some being also colorless. Similarly, conidial pigmentation varies from colorless to various green shades and sometimes gray or brown.

4.8 Mechanism of Enzyme Production by *T. reesei*

The secretion of cellulolytic enzymes from *T. reesei* is subjected to multiple levels of control in which most of the regulation occur at the level of transcription. The role of the cellulolytic enzymes are to break the cellulose; therefore, for expression of the enzyme producing genes in fungal cells, cellulose and/or soluble oligomers (as carbon source and for energy production) need to be provided to fungal cells.

Understanding the correct molecular basis of cellulolytic enzyme productions and how *T. reesei* sense cellulose initiates enzyme secretion is crucial for a successful fermentation process. As it was described earlier, metabolite adjustments are more important in enzyme generation than the gene manipulation. Fungal cells do not receive signal from the presence of cellulose itself, they need a soluble inducer that can be up taken inside the fungal cell and that creates signal for secretion of enzyme. In the absence of no soluble inducer (only available cellulose), a basal enzyme is produced which attacks to the insoluble cellulose that generates inducer (dimmers of sugars) or inducer precursors (sugar oligomers) that can be taken up to generate enzyme (Carle-Urioste et al. 1997).

Other than the above mechanism, ready inducers can be provided to fungi from the beginning and throughout the fermentation. Among the inducers, Sophorose is one of the most efficient cellulase-inducing sugars and is produced via transglycosylation of cellulose by initial cellulase enzyme secreted from *T. reesei* (Gritzali and Brown 1979; Vaheri et al. 1979). Other than the cellulose-derived inducer, lactose (dimer) and L-sorbose (sugar monomer) are the other inducers and by far lactose has been recognized as a more soluble and cheaper source of inducer compared to other disaccharide inducers such as cellobiose and sophorose (Kubicek et al. 2009; Seiboth et al. 2007).

In *T. reesei,* expression of a majority of cellulases does not occur during the growth on glucose; however, as is known, increase of microbial population also requires providing mono sugars to fungi. This causes a lag between the times required for generation of microbial population and the time spent on enzyme generation. As a result, one of the first efforts for improvement of cellulase generation in *T. reesei* was to eliminate the effect of carbon catabolite repression. From this effort, a publically available strain of RUT C-30 with strong cellulose generating capability was produced that has truncation in CREA-1 gene and is carbon catabolite depressed (Ilmen et al. 1996; Seidl et al. 2008). However, despite of the effort, still the utilization of glucose or other carbon sources showed to result in low

cellulase yield; this was also proved by deleting CREA-1 gene from wild type. These results showed that even the carbon catabolite depressed strains are still inducer dependent and carbon catabolite sensitive (Nakari-Setälä et al. 2009).

4.9 Strategies for Improvement of Enzyme Activity

Improvement of enzyme activity for commercialization of biomass to sugars would require either maintaining the activity of enzyme for a longer period of time or reusing the enzyme by recycling. As it was mentioned in previous sections, a natural drop in hydrolysis rate is expected due to change in cellulose reactivity; however, fraction of the enzyme activity that has been lost to different soluble and insoluble inhibitory compounds can be recovered when the inhibitory effect is eliminated (Cantarella et al. 2014).

Several methods have been proposed for the reduction of enzyme utilization that includes enzyme immobilization (Yang et al. 2009; Pavani and Basil 2010), Enzyme recycling (Steele et al. 2005; Qi et al. 2011), washing off the biomass with sodium chloride solution to release the adsorbed enzyme (Yang and Wyman 2006), and application of surfactants, lipids, or metal ions prior to application of enzymes to prevent from their irreversible adsorption (Yang and Wyman 2006; Zhang et al. 2008; Borjesson et al. 2007).

4.10 Enzyme Recycling, an Approach for Reduction of Process Cost

After an effective pretreatment, lignocellulosic biomass can be fairly digestible to fermentable sugars; however, the enzymatic hydrolysis process is still slow and the enzyme requirement remains high (up to 15–30 FPU/g glucan) from a commercially desirable standpoint (Ferreira et al. 2000; Hambrid et al. 2011; Eckard et al. 2013b).

