

Cellulase recycling in biorefineries—is it possible?

Daniel Gomes¹ · Ana Cristina Rodrigues¹ · Lucília Domingues¹ · Miguel Gama¹

Received: 8 January 2015 / Revised: 9 March 2015 / Accepted: 11 March 2015 / Published online: 29 March 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract On a near future, bio-based economy will assume a key role in our lives. Lignocellulosic materials (e.g., agroforestry residues, industrial/solid wastes) represent a cheaper and environmentally friendly option to fossil fuels. Indeed, following suitable processing, they can be metabolized by different microorganisms to produce a wide range of compounds currently obtained by chemical synthesis. However, due to the recalcitrant nature of these materials, they cannot be directly used by microorganisms, the conversion of polysaccharides into simpler sugars being thus required. This conversion, which is usually undertaken enzymatically, represents a significant part on the final cost of the process. This fact has driven intense efforts on the reduction of the enzyme cost following different strategies. Here, we describe the fundamentals of the enzyme recycling technology, more specifically, cellulase recycling. We focus on the main strategies available for the recovery of both the liquid- and solid-bound enzyme fractions and discuss the relevant operational parameters (e.g., composition, temperature, additives, and pH). Although the efforts from the industry and enzyme suppliers are primarily oriented toward the

development of enzyme cocktails able to quickly and effectively process biomass, it seems clear by now that enzyme recycling is technically possible.

Keywords Biorefineries · Enzyme recycling · Cellulase-substrate interactions · Lignocellulosic bioethanol · Cellulases cost

Introduction

Over the last decades, an increasing number of economic and environmental issues associated to fossil fuel utilization has claimed an equally higher role for biofuels in the overall energetic picture. Although referring to several compounds (bioethanol, biodiesel, biogas, biobutanol, etc.), first generation bioethanol (1G) represents nowadays most of biofuel production worldwide, already established in several countries such as USA, Brazil, China, India, Canada, among others (Gupta and Verma 2015). On the other hand, the number of facilities for second generation (2G) production is still very small, even though the widely recognized advantages regarding the substrate cost. Rather than corncob (USA) or sugarcane (Brazil), 2G bioethanol employs cellulosic materials, which can be residues from the plant used in 1G, crops purposely cultivated, forestry residues (e.g., from cleaning activities), industrial residues, or solid wastes (Hayes 2013).

Contrarily to first generation, 2G bioethanol employs a more complex substrate, not easily accessible for microbial fermentation. Lignocellulosic materials are mainly composed by cellulose, hemicellulose, and lignin in variable amounts (Table 1).

✉ Miguel Gama
fmgama@deb.uminho.pt

Daniel Gomes
danielg_gomes@deb.uminho.pt

Ana Cristina Rodrigues
ana_cris@deb.uminho.pt

Lucília Domingues
luciliad@deb.uminho.pt

¹ CEB—Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Table 1 Percentage of cellulose, hemicellulose, and lignin in the composition of different lignocellulosic materials (on a dry basis)

Material	Cellulose	Hemicellulose	Lignin
Corn cob	45	35	15
Grass	25–40	35–50	10–30
Hardwood steam	40–55	24–40	18–25
Newspaper	40–55	25–40	18–30
Paper	85–99	0	0–15
Primary wastewater solids	8–15	NA	24–29
Switch grass	45	31	12
Wheat straw	30	50	15

Adapted from Sun and Cheng (2002)

NA not available

Cellulose structure consists of long and linear chains of glucose units (usually in the range of several thousands) linked by $\beta(1-4)$ glycosidic bonds. It presents a high crystallinity degree as a result of the hydrogen bounds established between different layers of cellulose chains. This contributes to a more robust and hard to digest component in lignocellulosic materials. Hemicellulose, on the other hand, is composed by shorter (usually ranging from hundreds to some thousands of units) and not strictly linear chains of glucose, but also xylose, mannose, galactose, rhamnose, and arabinose. Differently from cellulose, it presents an amorphous structure with a frequent presence of ramifications, rendering a more fragile structure and more suitable to digestion. Finally, lignin is a polymer composed by three main aromatics: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Horn et al. 2012). Cellulose holds the larger share of the energetic potential available in this kind of materials, its hydrolysis rendering glucose molecules only, which can be easily used by most of the traditional fermentation microorganisms (e.g., *Saccharomyces cerevisiae*). Hemicellulose, which is present in smaller amounts for most of lignocellulosic materials (with some exceptions such as grass), results in different sugars that in some cases cannot be directly used by the traditional fermenting microorganisms (C5 sugars). However, the current existence of some industrial options already able to coferment pentoses (e.g., DSM, Abengoa) together with recent encouraging results from metabolic engineer strategies toward C5 fermentation (e.g., Romani et al. 2015) suggests that, on a near future, hemicellulose may equally hold a high energetic potential. Lignin is usually burned for energy production, although many other applications—largely remaining to be explored—exist for this material, such as the synthesis of different aromatic compounds (Demirbas 2008).

Acting as the skeleton of vegetable materials, lignocellulose presents a very solid and robust structure, hard to digest by cellulolytic microorganisms (Himmel et al. 2007). Thus, a pretreatment is usually required to facilitate saccharification,

which in most of the cases means increasing the accessibility of cellulases to the substrate. Several pretreatments have been developed over the years: autohydrolysis, acid hydrolysis, steam explosion, organosolv, etc. (Sánchez and Cardona 2008). While facilitating enzyme's action, these processes usually result in the production of compounds inhibitory for microbial growth, such as furfural, hydroxymethylfurfural (HMF), and acetic acid (Almeida et al. 2009). Naturally robust industrial isolates able to resourcefully degrade furfural and HMF inhibitors were recently identified (Pereira et al. 2014).

Following a pretreatment process (which depends on the structure of the lignocellulosic material), hydrolysis is conducted in order to release sugars for fermentation. Enzymatic hydrolysis relies on the action of cellulases, enzymes with the ability to break down cellulose to simpler monomers. These enzymes are usually produced by cellulolytic organisms such as *Aspergillus niger*, *Trichoderma reesei*, *Clostridium thermocellum*, and others. Cellulases contribute to a significant part of the final bioethanol cost (Aden and Foust 2009), resembling as the second most expensive element (following the raw material) in the overall process. According to Klein-Marcusschamer et al. (2012), the current cost of cellulases on bioethanol production is approximately \$ 0.68 per gallon. In recent years, intense efforts have been made to modify this scenario, a goal pursued by three main strategies: reducing cellulase production cost; creating more efficient cellulases; and reducing the amounts of required cellulases by recycling them over several rounds of hydrolysis (Pribowo et al. 2012).

Achieving a significant reduction on the cost of cellulases will allow an important improvement on the economics of second-generation biofuels, facilitating their competition with fossil fuels, but also of other processes based on lignocellulosic materials through the biorefineries concept (Fig. 1). These are platforms for the production of a wide range of compounds, in some cases currently produced by chemical synthesis, using different types of biomass as feedstock (e.g., sugar/starch crops, vegetable oil, and micro-algae). Through different possible conversion techniques, such as fermentation, transesterification, gasification, hydrogenation, or anaerobic digestion, biomass can be converted into either energy or chemicals, in a cleaner and sustainable route. However, as for second-generation bioethanol, the integration of lignocellulosic materials into biorefineries processes is currently hampered by the high cost of enzymes, which considerably affects the final cost of the products.

Enzymatic hydrolysis of lignocellulosic materials

Cellulose conversion into fermentable sugars is a complex process, involving several types of cellulases (Klein-Marcusschamer et al. 2012). In fact, cellulolytic organisms usually code and secrete a huge number of different cellulases.

