A review of biological delignification and detoxification methods for lignocellulosic bioethanol production

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A review of biological delignification and detoxification methods for lignocellulosic bioethanol production

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Abstract

Future biorefineries will integrate biomass conversion processes to produce fuels, power, heat and value-added chemicals. Due to its low price and wide distribution, lignocellulosic biomass is expected to play an important role toward this goal. Regarding renewable biofuel production, bioethanol from lignocellulosic feedstocks is considered the most feasible option for fossil fuels replacement since these raw materials do not compete with food or feed crops. In the overall process, lignin, the natural barrier of the lignocellulosic biomass, represents an important limiting factor in biomass digestibility. In order to reduce the recalcitrant structure of lignocellulose, biological pretreatments have been promoted as sustainable and environmentally friendly alternatives to traditional physico-chemical technologies, which are expensive and pollute the environment. These approaches include the use of diverse white-rot fungi and/or ligninolytic enzymes, which disrupt lignin polymer and facilitate the bioconversion of the sugar fraction into ethanol. As there is still no suitable biological pretreatment technology ready to scale up in an industrial context, white-rot fungi and/or ligninolytic enzymes have also been proposed to overcome, in a separated or *in situ* biodetoxification step, the effect of the inhibitors produced by non-biological pretreatments. The present work reviews the latest studies about the application of different microorganisms or enzymes as useful and environmentally friendly delignification and detoxification technologies for lignocellulosic biofuel production. This review also points out the main challenges and possible ways to make these technologies a reality for the bioethanol industry.

Keywords: lignocellulose, bioethanol, biodelignification, biodetoxification, white-rot fungi, ligninolytic enzymes, biorefinery.

Introduction

The continuous increase in the world energy demand requires the development of sustainable alternatives to non-renewable sources of energy. Biomass facilities and biorefineries to produce renewable fuels and products represent alternatives to gradually replace the present industry based on fossil fuels (Ragauskas *et al.*, 2006; Himmel *et al.*, 2007; Martínez *et al.*, 2009; FitzPatrick *et al.*, 2010). In 2011, the bioethanol production was worldwide more than 10^{11} liters (Lichts, 2012). Most of this production, however, comes from sugar and starch-based raw materials such as sugarcane and cereal grain, whilst lignocellulosic bioethanol plays a minor role. Lignocellulosic raw materials include agricultural wastes, forest products or energy crops and constitute abundant, widely distributed and cheap feedstocks for biofuels production (Berndes *et al.*, 2001; Taherzadeh and Karimi, 2008).

Lignocellulose production is estimated on more than 10^9 tons per year, being the major renewable organic matter in nature (Reddy and Yang, 2005). The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. From the biochemical point of view, high amount of sugars present in cellulose and hemicellulose can be chemically, using acid as catalyst, or enzymatically hydrolysed and converted into biofuels by a fermentation process (Taherzadeh and Karimi, 2007a, 2007b; Tomás-Pejó *et al*., 2008). In contrast, the third major component of lignocellulosic materials, lignin, is not constituted of fermentable sugars and plays an important role providing a recalcitrant structure difficult to disrupt (Brett and Waldron, 1996). These structural properties of lignocellulose make a pretreatment step essential to improve its digestibility and increase the release of fermentable sugars. Current leading pretreatment technologies are based on physico-chemical processes, which in most cases involve high-energy demand, high-capital investment, some sugar degradation and

generation of inhibitory compounds that affect the downstream hydrolysis and fermentation steps (Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Klinke *et al*., 2004; Panagiotou and Olsson, 2007; Alvira *et al*., 2010; Tomás-Pejó *et al*., 2011). In this context, different biological approaches have been developed as environmentally friendly alternatives to alter lignin (biodelignification) and to reduce the amount of inhibitors (biodetoxification) produced after the physico-chemical pretreatment during lignocellulosic bioethanol production (Isroi *et al*., 2011; Parawira and Tekere, 2011). These technologies represent interesting approaches to improve the efficiency of the bioconversion processes and to overcome barriers in the scale-up and commercialization of renewable biorefineries.

Biological delignification

Breaking down lignocellulosic sugars enzymatically is advantageous compared to acid hydrolysis due to its higher conversion efficiency, lower process energy requirements and lower formation of fermentation inhibitors (Taherzadeh and Karimi, 2007b; Tomás-Pejó et al., 2008). Many physico-chemical, structural, and compositional factors, however, make the native lignocellulosic biomass recalcitrant and difficult to be hydrolyzed by enzymes. Among these factors, lignin plays a fundamental role, being the physical barrier that hinders the accessibility of sugar fraction (Jørgensen *et al.*, 2007). To improve the efficiency of the enzymatic hydrolysis, a delignification process may enhance the accessibility of enzymes by increasing the number of pores and the available surface area (Yu *et al.*, 2011). In this context, several pretreatment technologies have been developed to overcome the lignin barrier. These pretreatments produce other effects apart from increasing the digestibility of lignocellulose, such as hemicellulose solubilisation and/or degradation (wet oxidation, acid pretreatment and steam explosion) and cellulose decrystallization (ammonia fiber explosion)

(Alvira *et al.*, 2010; Tomás-Pejó *et al.*, 2011). Biological delignification is a promising technology due to the low environmental impact, higher product yield, mild reaction conditions, few side reactions, less energy demand and less reactor requirements to resist pressure and corrosion. Moreover, biodelignification also avoids the formation of degradation compounds that inhibit the subsequent steps.