During hydrolysis, enzymes are partitioned between solid and liquid phase. Several studies in the past evaluated the enzyme recycling with recycles of solid residues as well as the liquor from hydrolysis (Lee et al. 1995; Tu et al. 2009, 2007; Eckard et al. 2013a). Most of these studies were conducted using low total solid of 2–5 % and supplementation of beta-glucosidase in new hydrolysis cycle (Lee et al. 1995; Tu et al. 2007, 2009). The results specifically shown that recycling is much less effective with pretreatment methods that do not remove lignin and is more efficient when pretreated materials contained less lignin (Tu et al. 2007, 2009). This is because lignin unproductively adsorbs a large fraction of the cellulase, making it unavailable for enzymatic hydrolysis of cellulose (Uribe and Sampedro 2003; Taherzadeh and Karimi 2009).

For recycling of free enzyme from liquid phase, a re-adsorption technique was first proposed by Sinistyn et al. (1986) as an alternative to costly ultrafiltration techniques. In this method, the slurry of hydrolysate (liquid after enzymatic hydrolysis of biomass) or fermentation broth is centrifuged or filtered, and the supernatant containing soluble enzymes is incubated with fresh biomass for 2 h to allow the adsorption of free enzymes from cycle 1 onto fresh biomass. After a second separation step, the insoluble solids of biomass are then re-solubilized in fresh buffer and additional β-glucosidase is added for another cycle (2nd) of hydrolysis or fermentation (Tu et al. 2007, 2009). It has been suggested that the re-adsorption technique with two separation steps prevents the accumulation of lignin degradation by-products and sugars to toxic levels (Tu et al. 2007; Palmqvist et al. 1996; Xue et al. 2012). However, it should be noted that an additional separation is costly and due to the advantage of the SSF process that assimilates the inhibitors and sugars generated, the additional re-adsorption step might not be necessary (Eckard et al. 2013a).

As it was mentioned earlier, non-specific adsorption of enzymes to lignin and maybe crystalline cellulose limits the efficacy of enzyme recycling greatly. One of the strategies for improvement of this problem is the use of amphiphiles prior or during the enzymatic hydrolysis that prevent irreversible adsorption of enzyme on substrate and free enzymes can be then recycled for hydrolysis of fresh substrate (Tu et al. 2007; Eckard et al. 2013a). The ability of amphiphiles to adhere to nonproductive sites of lignocelluloses such as lignin and prevent irreversible enzyme adsorption has been clearly demonstrated before. According to Errikson et al. (2002), the adsorption of cellobiohydrolase decreased by 60–70 % onto steam-exploded lodgepole pine (SELP) using Tween 20. Likewise, desorption of cellulases (cellobiohydrolase and endo-glucanase) into the liquid phase was improved from 46 to 73 % during the hydrolysis of SELP when Tween 80 was used as an enzyme stabilizer (Tu et al. 2007). This property of amphiphiles can be exploited for recycling of cellulase if the revenue made from the additional amount of sugar and ethanol is higher than the cost paid for the amphiphiles. Moreover, when surfactant was applied to ethanol pretreated-lodgepole pine (EPLP), it increased the free enzyme levels of cellulase from 71 % of the original amount to 96 %. Similarly, in another study, it was found that the efficiency of enzyme recycling (using re-adsorption technique) was significantly higher in lower lignin content substrates, compared to feedstock's such as SELP that contained higher lignin levels (Tu et al. 2007). Differences in enzyme recycling also may be related to the source of enzymes. For instance, under similar experimental conditions (similar substrate and surfactant), enzymes from *T. reesei* were successfully recycled for 4 cycles, while penicillium-derived enzyme was only recycled once successfully (Tu et al. 2007).

According to Eckard et al. (2013a), after two recycling of fermentation liquor containing enzymes, the ethanol yield was improved by 80 and 130 % with the aid of Tween 20 and liquid casein micelles, respectively. polymeric micelles (PMs) of PEG–Tween and PEG–casein improved enzyme recycling further, such that the ethanol yield was improved by 50 and 108 % beyond that obtained with only

Tween and casein, respectively. Amphiphiles of acid casein were also found to improve the sugar recovery and fermentability of dilute acid, lime, alkali, and extrusion pretreated corn stover by up to 31 and 33 %, respectively. Neither of Tween 20, nor the accumulated sugars showed toxicity to microbial or enzyme activity (Eckard et al. 2012).