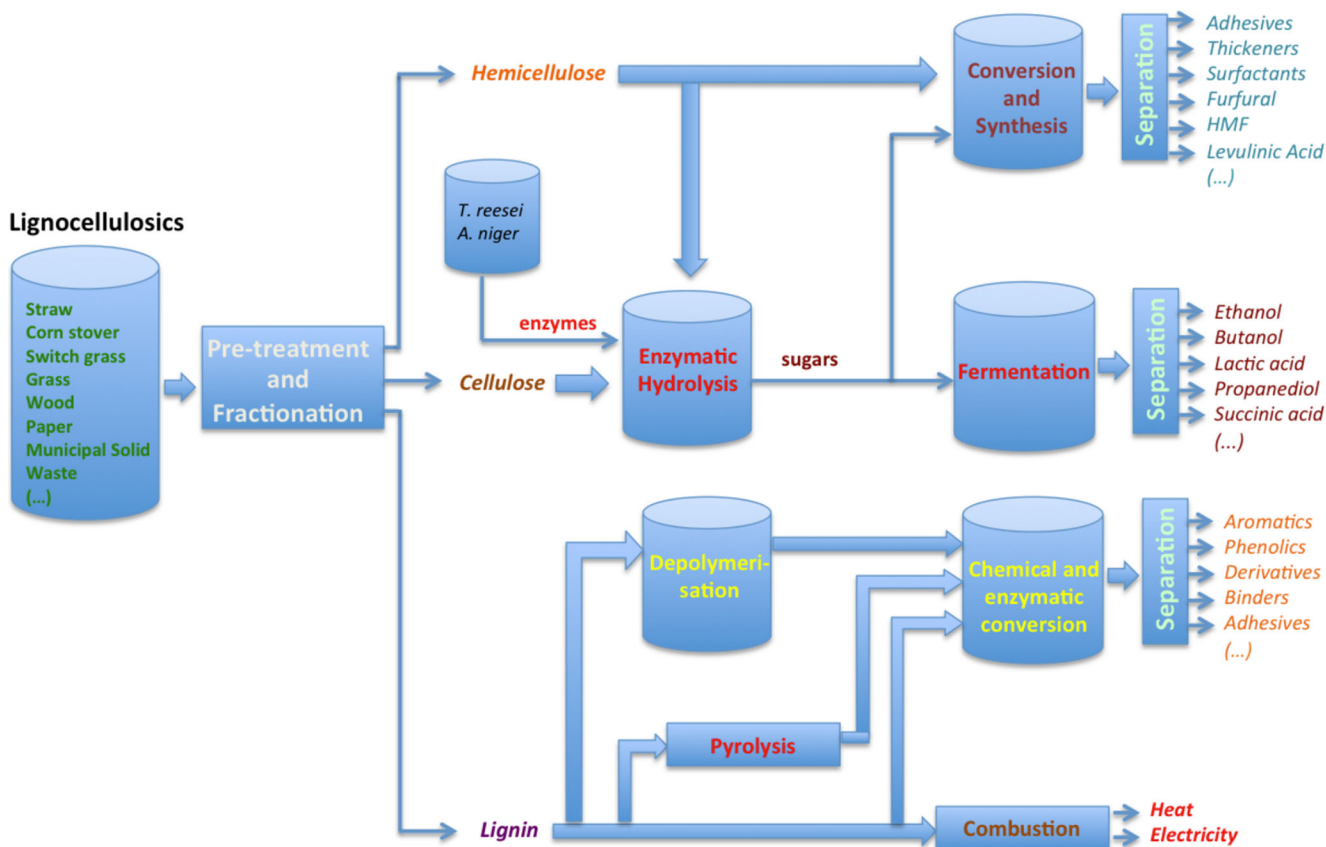


Fig. 1 Simplified schematic representation of a lignocellulosic biorefinery

Cellulose hydrolysis is usually conducted synergistically by two main classes of cellulases: endoglucanases (EGs) randomly cleave internal β -1,4-glycosidic bonds of cellulose chains and exoglucanases such as cellobiohydrolases (CBHs), which form cellobiose units by acting either on the reducing or on nonreducing ends of cellulose chains. Finally, one additional class of enzymes, β -glucosidases, hydrolyzes cellobiose into glucose (Segato et al. 2014).

Among the several cellulolytic systems reported to date, *T. reesei* presents probably one of the most studied ones. This fungus, which was initially isolated from cotton tents during the World War II (Reese 1976), is currently the most employed organism in the production of commercial enzymes for biomass hydrolysis (Kumar et al. 2008; Horn et al. 2012). Its cellulolytic system encompasses CBHs (EC 3.2.1.91), EGs (EC 3.2.1.4), and β -glucosidases (EC 3.2.1.21) (Seiboth et al. 2011). According to Suominen et al. (1993), approximately 60 and 20 % of the secreted proteins in *T. reesei* correspond to the CBHs Cel7A (formerly CBH I) and Cel6A (formerly CBH II), respectively, which constitute the only CBHs of this cellulolytic system. They both act processively on cellulose chains, but while Cel6A forms cellobiose from the nonreducing ends, Cel7A acts on the reducing ends. Although their processive way of action, they both present a reversible binding to the substrate, which represents an important feature especially in a

context of enzyme recycling. Palonen et al. (1999) have observed some differences in this regard: while Cel6A exhibits a binding reversibility between 60 and 70 %, this feature is considerably increased in the case of Cel7A to a minimum of 90 %. Their action is complemented by the synergetic action of several EGs, produced in considerable smaller amounts: Cel5A (formerly EG II), Cel5B, Cel7B (formerly EG I), Cel12A (formerly EG III), Cel45A (formerly EG V), Cel61A (formerly EG IV), and Cel61B and Cel75A (formerly EG VI) (Seiboth et al. 2011). Finally, seven β -glucosidases Cel1A (formerly BGL II), Cel1B, Cel3A (formerly BGL I), Cel3B, Cel3C, Cel3D, and Cel3E are usually produced in very small amounts (approximately 0.5 % of total secreted proteins), which in some cases forces the supplementation with β -glucosidases from another organism (e.g., Novozymes 188 from *A. niger*).

Interestingly, in spite of the notorious efficiency of *T. reesei* cellulolytic system, the number of cellulases produced by this organism was found to be considerably smaller comparatively to others (Martinez et al. 2008). According to Seiboth et al. (2011), this fact suggests that part of the high cellulose-degrading capacity of *T. reesei* may be due to an efficient gene transcription and increased protein production and secretion, rather than the number of coded cellulases. Furthermore, this high cellulolytic performance of *T. reesei* has also been

assigned to a high adsorption efficiency to the substrate (e.g., Tu et al. 2007b).

The concerted action of endoglucanases and exoglucanases for cellulose hydrolysis, a heterogeneous catalysis process, implies the following events orchestrated in a well-established order: cellulase binding onto the substrate; formation of the complex cellulase-substrate; cleavage of the glycosidic bond and displacement of enzyme to the next cleavage zone; desorption of the enzyme (Fig. 2).

As in every enzyme-mediated process, the reaction rate is governed by several environmental factors, e.g., pH, temperature, and presence of surfactants (Kumar et al. 2008). Also, each cellulase can present very distinct substrate affinity, thermostability, reaction kinetics, etc. (Tu et al. 2007a). Furthermore, the properties of the lignocellulosic material influence the degradation process, namely, its structure and composition, cellulose crystallinity, surface area (Bommarius et al. 2008). Altogether, the degradation of cellulose is thus a rather complex process.

Is cellulase recycling possible?

Enzyme recycling has been one of the most pursued routes to reduce cellulase cost on 2G bioethanol, given the high amounts of enzymes that are currently required for efficient hydrolysis (Himmel et al. 2007) but also considering the fact that cellulases have shown remarkable stability (Maheshwari et al. 2000). Over the last 30 years, and especially on the last decade, a suitable recycling process has been investigated in several studies. Nevertheless, considerable research is still required for a mature technology to be developed. This process will probably be driven by the biorefineries industry and the academy, since the manufacturers do not benefit from the

development of enzyme's recycling. However, competition between enzyme producers will eventually make it happen. At this point, it must be stated that—technically—enzyme recycling is indeed possible.

An efficient cellulase recycling process would largely depend on three major requirements: (1) a highly stable cellulase, (2) a high hydrolysis efficiency, and (3) good control over the substrate adsorption/desorption processes.