Different microorganisms, including bacteria and fungi, can be involved in lignin degradation but only the so-called "white-rot" basidiomycetes are able to depolymerize and mineralize lignin efficiently (Martínez *et al.*, 2005; Isroi *et al*., 2011). These fungi have developed an extracellular and unspecific oxidative enzymatic system for lignin degradation (Figure 1). The process involves different enzymatic activities such as peroxidases, oxidases and reductases but also low molecular mass compounds that mediate the action of these enzymes (Martínez *et al.*, 2005).

Laccases have been described for many years in plants, fungi and bacteria (Mayer and Staples, 2002). These enzymes are multicopper oxidases that catalyze the oxidation of substituted phenols, anilines, and aromatic thiols to their corresponding radicals by the extraction of one electron, used to reduce oxygen to water. The low redox potential of laccases only allows the direct oxidation of phenolic lignin units, which represent a small percentage of the polymer (Mayer and Staples, 2002). However, in the presence of low molecular weight compounds that form stable radicals and act as redox mediators, laccases can also oxidise non-phenolic lignin units (Bourbonnais and Paice, 1990).

Ligninolytic peroxidases are high redox potential hemeperoxidases that require H_2O_2 as co-substrate for the enzymatic catalysis and include lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). The LiPs and MnPs were first described in *Phanerochaete chrysosporium* (Martínez, 2002). LiPs are able to oxidize directly non-phenolic and phenolic lignin units, whereas MnPs generate Mn^{3+} acting preferentially on

phenolic units, but also on non-phenolic units via lipid peroxidation reactions (Martínez *et al.*, 2005). VP was described in *Pleurotus eryngii* as a new peroxidase which shares catalytic properties with LiP and MnP (Ruiz-Dueñas *et al.*, 1999). The H_2O_2 required for ligninolytic peroxidases is produced by oxidases, such as glyoxal oxidase, a copper radical enzyme described in *P. chrysosporium* (Kersten, 1990) and arylalcohol oxidase described in *P. eryngii* (Guillén *et al.*, 1992). Finally, reductases such as aryl-alcohol dehydrogenases and quinone reductases catalyze the reduction of phenolic products derived from lignin degradation, avoiding their subsequent repolymerization (Guillén *et al.*, 1997).

Lignin degradation by this non-specific oxidative system makes "white-rot" fungi useful for a wide range of biotechnological applications in industrial uses of cellulosic biomass. Commonly used in the pulp and paper industry for biopulping or biobleaching, these fungi and their ligninolytic enzymes are currently attracting much attention as an alternative or an additional pretreatment step to traditional physico-chemical methods for enhancing enzymatic saccharification of lignocellulosic biomass (Ruiz-Dueñas and Martínez, 2009).

Microbial delignification processes

The biological pretreatment where the microbial delignification takes place, consists of a solid state fermentation process in which microorganisms grow on the lignocellulosic biomass selectively degrading lignin (and in some cases hemicellulose), while cellulose is expected to remain intact. For an efficient solid state fermentation, there are different factors to be considered such as nutrient addition, moisture content, aeration, pH, temperature, inoculum size or the microorganism strain (Isroi *et al*., 2011). In terms of nutrient requirements, nitrogen, Mn^{+2} and Cu^{+2} have an important role modifying the expression of different ligninolytic activities. For instance, the presence of Mn^{+2} can influence the production levels of MnP and LiP in favour of MnP as the dominant enzyme. On the other

144 hand, solid state fermentations are usually performed at moisture content about 45-85% with
145 an inoculum level of 1-10 mg/g substrate (dry weight), at pH 4-5 and temperatures between
146 15 and 40 ºC for 1-12 weeks, depending on the strain used (Itoh *et al*., 2003; Lee *et al*., 2007;
147 Muñoz *et al*., 2007; Singh *et al*., 2008; Wan and Li, 2010 Canam *et al*., 2011; Salvachúa *et*
148 *al*., 2011).

149 The patterns of cell wall deconstruction by "white-rot" fungi vary among species and
150 strains. Several fungi have been tested using different lignocellulosic feedstocks as a
151 pretreament method for bioethanol production, obtaining delignification efficiencies from 6%
152 to 92% (Supplementary Table 1).