Several mechanism of action have been suggested to describe how surfactants enhance the cellulose activity and enzymatic hydrolysis of pure cellulose or lignocellulosic biomass: (1) Surfactants can extract hydrophobic degradation products from lignin and hemicellulose by forming emulsions, thereby enhancing the removal of lignin and increasing the access of feedstock's reaction sites to the cellulolytic enzyme (Kaar et al. 1998; Tu et al. 2007; Seo et al. 2011); (2) Surfactants lessen irreversible, nonproductive adsorption of cellulase to nonproductive sites of biomass (e.g., crystalline cellulose and lignin), which allows the enzyme to be available in solution and have higher activity (Castanon et al. 1981; Errikson et al. 2002; Parke et al. 1992); (3) Improved electrostatic interaction between surfactant monomer or micelles and enzyme causes an enhanced enzyme activity by activating a certain amino acid in the enzyme or reforming enzyme secondary structure, specially the α-helixes (Eckard et al. 2013a, 2014); (4) Surfactants protect enzyme from thermal deactivation after extended incubation period (Kim et al. 1982) and denaturation by reducing the surface tension and viscosity of liquid that in turn diminishes the contact of enzyme with air–liquid interface (Kim et al. 1982). Overall, in a solution of surfactants, enzymatic reactions occur either inside the surfactant micelle core or at the interface of the micelles or monomers and the pseudo-phase of the liquid, depending on the enzyme hydrophobicity (Biasutti et al. 2008). In spite of the above hypotheses, a mechanism that can consistently explain how surfactants improve enzymatic hydrolysis has yet to be developed.

References

Bailey MJ, Tahtiharju J (2003) Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. Appl Microbiol Biotechnol 62(8):156–162

Baker JO, Adney WS, Nieves RA, Thomas SR, Wilson DB, Himmel ME (1994) A new thermostable endoglucanase, Acidothermus cellulolyticus E1—synergism with *Trichoderma reesei* CBH-I and comparison to *Thermomonospora fusca*. Appl Biochem Biotechnol 245 (4):45–46

Bayer EA, Chanzy H, Lamed R, Shoham Y (1998) Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 8(5):548–557 (ISSN 0959-440X)

Beguin P, Aubert JP (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13 (1):25–58 (ISSN 0168-6445)

Belarbi C (2000) A Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism. Enzyme Microb Technol 27:612–618

Biasutti MA, Abuin EB, Silber JJ, Correa NM, Lissi EA (2008) Kinetics of reactions catalyzed by enzymes in solutions of surfactants. Adv Colloid Interface Sci 136:1–24

Borjesson J, Engqvist M, Slipos B, Tjerneld F (2007) Effect of polyethylene glycol on enzymatic hydrolysis and adsorption of cellulose enzymes to pretreated lignocellulose. Enzyme Microb Technol 41:186–195

Brownell HH, Saddler JN (1987) Steam pretreatment of lignocellulosic material for enhanced enzymatic-hydrolysis. Biotechnol Bioeng 29:228

Carle-Urioste JC, Escobar-Vera J, El-Gogary S, Henrique-Silva F, Torigoi E, Crivellaro O, Herrera-Estrella A, El-Dorry H (1997) Cellulase induction in *Trichoderma reesei* by cellulose requires its own basal expression. J Biol Chem 272(15):10169–10174 (ISSN 0021-9258)

Castanon M, Wilke CR (1981) Effect of the surfactant tween 80 on enzymatic hydrolysis of newspaper. Biotechnol Bioeng 23:1365–1372

Cantarella M, Mucciante C, Cantarella L (2014) Inactivating effects of lignin-derived compounds released during lignocellulosic biomass pretreatment on the endo-glucanase catalyzed hydrolysis of carboxymethylcellulose: a study in continuous stirred ultrafiltration-membrane reactor. Bioresour Technol 156:48–56

Chang CS, Holtzapple MT (2000) Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotechnol 84–86(1–9):5–37

Collen A, Saloheimo M, Bailey M, Penttilä M, Pakula TM (2005) Protein production and induction of the unfolded protein response in *Trichoderma reesei* strain Rut-C30 and its transformant expressing endoglucanase I with a hydrophobic tag. Biotechnol Bioeng 89 (3):335–344

Coughlan MP, Moloney AP, McCrae SI, Wood TM (1987) Cross-synergistic interactions between components of the cellulase systems of *Talaromyces emersonii*, *Fusarium solani, Penicillium funiculosum*, and *Trichoderma koningii*. Biochem Soc Trans 15:263–268

Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles JK, Teeri TT, Jones TA (1994) The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science 265(5171):524–528 (ISSN 0036-8075)

Domingues FC, Queiroz JA, Cabral JM, Fonseca LP (2000) The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme Microb Technol 26:394–401

Eckard AD, Muthukumarappan K, Gibbons W (2011) Pretreatment of extruded corn stover with polyethylene glycol to enhance enzymatic hydrolysis: optimization, kinetics, and mechanism of action. Bioenergy Res 5:424–438