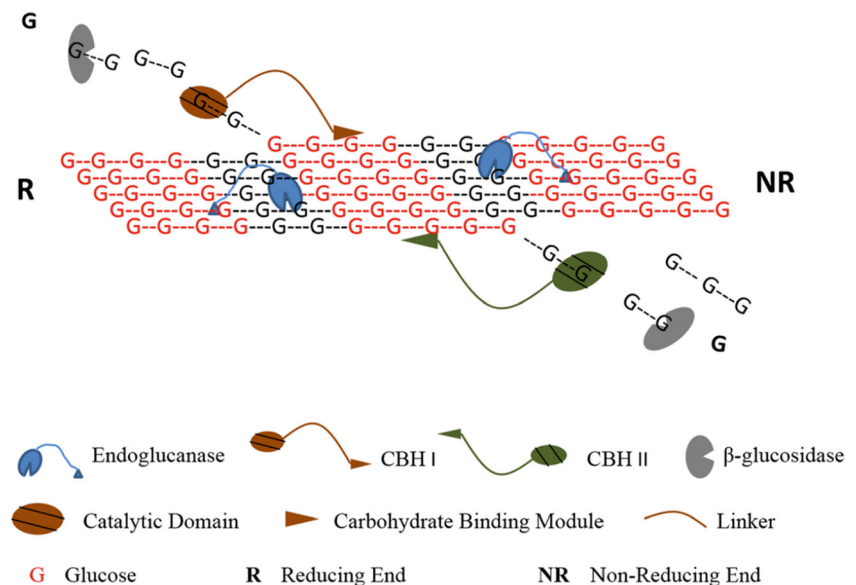
Cellulase-substrate interactions

During the process of enzymatic hydrolysis, three main actors can be distinguished: the cellulases, the lignocellulosic fibers, and the liquid phase. The relative amount of free cellulases on the liquid phase is not constant, it rather changes over the extension of hydrolysis. The final composition of the system determines which fraction of the enzyme will be free in solution, and easily available to be reused, and which fraction will remain bound to the final solid, requiring an additional step of desorption to be reused. This dynamic process strongly depends on the affinity of each cellulase (e.g., Cel7A, Cel5A, Cel7B, β -glucosidase) for cellulose and lignin (Pribowo et al. 2012), on the structure and composition of the substrate (Tu et al. 2007a), and finally on multiple environmental factors (e.g., pH, presence of surfactants) (Shang et al. 2014; Seo et al. 2011).

The role of cellulose-binding domains

A considerable number of studies have previously demonstrated that different cellulases can present distinct affinities for the substrate. Furthermore, for a given cellulase, diverse affinities can also be found for different substrates. Cellulose-binding domains (CBDs) play an important role in defining

Fig. 2 Simplified schematic of the enzymatic hydrolysis of lignocellulose (reproduced from Kumar and Murthy (2013))



the affinity and specificity of cellulases toward the insoluble fibers.

CBDs are part of a wider class of protein components designated carbohydrate-binding modules (CBMs). These are specific amino acid sequences (between 30 and 200 amino acids), present in many carbohydrate-hydrolyzing enzymes, and which have a carbohydrate-binding activity (Boraston et al. 1998). CBMs are particularly common on cellulases, which usually present a modular structure. In addition to the catalytic module, they also present, at least, one carbohydrate-binding module (Gilkes et al. 1991). These noncatalytic modules can either be linked to the C- or the N-terminal of the protein's structure and seem to facilitate the adsorption to specific carbohydrates. According to Arantes and Saddler (2010), CBM action on cellulose may occur by three distinct ways: increasing cellulase concentration on the surface of cellulose and promoting substrate selectivity and disruption of crystalline substrate. For the particular case of CBDs, they are reported to enable an efficient adsorption of the enzyme to cellulose and its processive hydrolysis. After the cleavage of a glycosidic bond, cellulase does not separate from the substrate, but rather slides for the next hydrolysis.

In addition to their evident role on substrate hydrolysis, CBDs may also play a critical role on enzyme recycling. As an important element on the process of cellulase adsorption to the substrate, it will significantly dictate the equilibrium between free and bound cellulases and their desorption from solid residue. With exception of Cel12A, all major EGs and CBHs in *T. reesei* present a CBD (Viikari et al. 2007) suggesting a possible high affinity toward cellulose. Reverse wise, as β -glucosidases do not present this binding domain, its capacity to bind cellulose will probably be considerably reduced. In fact, on early studies conducted by Ishihara et al. (1991), the higher binding affinities for a delignified substrate were observed for CBH, followed by EG, and finally β -glucosidase. Also, using an electrophoretic analysis, Tu et al. (2007a) verified that, differently from Cel7A, Cel6A, Cel7B, and Cel5A, β -glucosidase levels on the liquid fraction remained constant during hydrolysis, suggesting a low adsorption to substrate. Similar results were also observed by Pribowo et al. (2012) and Lindedam et al. (2013) who have analyzed the adsorption profiles of the different enzyme components applying an SDS-PAGE analysis. In still another example, Tu et al. (2007b) observed that *T. reesei* cellulases (Celluclast and Spezyme CP) presented higher substrate affinity than those from *Penicillium* sp., because the later do not have a CBD (Jorgensen et al. 2003).

The influence of substrate composition on the equilibrium of free versus bound cellulases

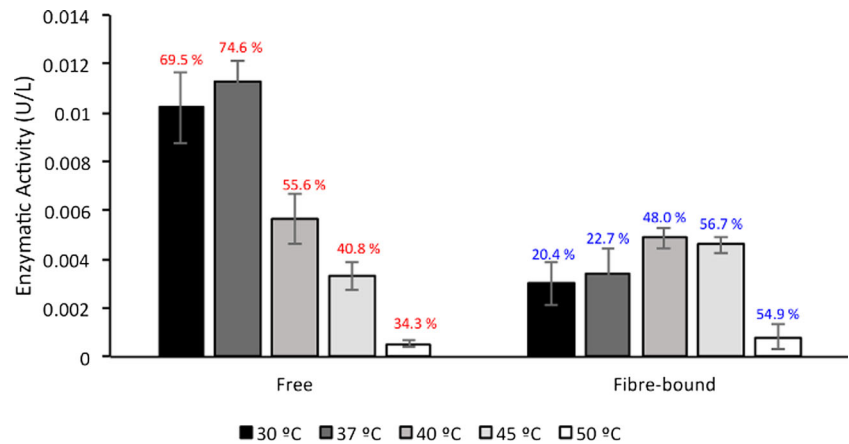
Cellulases have a high affinity for both cellulose and lignin. However, while they return into the liquid fraction once

cellulose is fully hydrolyzed, lignin-bound cellulases remain adsorbed in a nonproductive way (Yang and Wyman 2006). Early studies by Desphande and Erikson (1984) showed that after a 24-h hydrolysis of Avicel (almost pure cellulose), most of the endo-1,4- β -glucanases were free on the liquid fraction (around 85 %). However, when lignin-containing substrates were employed, this value decreased to less than 50 %. More recently, Lu et al. (2002) analyzed the Langmuir isotherms for the adsorption of cellulases to Avicel and two lignin-containing substrates. While the maximum cellulase adsorption of 80 mg/g_{substrate} was found for Avicel, in the case of a substrate with 46 % of lignin, it was nearly 160 mg/g_{substrate}. After a 48-h hydrolysis, the protein content on the liquid fraction was 85 % for the case of Avicel, contrasting with only 30 % for the 46 % lignin substrate. Another example was provided by Qi et al. (2011), who obtained around 30 % of the proteins in the supernatant after a 48-h hydrolysis of a 20 % lignin-substrate, and 65 % when the lignin content decreased to 3.6 %.

The referred results suggest a clear influence of lignin on the final fraction of free enzymes. While the influence of lignin on enzyme adsorption to cellulose, and on its subsequent hydrolysis, is more or less consensual, the underlying mechanisms for such effects are still not very clear and seem to be case-dependent. According to the most traditional assumptions, lignin can either competitively bind cellulases, reducing the ability for adsorption on cellulose, or block the access of cellulases to cellulose by forming a physical barrier (Kumar et al. 2012). The last one is the most accepted theory, as is supported by the well-known lignin-holocellulose interaction. It is well established that lignin forms a physical hydrophobic barrier to the holocellulose present on the substrate. Nevertheless, the application of different physical or chemical treatments, very common on 2G processes, has been suggested to be able to decrease this lignin barrier (Barsberg et al. 2013). A different case corresponds to the competitive binding of cellulase to lignin, as whether it may really occur or not, seems to be dependent namely on the chemical structure of lignin, specifically its hydrophobicity, since lignin-cellulase interactions are mainly hydrophobic (Schmaier et al. 1984; Wang et al. 2015).

On the other hand, one of our recent studies suggests that cellulose content on the final solid residue may have an even higher role on the enzyme desorption. In one of these studies, wheat straw was hydrolyzed at different temperatures. Following hydrolysis, solid-bound cellulases were recovered by applying an alkaline wash (at pH 9), and the remaining activity bound to the solid and liquid fractions was quantified (Rodrigues et al. 2012). We verified that the percentage of enzyme recovery from the solid was higher for the cases where lower temperatures were employed in the hydrolysis step, such as 30 or 37 °C, rather than higher temperatures, such as 45 or 50 °C (Fig. 3).

Fig. 3 Distribution of 4-methylumbelliferyl- β -D-cellobioside (MUC) activity and percentage of enzyme recovered and fiber-bound after an alkaline wash (pH 9) performed over the final solid obtained from the hydrolysis of wheat straw at different temperatures: activity of cellulases released from the solid (free); activity of cellulases remaining adsorbed to the solid (fiber-bound) (adapted from Rodrigues et al. (2012))



We concluded that the lower cellulose conversion that occurred at higher temperatures (a surprising finding that was assigned to the enzyme denaturation at higher temperatures) resulted in a higher amount of residual cellulose, consequently increasing the difficulty to desorb cellulases from the final residue. Indeed, we have also observed that, contrarily to pure lignin-bound cellulases, the cellulose-bound cellulases were not fully desorbed from the final solid when applying an alkaline wash (Rodrigues et al. 2012).

The influence of crystallinity degree of the lignocellulosic substrates

Taking into account how important a complete cellulose conversion seems to be for a proper cellulase recycling, another issue that should be addressed is the substrate crystallinity. This property translates the prevalence of crystalline and amorphous domains on cellulose chains as well as the distribution of the different crystalline forms.

Crystalline domains are usually well-structured and organized regions, highly resistant to chemical and enzymatic hydrolysis. These alternate with amorphous regions, which are less ordered and thus, more susceptible to enzymatic or chemical hydrolysis (Adsul et al. 2011) (Fig. 4). Furthermore, crystalline domains can appear in different forms (allomorphs), with different stabilities and levels of organization. Cellulose I is the most common form found in nature, and thus, on lignocellulosic materials, however, cellulose II is the most resistant form (Segato et al. 2014). Other forms (celluloses III and IV) can still be obtained when chemical or physical treatments are applied over the main ones.

Mittal et al. (2011) obtained materials with an improved digestibility by applying a treatment with sodium hydroxide or liquid ammonia and demonstrated that the content of amorphous regions on substrate structure strongly influences its digestibility in the first 24 h. Furthermore, it was also observed that, while digestibility have a weak correlation with the allomorph type on the first 24 h, this considerably increases for

later digestion times. These results suggest that the interaction of cellulases with different types of cellulose is not exclusively dependent on the type of cellulose itself, but also relies on other factors such as the current extension of the hydrolysis.

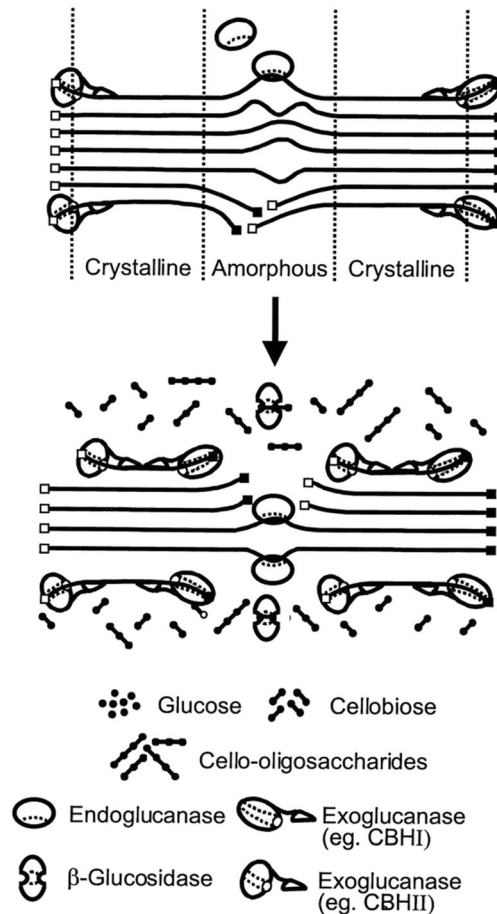


Fig. 4 Schematic representation of the hydrolysis of different regions on cellulose chains (amorphous and crystalline) by a noncomplex cellulase system (Reprinted from Microbiology and Molecular Biology Reviews, Volume 66, Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS, Microbial Cellulose Utilization: Fundamentals and Biotechnology, pp 506-577, 2012, with permission from American Society for Microbiology)

Another important factor refers to the way the degree of crystallinity, and the adsorption efficiency are related and to what extent this may affect hydrolysis efficiency. It is a well-known fact that amorphous regions are more easily digested, but the exact reason for that remains unknown. As already observed on several studies, cellulases present a higher adsorption toward less crystalline materials (Klyosov et al. 1986; Lee et al. 1982). This may be partially explained by the fact that different affinities have been reported for a specific CBM toward substrates with a different crystallinity degree (McLean et al. 2002). Such assumption was inclusively the base for specific methods employed for measuring crystallinity changes of a material using CBMS (e.g., Široký et al. 2012; Gourlay et al. 2012). This would suggest that decreasing crystallinity would increase substrate digestion since cellulase adsorption could be enhanced. However, the results obtained by Hall et al. (2010) indicated that this might not be completely true. The authors observed that, reaching a specific enzyme concentration, the substrate crystallinity continued to influence the initial rate of enzymatic hydrolysis while the amount of enzyme bound to the substrate remained unchanged. This result seems to suggest that the influence of crystallinity on hydrolysis is much broader than its effect on cellulase adsorption.

Recycling strategies

As described above, cellulases can either bind reversibly to the substrate, being posteriorly released to the liquid fraction, or remain adsorbed on the final residue after hydrolysis. An efficient strategy for cellulase recycling will therefore require the recovery of both fractions (Shang et al. 2014).

As mentioned before, the utilization of a particular lignocellulosic substrate and a specific cellulase mixture strongly dictate the distribution of cellulase activity between the solid and liquid fractions. Consequently, these same factors will equally influence the efficiency of cellulase recovery, and ultimately, the adopted method to achieve it.

Free cellulases on the liquid fraction

Soluble cellulases on the final hydrolysate have been efficiently recovered using two main methods: (i) ultrafiltration of the supernatant collected from the final hydrolysate (Lu et al. 2002; Yang et al. 2010; Qi et al. 2011, 2012; Chen et al. 2013; Rodrigues et al. 2012, 2014); (ii) re-adsorption of free cellulases onto fresh substrate (Tu et al. 2007a, b, 2009; Tu and Saddler 2010; Waeonukul et al. 2013; Ouyang et al. 2013; Eckard et al. 2013; Shang et al. 2014).

Usually, for the first case, the final hydrolysate is initially filtered or centrifuged to separate the solid residue (together with bound cellulases) from the liquid fraction containing free cellulases (Lu et al. 2002). Next, an ultrafiltration unit is

employed using a membrane with a cutoff of 10 kDa (Yang et al. 2010; Qi et al. 2011), in order to enable cellulase retention. The final retentate, consisting of cellulases and β -glucosidases, is then added to fresh substrate and buffer to conduct the next hydrolysis round. Using this methodology for three consecutive rounds, Lu et al. (2002) observed a decrease on the saccharification efficiency of only 25 % after the third round.

More recently, Chen et al. (2013) reported a similar methodology, aiming to enhance ultrafiltration flux through the utilization of electric fields. The authors observed that the application of an electric field over the membrane caused a decrease on the concentration polarization, leading consequently to an increased ultrafiltration flux. They observed that the buffer concentration of the hydrolysate, the temperature, and the applied current directly affected the strength of the electric field, therefore rising as major determinants on this technology. Using a specific set of conditions, consisting of a current of 150 mA, 5-mM buffer concentration, and room temperature, an optimum 836-V/m electric field was obtained, which allowed increasing the ultrafiltration flux by a factor of 4.4.