Eckard AD, Muthukumarappan K, Gibbons W (2012) Enhanced bioethanol production from pretreated corn stover via multi-positive effect of casein micelles. Bioresour Technol 135:93–102

Eckard AD, Muthukumarappan K, Gibbons W (2013a) Enzyme recycling in a simultaneous and separate saccharification and fermentation of corn stover: comparing polymeric micelles of surfactants and polypeptides. Bioresour Technol 132:202–209

Eckard AD, Muthukumarappan K, Gibbons W (2013b) A review of the role of amphiphiles in biomass to ethanol conversion. Appl. Sci 3:396–419

Eckard AD, Muthukumarappan K, Gibbons W (2014) Evaluation of the role of polymerized micelles on yield of hydrolysis, chemical changes of biomass and cellulase structure and adsorption. Bioenergy Res 7(1):389–407

El-Gogary S, Leite A, Crivellaro O, El-Dorry H, Eveleigh DE (1990) *Trichoderma reesei* cellulase —from mutants to induction. In: Kubicek CP, Eveleigh DE, Esterbauer H, Steiner W, Kubicek-Pranz EM (eds) *Trichoderma reesei* cellulases. Royal Society of Chemistry, Cambridge, pp 200–211

Erriksson T, Karlsson J, Tjerland F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb Technol 3:353–364

Ferreira SMP, Duarte AP, Queiroz JA, Domingues FC (2000) Influence of buffer systems on *Trichoderma reesei* Rut C-30 morphology and cellulase production. Electron J Biotechnol 12:1–9

Geysens S, Pakula T, Uusitalo J, Dewerte I, Penttilä M, Contreras R (2005) Cloning and characterization of the glucosidase II alpha subunit gene of *Trichoderma reesei*: a frameshift mutation results in the aberrant glycosylation profile of the hypercellulolytic strain Rut-C30. Appl Environ Microbiol 71:2910–2924

Gamms W, Bissets J (1998) Morphological and identification of Thrichoderma. In: Harmann GE, Kubicek CP (eds) Thrichoderma and Gliocladium. Taylor and Francis, UK

Gilligan W, Reese ET (1954) Evidence for multiple components in microbial cellulases. Can J Microbiol 1:90

Gritzali M, Brown RDJ (1979) The cellulase system of Trichoderma: relationship between purified extracellular enzymes from induced or cellulose-grown cells. Adv Chem Ser 181:237–260 (ISSN 0065-2393)

Humbird R, Davis L, Tao C, Kinchin D, Aden A (2011) NREL Report

Holtzapple M, Cognata M, Shu Y, Hendrickson C (1990) Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. Biotechnol Bioeng 36:275–287

Himmel ME, Ruth MF, Wyman CE (1999) Cellulase for commodity products from cellulosic biomass. Curr Opin Biotechnol 10:358–364

Igarashi K, Koivula A, Wada M, Kimura S, Penttilä M, Samejima M (2009) High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. J Biol Chem 284(52):36186–36190 (ISSN 1083-351X)

Ilmen M, Thrane C, Penttilä M (1996) The glucose repressor genecre1 of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. Mol Gen Genet 251(4):451–460 (ISSN 0026-8925)

Kaar WK, Holtzapple MT (1998) Benefits from Tween during enzymatic hydrolysis of corn stover. Biotechnol Bioeng 59:419–427

Kadla JF, Gilbert RD (2000) Cellulose structure: a review. Cellul Chem Technol 34:197

Kim MH, Lee SB, Ryu DDY (1982) Surface deactivation of cellulose and its prevention. Enzyme Microb Technol 4:99–103

Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2:19 (ISSN 1754-6834)

Kumar R, Wyman C (2008) Effect of additive on the digestibility of corn stover solids following pretreatment by leading technologies. Biotechnol Bioeng 102:1544–1557

Kurakake M, Shirasawa T, Ooshima H, Converse AO, Kato J (1995) An extension of the Harano-Ooshima rate expression for enzymatic-hydrolysis of cellulose to account for changes in the amount of adsorbed cellulase. Appl Biochem Biotechnol 50:231

Levasseur A, Saloheimo M, Navarro D, Andberg M, Monot F, Nakari-Setala T, Asther M, Record E (2006) Production of a chimeric enzyme tool associating the *Trichoderma reesei* swollenin with the aspergillus niger feruloyl esterase A for release of ferulic acid. Appl Microbiol Biotechnol 73:872–880