Alternatively, cellulases may also be recovered by simple exposure to fresh substrate, relying on their high capacity to adsorb the solid residue (excepting for β -glucosidases). Fresh substrate (usually the same amount used in the initial round) is added to the free cellulase suspension, and the adsorption process is allowed to occur for a period of approximately 2 h, under adsorption-promoting conditions (e.g., agitation) (Tu et al. 2007b; Ouyang et al. 2013). Afterward, the overall suspension is either filtered (Shang et al. 2014) or centrifuged (Tu et al. 2009), separating the fresh substrate with bound cellulases from the products of hydrolysis. The solid is finally resuspended in buffer and supplemented with fresh β -glucosidase, allowing a next round of hydrolysis. The addition of fresh β -glucosidase is a mandatory requirement on this case since, as was described above, they adsorb with a very low efficiency to the solid residue, which hinders their recovery by adsorption with fresh substrate (Lee et al. 1995). Therefore, β -glucosidase must be recovered from the liquid phase. With such strategy, Tu et al. (2007a) reported a recovery of 88 % of the free cellulases at the end of hydrolysis (51 % of the original load) of mixed softwood. More recently, Shang et al. (2014), following the same method, were able to obtain 46.7 % of the glucose yield achieved on the initial round of hydrolysis, suggesting that a significant part of the enzyme was recycled from the liquid.

When the hydrolysis efficiencies of these two recovery methods were compared by Qi et al. (2011), no significant differences were found. According to the authors, the only difference between these methodologies seems to be the requirement of β -glucosidase supplementation for the case of adsorption into fresh substrate. Although this may constitute a

significant economic barrier, the viability and complexity for an industrial-scale implementation of an ultrafiltration process should also be considered.

Fiber-adsorbed cellulases

Even though most cellulases are found free on liquid fraction at the end of hydrolysis, solid-bound cellulases may also be worth recovering. As already observed in one of our studies, this fraction of enzymes is still active and retains its capacity to efficiently adsorb onto fresh substrates (Rodrigues et al. 2012). However, the direct recycling of the final solid with the bound enzyme may not be feasible as it would probably lead to a significant buildup of lignin-rich residues that would ultimately have an adverse effect on the hydrolytic ability of the recovered enzyme, in subsequent hydrolysis of fresh substrates (Lee et al. 1995; Tu et al. 2007a, b; Qi et al. 2011). For this reason, and contrasting with the soluble cellulase scenario, the recycling of bound enzymes is complex since it requires desorption from the solid residue, followed by recovery.

Solid-bound cellulases are adsorbed either to residual cellulose or lignin, bearing higher affinity for the former. The interaction with cellulose is driven by specific recognition mediated by the cellulose-binding domains, while the adsorption onto lignin represents an unspecific interaction. The adsorption of proteins in hydrophobic materials, such as lignin, is often associated to denaturation. However, as it has been demonstrated in our group, this is not the case for the cellulase-lignin interaction. Indeed, it was clearly demonstrated that the exposure of an enzyme suspension to 2 % pure lignin (room temperature, 76 h) led to no significant alteration in the activity of Cel7A, suggesting therefore that recycling is not compromised by this interaction (Rodrigues et al. 2012).

Most desorption methods involve either a pH shift or the addition of chemicals such as alcohols or surfactants, as discussed in the next sections.

Effect of pH on the desorption of fiber-bound cellulases

As proteins are composed by amino acids, many of them bearing a side chain with a pH titratable group, their structure, and consequently their interactions with other materials are strongly influenced by the pH of the medium. The control of pH allows indeed a substantial control over the cellulase adsorption/desorption onto the substrate. Thus, the application of pH shifts becomes an efficient option to desorb bound cellulases.

Early reports by Otter et al. (1984, 1989) suggested alkaline wash as a possible method to recover bound cellulases; however, enzyme activity seemed to be affected above specific pH values. Otter et al. (1984) observed that Avicelase was significantly desorbed (40–45 %) through an increase on the pH

value to 10. A further increase in pH led to an even higher desorption but caused a severe decrease on cellulase activity. Among several methods tested by Zhu et al. (2009) for bound cellulase desorption, a pH shift to an alkaline environment was shown as one of the best options. Increasing the pH from 8 to 13 led to an increase on cellulase desorption efficiency, which reached 85 and 94 % for Avicel and diluted acid pretreated corn stover, respectively; however, no information was provided regarding whether cellulases were able to maintain their activity under such alkaline pH.

In addition to the above-mentioned works, some studies have reported that beyond facilitating cellulase desorption, the alkaline wash also allowed for high cellulase activity recovery. Du et al. (2012) reported the maintenance of 97 % of cellulase activity after 2-h incubation at pH 10. More recently, Shang et al. (2014) compared the efficiency of bound cellulase desorption conducted at different pH values. The amount of desorbed cellulase significantly increased from less than 20 % with an acidic neutral pH (4.8 and 7) to nearly 85 % with an alkaline pH (10).

Relevant insights into the effects of pH on cellulase structure and stability were provided in the studies by Rodrigues et al. (2012). In addition to the fact that an alkaline wash (pH 9 or 10) allowed a considerable desorption of bound cellulases, analysis by intrinsic tryptophan fluorescence (ITF) and circular dichroism (CD) revealed significant conformational changes in the structure of Cel7A when the pH was altered from 4.8 to 9 or 10, which were reversed when pH was changed back to 4.8. Furthermore, the authors also observed that no loss of 4-methylumbelliferyl- β -D-cellobioside (MUC) activity arose from the pH alteration.

Addition of chemicals

Nonproductive and irreversible adsorption of cellulases on lignin residues remains nowadays as one of the main barriers to an efficient saccharification (Seo et al. 2011). Therefore, a decrease of lignin interference has been intensively pursued either by decreasing its content on the initial lignocellulosic material, applying suitable pretreatments (Sipponen et al. 2014; Pan et al. 2005), or by trying to control the adsorption and desorption of cellulases. Additionally, as was previously referred here and clearly demonstrated by one of our studies, cellulose also represents an important barrier for the recovery of the enzymes, as the affinity of cellulases for cellulose is even higher than that for lignin (Rodrigues et al. 2012).

Here, the utilization of some types of chemicals showed to significantly decrease the binding of cellulases to both lignin and cellulose, with the consequent improvement of both hydrolysis and cellulase recovery.

Otter et al. (1989) observed that, among several detergents tested, with exception of sodium dodecyl sulfate (SDS), all caused an increase on Avicelase desorption from Avicel.

Tween 80 was found to be the best option, enabling a 67 % enzyme desorption, which supports its wide application in several desorption protocols (e.g., Pribowo et al. 2012; Tu et al. 2009). More recently, Tu et al. (2007b) reported that the utilization of Tween 80 led to a significant increase in the amount of total free enzyme during hydrolysis of ethanol pretreated Lodgepole pine (EPLP) and steam exploded Lodgepole pine (SELP). The authors have also observed that the utilization of 0.2 % (*w/v*) Tween 80 enabled an increase in the fraction of protein released at the end of hydrolysis, from 71 to 96 % and 46 to 73 %, for EPLP and SELP, respectively. Furthermore, the application of Triton X-100, Tween 80, or Tween 20 improved the efficiency of a single round of cellulase recycling, using EPLP, by 50 %, while a negative effect was verified for SDS. According to Eriksson et al. (2002), it is possible that surfactants (e.g., Tween) may compete with cellulases for adsorption sites on lignin-rich residues.

Zhu et al. (2009) have also explored a wide range of compounds for this purpose: NaCl, ethylene glycol, glycerol, Tween 80, Triton X-100, sodium dodecyl sulfate. Polyhydric alcohols (ethylene glycol and glycerol) were found to be more efficient in cellulase desorption compared to surfactants (e.g., Tweens, Triton X-100), both from Avicel and diluted acid pretreated corn stover, the utilization of 72 % ethylene glycol enabling a 76 % recovery of adsorbed cellulase from pretreated corn stover. Sipos et al. (2010) were also able to increase the recovery of cellulase activity, after hydrolysis of steam-pretreated spruce, when polyethylene glycol was supplemented to the hydrolysis medium. More recently, Eckard et al. (2013) have observed that also casein micelles could work as lignin blockers, increasing glucose and ethanol yield by up to 32 and 34 %, respectively, as well as the final cellulase recycling.

The key relevance of temperature

Temperature is a major determinant of cellulase recycling efficiency as it is related with two critical aspects of this process: the maintenance of good levels of enzyme activity during extensive periods of hydrolysis and its effect on the desorption of solid-bound enzymes.

Several studies have been conducted addressing cellulase stability after exposure to high temperatures. Rosales-Calderon et al. (2014) reported that an incubation of cellulases (Celluclast+Novozym 188) for 78 h at 50 °C caused a decrease by 30 to 45 % (depending on the initial amount of enzyme) on the protein concentration in suspension (suggesting denaturation of proteins). According to Tu et al. (2009), the cellulase desorption increased when temperature raised from 25 to 45 °C (due to a shift in the thermodynamic equilibrium position), but dropped rapidly in the range of 50 to 75 °C (likely due to enzyme denaturation). Also, Shang et al.

(2014) observed higher desorption efficiencies for lower temperatures (4–37 °C), while temperatures above 50 °C rapidly decreased desorption. On a recent study, Lindedam et al. (2013) observed that, for a short period hydrolysis (6 h), the utilization of a temperature of 40 or 50 °C did not significantly compromise the recovery of cellulase activity. However, following incubation for a longer period of 96 h, cellulase recovery at 50 °C was significantly hampered.

Although higher temperatures may favor a faster reaction rate, it also leads to faster denaturation. Thus, as often observed in enzymology, a long stand enzyme activity (and therefore its recycling) may be achieved by using a moderate temperature that does not compromise its stability. Such fact was widely demonstrated in some of our recent studies (Rodrigues et al. 2012, 2014).

Rodrigues et al. (2012) observed that Cel7A, the most abundant component on *T. reesei* cellulase cocktails (Pakarinen et al. 2014), did not lose any MUC activity at 30, 37, and 40 °C over a period of 168 h, but a considerable decrease occurred for temperatures above 45 °C: only 37.5 % of the original activity was preserved at 50 °C, as compared with 89.7 % for a temperature of 45 °C. Also, the amount of active cellulases bound to the final solid, as suggested by MUC activity measurements, was found to be higher at lower temperatures (30, 37 °C), suggesting that a lower thermal denaturation of cellulases occurred. In a more recent study (Rodrigues et al. 2014), we evaluated the evolution of enzyme activity for three consecutive runs of hydrolysis and fermentation conducted both at 37 and 50 °C (Fig. 5).

For a temperature of 37 °C, no considerable changes were observed in the activity of Cel7A, Cel7B, and β -glucosidase on each separate run of hydrolysis, although a notorious reduction could be observed between the different rounds. On the other hand, when a temperature of 50 °C was employed, a clear reduction was observed for all enzyme activities, during the three consecutive rounds. Such reduction was specially observed on the initial 24 h of each run, and more significantly on the initial one. Considering the particular case of Cel7A and for a temperature of 37 °C, the activity remained constant around 0.8 IU/ml, while for 50 °C, the enzymatic activity decreased from approximately 0.87 to 0.62 IU/ml after 24 h. Following these initial 24 h, the activity levels continued to decrease, although at a considerably lower rate. This effect of temperature is also patent on the efficiencies of enzyme recovery (Table 2).

As for the levels of enzyme activity, a significant difference was observed for the percentage of cellulase recovery when different temperatures were utilized. However, these differences decreased from the initial to the last round of hydrolysis rendering similar recovery efficiencies on the last round.

In addition to the clear effect of temperature on the maintenance of enzyme activity during the entire process of enzymatic hydrolysis, it should be noted that it can also directly

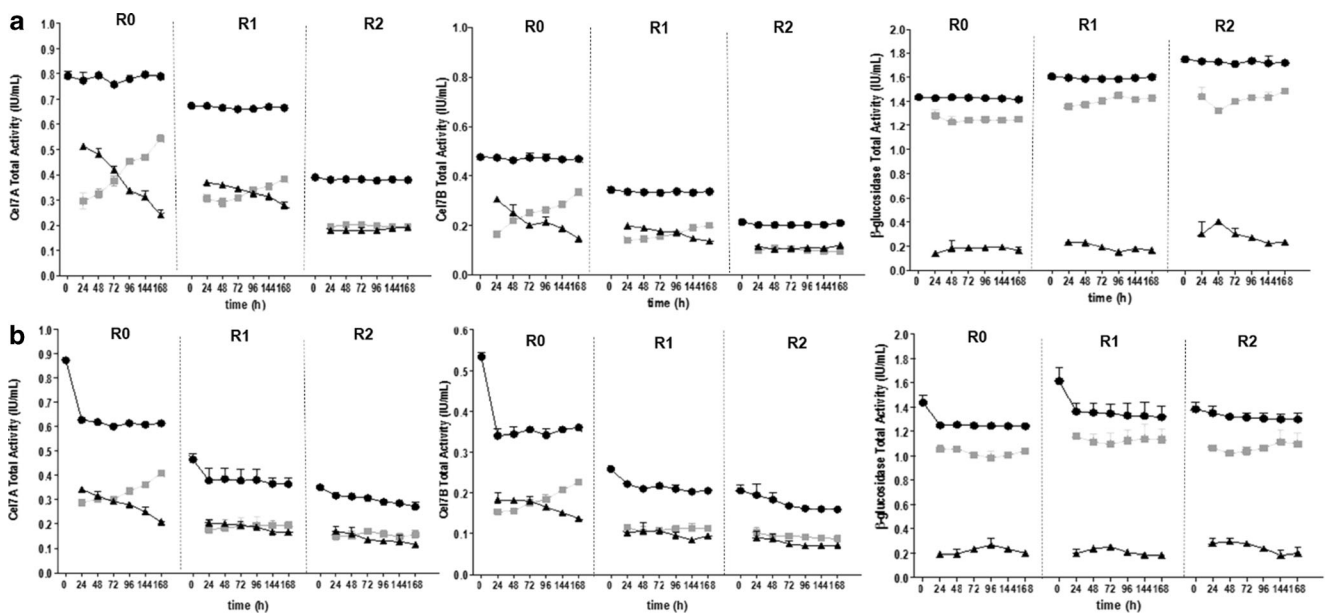


Fig. 5 Evolution of Cel7A, Cel7B, and β -glucosidase activities in solid and liquid fraction during wheat straw hydrolysis and fermentation at a 37 °C and b 50 °C using 20 FPU/g cellulose: (●) total activity; (■) activity in the liquid fraction; (▲) activity in the solid fraction. R0, R1, and R2 refer to the initial step of hydrolysis and to the first and second

rounds of enzyme recycling, respectively (Reprinted from Bioresource Technology, Volume 156, Rodrigues AC, Felby C, Gama M, Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw, pp 163-169, 2014, with permission from Elsevier)

affect cellulase recycling from the final solid, by influencing the extent of hydrolysis, and consequently the final solid composition (Rodrigues et al. 2012), as already discussed on a previous section. The higher the amount of residual cellulose, the more difficult the recovery of the enzymes will be.

Even with the evident benefits of operating at moderate temperatures (approximately 37 °C), this may not be viable on an industrial scale due to an increased risk of microbial contaminations (Lindedam et al. 2013). Such limitation, together with a wide range of potential advantages as consequence of increasing operating temperature, has recently

driven considerable efforts on the development of more thermostable cellulases (Viikari et al. 2007), which will most likely introduce significant improvements on lignocellulosic ethanol.

Conclusions and future perspectives

A significant reduction in the cost of cellulases is an urgent requirement to enable an economically sustainable utilization of lignocellulosic materials. Recycling enzymes is quite likely the solution to reduce this cost. This process has been intensively studied from more than 30 years, with special emphasis on the last decade. Several authors were already capable of successfully recovering both the free liquid cellulases and solid-bound cellulases. Ultrafiltration and addition of fresh substrate have been widely used for the recycling of the liquid fraction, while the addition of chemicals and the application of a pH shift have been shown equally efficient for the solid-bound fraction. A considerable amount of studies have been conducted characterizing the distribution of cellulases during the process of enzymatic hydrolysis. It was shown that the levels of cellulases change during hydrolysis between the liquid and solid fraction, toward a final equilibrium that dictates the amounts on each fraction. This equilibrium seems to strongly depend on the lignocellulosic material, more specifically the amounts of lignin and cellulose, the affinity of cellulases for the substrate, the pH, the presence of additives, and the temperature. Temperature most likely plays a key role on

Table 2 Activity recovered (% of original load) in each round after an ultrafiltration step compared to the activity recovered in the liquid after fermentation

Enzyme	Round	37 °C	50 °C
Cel7A	0	55	33
	1	46	28
	2	38	39
Cel7B	0	54	31
	1	47	31
	2	35	39
β -glucosidase	0	77	61
	1	77	67
	2	71	75

Adapted from Rodrigues et al. (2014)

R0, R1, and R2 refer to the initial step of hydrolysis and to the first and second rounds of enzyme recycling, respectively

this process since not only influences the maintenance of enzymatic activity over different rounds of hydrolysis, but also the degree of saccharification of the solid, and consequently, the amount of enzymes remaining bound to the final residue. Finally, it must be remarked that for each particular enzyme cocktail, operational features and biomass source, a customization of the recycling technology will have to be performed, given the specificity of each system.