Lee JH, Lee HB, Andrade JD (1995) Blood compatibility of polyethylene oxide surfaces. Prog Polym Sci 20:1043–1079

Lu Y, Yang B, Gregg D, Saddler JN, Mansfield SD (2002) Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl Biochem Biotechnol 100:641–654

Linder M, Mattinen ML, Kontteli M, Lindeberg G, Stahlberg J, Drakenberg T, Reinkainen T, Pettersson G, Annila A (1995) Functional and structural roles of conserved amino acids in the cellulose binding domain of *Trichoderma reesei* CBH I. Protein Sci 4:1056

Mansfield SD, Mooney C, Saddler JN (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol Prog 15:804

Mooney CA, Mansfield SD, Touhy MG, Saddler JN (1998) The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresour Technol 64:113

Nakari-Setälä T, Paloheimo M, Kallio J, Vehmaanperä J, Penttilä M, Saloheimo M (2009) Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. Appl Environ Microbiol 75(14):4853–4860 (ISSN 1098-5336)

Nutor JRK, Converse AO (1991) The effect of enzyme and substrate levels on the specific hydrolysis rate of pretreated poplar wood. Appl Biochem Biotechnol 28, pp. 29, 757

Ooshima H, Kurakake M, Kato J, Harano Y (1991) Enzymatic-activity of cellulase adsorbed on cellulose and its change during hydrolysis. Appl Biochem Biotechnol 31:253

Parke JW, Takahata Y, Kajuchi T, Akehata T (1992) Effects of non-ionic surfactant on enzymatic hydrolysis of used newspaper. Biotechnol Bioeng 39:117–120

Palmqvist E, Hahn-Hagerdel B, Galbe M, Zacchi G (1996) The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. Enzyme Microb Technol 19:470–476

Pavani M, Basil DK (2010) Immobilization of cellulase and hemicellulases on porous glass beads. ASTM 6:7–10

Puls J, Schuseil J (1993) Chemistry of hemicellulose: relationship between hemicellulose structure andenzymes required for hydrolysis. In: Coughlan MP, Hazlewood GP (eds) Portland Press, London, pp 1–27

Qing Q, Wyman CE (2011) Supplementation with xylanase and β-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. Biotechnol Biofuels 4:18

Qing Q, Yang B, Wyman CE (2010) Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresour Technol 101:9624–9630

Qi B, Chen X, Su Y, Wan Y (2011) Enzyme adsorption and recycling during hydrolysis of wheat straw lignocellulose. Bioresour Technol 102:2881–2889

Reese ET, Mandels M (1984) Rolling with the times: production and applications of *Trichoderma reesei* cellulases. Annu Rep Ferm Proc 7:1–20

Receveur W, Czjzek M, Schulein M, Panine P, Henrissat B (2002) Dimension, shape, and conformational flexibility of a two domain fungal cellulase in solution probed by small angle X-ray scattering. J Biol Chem 277:40887

Rouvinen J, Bergfors T, Teeri T, Knowles JK, Jones TA (1990) Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science 249(4967):380–386 (ISSNl 1095-9203)

Saddler JN, Brownell HH, Clermont LP, Levitin N (1982) Enzymatic-hydrolysis of cellulose and various pretreated wood fractions. Biotechnol Bioeng 24:1389

Seo DJ, Fujito H, Sakoda A (2011) Effects of a non-ionic surfactant, tween 20, on adsorption/desorption of saccharification enzymes onto/from lignocelluloses and saccharification rate. Adsorption 17:813–822

Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP (2008) The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 9:327 (ISSN 1471-2164)

Seiboth B, Ivanova C, Seidl-Seiboth V (2011) *Trichoderma reesei*: a fungal enzyme producer for cellulosic biofuels bernhard. Vienna University of Technology, Austria

Seiboth B, Pakdaman BS, Hartl L, Kubicek CP (2007) Lactose metabolism in filamentous fungi: how to deal with an unknown substrate. Fungal Biology Reviews 21:42–48

Seiboth B, Hakola S, Mach RL, Suominen PL, Kubicek CP (1997) Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei*. J Bacteriol 179:5318–5320

Selby K (1969) 'The purification and properties of the C1—component of the cellulase complex. In: Hajny GJ, Reese ET (eds) Cellulases and their applications. American Chemical Society, Washington, DC, pp 34–50

Shin CS, Lee JP, Lee JS, Park SC (2000) Enzyme production of *Trichoderma reesei* Rut C-30 on various lignocellulosic substrates. Appl Biochem Biotechnol 84:237–245