Acknowledgments The authors acknowledge the financial support from FEDER and “Fundação para a Ciência e a Tecnologia” (FCT): GlycoCBMs Project PTDC/AGR-FOR/3090/2012–FCOMP-01-0124-FEDER-027948 and Strategic Project PEst-OE/EQB/LA0023/2013, Project “BioInd-Biotechnology and Bioengineering for improved Industrial and Agro-Food processes, REF. NORTE-07-0124-FEDER-000028” Co-funded by the Programa Operacional Regional do Norte (ON.2–O Novo Norte), QREN, FEDER and the PhD grant to DG (SFRH/BD/88623/2012) and ACR (SFRH/BD/89547/2012).

Conflict of interest The authors declare that they have no conflict of interest.

References

- Aden A, Foust T (2009) Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose* 16:535–545. doi:10.1007/s10570-009-9327-8
- Adsul MG, Singhvi MS, Gaikawai SA, Gokhale DV (2011) Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. *Bioresour Technol* 102(6):4304–4312. doi:10.1016/j.biortech.2011.01.002
- Almeida JRM, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Liden G (2009) Metabolic effects of furaldehydes and impacts on biotechnological processes. *Appl Microbiol Biotechnol* 82:625–638. doi:10.1007/s00253-009-1875-1
- Arantes V, Saddler JN (2010) Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnol Biofuels* 3:4. doi:10.1186/1754-6834-3-4
- Barsberg S, Selig MJ, Felby C (2013) Impact of lignins isolated from pretreated lignocelluloses on enzymatic cellulose saccharification. *Biotechnol Lett* 35(2):189–195. doi:10.1007/s10529-012-1061-x
- Bommarius AS, Katona A, Cheben SE, Patel AS, Ragauskas AJ, Knudson K, Pu Y (2008) Cellulase kinetics as a function of cellulose pretreatment. *Metab Eng* 6:370–381. doi:10.1016/j.ymben.2008.06.008
- Boraston A, Bray M, Burn E, Creagh AL, Gilkes N, Guarna M, Jervis E, Johnson P, Kormos J, McIntosh L, McLean B, Sandercock L, Tomme P, Haynes C, Warren A, Kilburn D (1998) The structure and function of cellulose binding domains. In: Claeyssen M, Nerinx W, Piens K (eds) *Carbohydrate from Trichoderma reesei and other microorganisms*. The Royal Society of Chemistry, Cambridge, United Kingdom, pp 139–146
- Chen G, Song W, Qi B, Lu J, Wan Y (2013) Recycling cellulase from enzymatic hydrolyzate of acid treated wheat straw by electroultrafiltration. *Bioresour Technol* 144:186–193. doi:10.1016/j.biortech.2013.06.089
- Demirabas A (2008) Products from lignocellulosic materials via degradation process. *Energy Sources A* 30(1):27–37. doi:10.1080/00908310600626705
- Desphande MV, Erikson K-E (1984) Reutilization of enzymes for saccharification of lignocellulosic materials. *Enzym Microb Technol* 6:338–340. doi:10.1016/0141-0229(84)90045-0
- Du R, Su R, Li X, Tantai X, Liu Z, Yang J, Qi W, He Z (2012) Controlled adsorption of cellulase onto pretreated comcob by pH adjustment. *Cellulose* 19:371–380. doi:10.1007/s10570-012-9653-0
- Eckard AD, Muthukumarappan K, Gibbons W (2013) Enhanced bioethanol production from pretreated corn stover via multi-positive effect of casein micelles. *Bioresour Technol* 135:93–102. doi:10.1016/j.biortech.2012.07.100
- Eriksson T, Börjesson J, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb Technol* 31:353–364. doi:10.1016/S0141-0229(02)00134-5
- Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RAJ (1991) Domains in microbial β -1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol Rev* 55:303–315
- Gourlay K, Arantes V, Saddler JN (2012) Use of substructure-specific carbohydrate binding modules to track changes in cellulose accessibility and surface morphology during the amorphogenesis step of enzymatic hydrolysis. *Biotechnol Biofuels* 5:51. doi:10.1186/1754-6834-5-51
- Gupta A, Verma JP (2015) Sustainable bio-ethanol production from agro-residues: a review. *Renew Sust Energ Rev* 41:550–567. doi:10.1016/j.rser.2014.08.032
- Hall M, Bansal P, Lee JH, Realf MJ, Bommarius AS (2010) Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *FEBS J* 277:1571–1582. doi:10.1111/j.1742-4658.2010.07585.x
- Hayes DJM (2013) Second-generation biofuels: why they are taking so long. *WIREs Energy Environ* 2:304–334. doi:10.1002/wene.59
- Himmel ME, Ding S, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 313:804–807. doi:10.1126/science.1137016
- Horn SJ, Vaaje-Kolstad G, Westereng B, Eijsink VG (2012) Novel enzymes for the degradation of cellulose. *Biotechnol Biofuels* 5(1):45. doi:10.1186/1754-6834-5-45
- Ishihara M, Uemura S, Hayashi N, Jellison J, Shimizu K (1991) Adsorption and desorption of cellulase components during enzymatic hydrolysis of steamed shirakamba (*Betula platyphylla* Skatchev) wood. *J Ferment Bioeng* 72:96–100. doi:10.1016/0922-338X(91)90316-9
- Jorgensen H, Eriksson T, Borjesson J, Tjerneld F, Olsson L (2003) Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. *Enzyme Microb Technol* 32:851–861. doi:10.1016/S0141-0229(03)00056-5
- Klein-Marcusschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch WH (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng* 109:1083–1089. doi:10.1002/bit.24370
- Klyosov AA, Mitkevich OV, Sinitsyn AP (1986) Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose. *Biochemistry* 25(3):540–542. doi:10.1021/bi00351a003
- Kumar D, Murthy GS (2013) Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. *Biotechnol Biofuels* 6(1):63. doi:10.1186/1754-6834-6-63
- Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *J Ind Microbiol Biotechnol* 35(5):377–391. doi:10.1007/s10295-008-0327-8
- Kumar L, Arantes V, Chandra R, Saddler J (2012) The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresour Technol* 103:201–208. doi:10.1016/j.biortech.2011.09.091
- Lee SB, Shin HS, Ryu DD, Mandels M (1982) Adsorption of cellulase on cellulose: effect of physicochemical properties of cellulose on

- adsorption and rate of hydrolysis. *Biotechnol Bioeng* 24(10):2137–2153. doi:10.1002/bit.260241003
- Lee D, Yu AHC, Saddler JN (1995) Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. *Biotechnol Bioeng* 45:328–336. doi:10.1002/bit.260450407
- Lindendam J, Haven M, Chylenski P, Jørgensen H, Felby C (2013) Recycling cellulases for cellulosic ethanol production at industrial relevant conditions: potential and temperature dependency at high solid processes. *Bioresour Technol* 148:180–188. doi:10.1016/j.biortech.2013.08.130
- Lu YP, 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* 98–100:641–654. doi:10.1385/ABAB:98-100:1-9:641
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* 66(3):506–577. doi:10.1128/MMBR.66.3.506-577.2002
- Maheshwari R, Bharadwaj G, Bhat MK (2000) Thermophilic fungi: their physiology and enzymes. *Microbiol Mol Biol Rev* 64(3):461–488. doi:10.1128/MMBR.64.3.461-488.2000
- Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol* 26(5):553–560. doi:10.1038/nbt1403
- McLean BW, Boraston AB, Brouwer D, Sanaie N, Fyfe CA, Warren RA, Kilburn DG, Haynes CA (2002) Carbohydrate-binding modules recognize fine substructures of cellulose. *J Biol Chem* 277:50245–50254. doi:10.1074/jbc.M204433200
- Mittal A, Katahira R, Himmel ME, Johnson DK (2011) Effects of alkaline or liquid-ammonia treatment on crystalline cellulose: changes in crystalline structure and effects on enzymatic digestibility. *Biotechnol Biofuels* 4:41. doi:10.1186/1754-6834-4-41
- Otter DE, Munro PA, Scott GK, Geddes R (1984) Elution of *Trichoderma reesei* cellulase from cellulose by pH adjustment with sodium hydroxide. *Biotechnol Lett* 6:369–374. doi:10.1007/BF00138007
- Otter DE, Munro PA, Scott GK, Geddes R (1989) Desorption of *Trichoderma reesei* cellulase from cellulose by a range of desorbents. *Biotechnol Bioeng* 34:291–298. doi:10.1002/bit.260340303
- Ouyang J, Liu B, Zhang M, Zheng Z, Yu H (2013) Enzymatic hydrolysis, adsorption, and recycling during hydrolysis of bagasse sulfite pulp. *Bioresour Technol* 146:288–293. doi:10.1016/j.biortech.2013.07.019
- Pakarinen A, Haven MØ, Djajadi DT, Várnai A, Puranen T, Viikari L (2014) Cellulases without carbohydrate-binding modules in high consistency ethanol production process. *Biotechnol Biofuels* 7:27. doi:10.1186/1754-6834-7-27
- Palonen H, Tenkanen M, Linder M (1999) Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. *Appl Environ Microbiol* 65:5229–5233
- Pan X, Xie D, Gilkes N, Gregg DJ, Saddler JN (2005) Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. *Appl Biochem Biotechnol* 121–124:1069–1079. doi:10.1385/ABAB:124-1-3:1069
- Pereira FB, Romani A, Ruiz HA, Teixeira JA, Domingues L (2014) Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. *Bioresour Technol* 161:192–199. doi:10.1016/j.biortech.2014.03.043
- Pribowo A, Arantes V, Saddler JN (2012) The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolyzing steam pretreated corn stover. *Enzyme Microb Technol* 50:195–203. doi:10.1016/j.enzmictec.2011.12.004
- Qi B, Chen X, Su Y, Wan Y (2011) Enzyme adsorption and recycling during hydrolysis of wheat straw lignocelluloses. *Bioresour Technol* 102:2881–2889. doi:10.1016/j.biortech.2010.10.092
- Qi B, Luo J, Chen G, Chen X, Wan Y (2012) Application of ultrafiltration and nanofiltration for recycling cellulase and concentrating glucose from enzymatic hydrolyzate of steam exploded wheat straw. *Bioresour Technol* 104:466–472. doi:10.1016/j.biortech.2011.10.049
- Reese RT (1976) History of the cellulase program at the U.S. Army Natick development center. *Biotechnol Bioeng Symp* 6:9–20
- Rodrigues AC, Leitão AF, Moreira S, Felby C, Gama M (2012) Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. *Bioresour Technol* 110:526–533. doi:10.1016/j.biortech.2012.01.140
- Rodrigues AC, Felby C, Gama M (2014) Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw. *Bioresour Technol* 156:163–169. doi:10.1016/j.biortech.2014.01.019
- Romani A, Pereira F, Johansson B, Domingues L (2015) Metabolic engineering of *Saccharomyces cerevisiae* ethanol strains PE-2 and CAT-1 for efficient lignocellulosic fermentation. *Bioresour Technol* 179:150–158. doi:10.1016/j.biortech.2014.12.020
- Rosales-Calderon O, Trajano HL, Duff SJB (2014) Stability of commercial glucanase and β -glucosidase preparations under hydrolysis conditions. *Peer J* 2:e402. doi:10.7717/peerj.402
- Sánchez OJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* 99(13):5270–5295. doi:10.1016/j.biortech.2007.11.013
- Schmaier AH, Silver L, Adams AL, Fischer GC, Munoz PC, Vroman L, Colman RW (1984) The effect of high molecular weight kininogen on surface-adsorbed fibrinogen. *Thromb Res* 33:51–67. doi:10.1016/0049-3848(84)90154-3
- Segato F, Damásio ARL, de Lucas RC, Squina FM, Prade RA (2014) Genomics review of holocellulose deconstruction by *Aspergilli*. *Microbiol Mol Biol Rev* 78(4):588–613. doi:10.1128/MMBR.00019-14
- Seiboth B, Ivanova C, Seidl-Seiboth V (2011) *Trichoderma reesei*: a fungal enzyme producer for cellulosic biofuels. In: Bernardes MAS (ed) *Biofuel Production-Recent Developments and Prospects*, In Tech, pp 309–340
- Seo D-J, Fujita 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. doi:10.1007/s10450-011-9340-8
- Shang Y, Su R, Huang R, Yang Y, Qi W, Li Q, He Z (2014) Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass. *Appl Microbiol Biotechnol* 98(12):5765–5774. doi:10.1007/s00253-014-5761-0
- Sipos B, Dienes D, Schleicher Á, Perazzini R, Crestini C, Siika-aho M, Réczey K (2010) Hydrolysis efficiency and enzyme adsorption on steam-pretreated spruce in the presence of poly(ethylene glycol). *Enzyme Microb Technol* 47:84–90. doi:10.1016/j.enzmictec.2010.05.010
- Sipponen MH, Pihlajaniemi V, Pastinen OLS (2014) Reduction of surface area of lignin improves enzymatic hydrolysis of cellulose from hydrothermally pretreated wheat straw. *RSC Adv* 4:36591–36596. doi:10.1039/C4RA06926A
- Široký J, Benians TAS, Russell SJ, Bechtold T, Knox JP, Blackburn RS (2012) Analysis of crystallinity changes in cellulose II polymers using carbohydrate-binding modules. *Carbohydr Polym* 89:213–221. doi:10.1016/j.biortech.2014.01.027

- Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol* 83(1):1–11. doi:10.1016/S0960-8524(01)00212-7
- Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen KMH (1993) High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. *Mol Gen Genet* 241:523–530. doi:10.1007/BF00279894
- Tu M, Saddler JN (2010) Potential enzyme cost reduction with the addition of surfactant during the hydrolysis of pretreated softwood. *Appl Biochem Biotechnol* 161:274–287. doi:10.1007/s12010-009-8869-4
- Tu M, Chandra RP, Saddler JN (2007a) Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. *Biotechnol Progr* 23:398–406. doi:10.1021/bp060354f
- Tu M, Chandra RP, Saddler JN (2007b) Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. *Biotechnol Progr* 23:1130–1137. doi:10.1021/bp070129d
- Tu M, Zhang X, Paice M, MacFarlane P, Saddler JN (2009) The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. *Bioresour Technol* 100:6407–6415. doi:10.1016/j.biortech.2009.06.108
- Viikari L, Alapuranen M, Puranen T, Vehmaanperä J, Siika-Aho M (2007) Thermostable enzymes in lignocellulose hydrolysis. *Adv Biochem Eng Biotechnol* 108:121–45. doi:10.1007/10_2007_065
- Waeonukul R, Kosugi A, Prawitwong P, Deng L, Tachaapaikoon C, Pason P, Ratanakhanokchai K, Saito M, Mori Y (2013) Novel cellulase recycling method using a combination of *Clostridium thermocellum* cellulosomes and *Thermoanaerobacter brockii* β -glucosidase. *Bioresour Technol* 130:424–430. doi:10.1016/j.biortech.2012.12.059
- Wang W, Zhu Y, Du J, Yang Y, Jin Y (2015) Influence of lignin addition on the enzymatic digestibility of pretreated lignocellulosic biomasses. *Bioresour Technol* 181:7–12. doi:10.1016/j.biortech.2015.01.026
- Yang B, Wyman CE (2006) BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. *Biotechnol Bioeng* 94:611–617. doi:10.1002/bit.20750
- Yang J, Zhang X, Yong Q, Yu S (2010) Three-stage hydrolysis to enhance enzymatic saccharification of steam-exploded corn stover. *Bioresour Technol* 101:4930–4935. doi:10.1016/j.biortech.2009.09.079
- Zhu Z, Sathitsuksanoh N, Zhang Y-HP (2009) Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulose desorption for potential recycling. *Analyst* 134:2267–2272. doi:10.1039/b906065k