Sinitsyn AP, Mitkevich OV, Klesov AA (1986) Inactivation of cellulolytic enzymes by stirring and their stabilization by cellulose. Prikladnaya Biokhimiya i Mikrobiologiya 22:759–765

Steele BR, Nghiem SJ, Stowers M (2005) Enzyme recovery and recycling following hydrolysis of ammonia fiber explosion-treated corn stover. Appl Biochem Biotechnol 901:121–124

Stone JE, Scallan AM (1969) Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. Adv Chem Sci 95:219

South CR, Hogsett DAL, Lynd LR (1995) Modeling simultaneous saccharification and fermentation of lignocelluloses to ethanol in batch and continuous reactors. Enzyme Microb Technol 17:797

Sutcliffe R, Saddler JN (1986) The role of lignin in the adsorption of cellulases during enzymatic treatment of lignocellulosic material. Biotechnol Bioeng 17:749–762

Taherzadeh MJ, Karimi K (2009) Enzyme based hydrolysis processes for ethanol from lignocellulosic materials: a review. Bioresources 4:707–738

Tomme P, Warren RA, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microb Physiol 37:1–81 (ISSN 0065-2911)

Tu M, Chandra RP, Sadller JN (2007) Recycling cellulase during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. Biotechnol Prog 65:1130–1137

Tu M, Zhang X, Piace M, McFarlance P, Saddler N (2009) Effect of surfactants on separate hydrolysis fermentation and simultaneous saccharification fermentation of pretreated lodgepole pine. Biotechnol Prog 25:1122–1129

Uribe S, Sampedro JG (2003) Measuring solution viscosity and its effect on enzyme activity. Biol Proced Online 5:108–115

Vaheri M, Leisola M, Kaupinnen V (1979) Transglycosylation products of the cellulase system of *Trichoderma reesei*. Biotechnol Lett 1:41–46 (ISSN 0141-5492)

Wingren A, Galbe M, Roslander C, Rudolf A, Zacch G (2005) Effect of reduction in yeast and enzyme concentrations in a simultaneous-saccharification-and fermentation-based bioethanol process: technical and economic evaluation. Appl Biochem Biotechnol 121:485–499

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153–157

Wyman CE, Decker SR, Himmel ME, Brady JW, Skopec CE, Viikari L, Wyman CE, Decker SR, Himmel ME, Brady JW, Skopec CE, Viikari L (2005) Hydrolysis of Cellulose and Hemicellulose. Marcel Dekker

Whitney SE, Gothard MG, Mitchell JT, Gidley MJ (1999) Roles of cellulose and xyloglucan in determining the mechanical properties of primary plant cell walls. Plant Physiol 121:657

Xue Y, Jameel H, Park S (2012) Strategies to recycle enzyme and their impact on hydrolysis for bioethanol production. Bioresources 7:602–615

Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2011) Deactivation of cellulases by phenols. Enzyme Microb Technol 48:54–60

Xiao ZZ, Zhang X, Gregg DJ, Saddler JN (2004) Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 113–16:1115–1126

Yang B, Wyman CE (2006) BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrate. Biotechnol Bioeng 94(4):611–617 (Wiley Interscience)

Yang SWZ, Quian JQY, Zhou YG, Xia XH (2009) Simple approach for efficient encapsulation of enzyme in silica matrix with retained bioactivity. Anal Chem 7:3478–3484

Zheng Y, Pan Z, Zhang R (2008) Non-ionic surfactants and noncatalytic protein treatment on enzymatic hydrolysis of pretreated creeping wild ryegrass. Appl Biochem Biotechnol 146:231–248

Zhang S, Wolfgang DE, Wilson DB (1999) Substrate heterogeneity causes the nonlinear kinetics of insoluble cellulose hydrolysis. Biotechnol Bioeng 66:35–41

Zhang Q, Lo CM, Ju LK (2007) Factors affecting foaming behavior in cellulase fermentation by *Trichoderma reesei* Rut C-30. Bioresour Technol 98:753–760

Zhang YH (2008) Reviving the carbohydrate economy via multi product lignocellulosic biorefineries. J Ind Microbiol Biotechnol 35:367–375

Zheng Y, Pan Z, Zhang RH (2009) Non-ionic surfactants and non-catalytic protein treatment on enzymatic hydrolysis of pretreated Creeping Wild Ryegrass. Appl Biochem Biotechnol 146:231–248