153 Microbiological delignification can alter or remove lignin, which leads to an increased
154 in the number of pores and the available surface area and also to a reduction in non-
155 productive binding of cellulases. Consequently, the subsequent acid or enzymatic hydrolysis
156 can be improved. Lee *et al.* (2007) reported an increase of 21% in the enzymatic hydrolysis
157 yield of Japanese red pine chips (*Pinus densiflora*) after a delignification of 14.5% by *S.*
158 *hirsutum* treatment compared to non-pretreated samples. On the other hand, Gupta *et al.*
159 (2011) described a delignification of 7.7-11.9% and 6.9-8.4% of mesquite (*Prosopis juliflora*)
160 and Spanish flag (*Lantana camara*), respectively, after a solid state fermentation using the
161 fungus *P. cinnabarinus.* In those cases, microbial delignification increased 21.4-42.4% the
162 sugar recovery after the acid hydrolysis and 21.1–25.1% after the enzymatic hydrolysis.
163 Moreover, the phenolic content measured after the acid hydrolysis decreased 18.5-19.9% in
164 both materials.

165 The positive effect observed in the fermentable sugars yield together with the lower
166 amount of inhibitory compounds formed during microbial delignification could also result in
167 an increase of the final ethanol production. In this context, Kuhar *et al.* (2008) pretreated
168 wheat straw and mesquite by solid state fermentation with the isolated basidiomycete fungus

RCK-1, followed by an acid hydrolysis and the fermentation with *Pichia stipitis*, increasing 33% and 10% the ethanol yield, and 80% and 57% the volumetric productivity, respectively.

Although only "white-rot" basidiomycetes can degrade lignin extensively, some ascomycetes can also colonize lignocellulosic biomass. For instance, *Trichoderma reesei* and *Aspergillus terreus* have been employed obtaining a delignification performance of 60% and 92% respectively (Singh *et al.*, 2008). Besides fungi, certain bacterial strains such as *Bacillus macerans*, *Cellulomonas cartae*, *Cellulomonas uda* and *Z. mobilis* have also shown delignification abilities yielding lignin degradation up to 50% (Singh *et al.*, 2008).

In addition to those factors mentioned before, the incubation time and the cellulolytic activity of the microorganism are important elements that must be taken into account for an adequate microbial delignification. Residence time can vary from 7 to 84 days (Canam *et al.*, 2011; Salvachúa *et al.*, 2011). The lowest residence time has been reported by Salvachúa *et al.* (2011) who observed 17% and 24% lignin reduction in wheat straw, using the fungi *P. tigrinus* and *T. versicolor*, respectively, after 7 days of treatment. However, after increasing the residence time from 7 to 21 days, the authors observed a decrease in the lignin content up to 47% and 46%, respectively with these fungi. On the other hand, the cellulolytic activity of the microorganism should be low in order to reduce the sugar loss during biological pretreatment. In this context, the use of strains such as *C. subvermispora* which consumes less than 6% of total sugars (Wan and Li, 2010), or the employment of genetically modified microorganisms such as the cellobiose dehydrogenase-deficient *T. versicolor* strain (Canam *et al.*, 2011), are promising options for an optimal microbial pretreatment.

By combining microbial delignification with other pretreatment methods, the delignification efficiency can be improved, whilst the severity conditions, the overall pretreatment time and the chemical and energy requirements of non-biological pretreatment can be reduced. Using this synergistic strategy, Zhang *et al.* (2008) combined steam explosion

and microbial delignification with *T. versicolor*, decreasing the lignin content of wheat straw up to 75% compared with the 31% obtained after the treatment with *T. versicolor* alone. Microbial delignification has also been combined with mild alkaline pretreatment, in which case, *I. lacteus* increased the lignin loss of cornstalks from 76% to 80% and improved significantly the enzymatic saccharification (Yu *et al.*, 2010). Regarding the ethanol production, Muñoz *et al.* (2007) combined organosolv pretreatment and biological delignification of wood chips from Monterey pine (*Pinus radiata*) and silver wattle (*Acacia dealbata*). The combined treatment increased the ethanol yield (calculated as percentage of the theoretical) obtained with *Saccharomyces cerevisiae* as fermenting microorganism from 38% to 55% and from 62% to 69%, respectively, in a separated hydrolysis and fermentation (SHF) process. Moreover, in a simultaneous saccharification and fermentation (SSF) process the ethanol yield increased from 10% to 65% and 77% to 82%, respectively, compared to the organosolv pretreatment alone. On the other hand, the combination of microbial delignification and ethanolysis increased 1.6 times the ethanol yield (calculated as percentage of the theoretical) and allowed save 15% of the electricity needed for the ethanolysis (Itoh *et al*., 2003).

In spite of the remarkable advantages in saccharification and ethanol production of microbial delignification, the long time required for this pretreatment as well as the sugars consumption are main challenges to overcome. Exploring new microorganisms and improving process conditions are important aspects to achieve better results. Although, delignification by using microorganisms is still far away from the industrial scale, the combination with other physico-chemical pretreatment can become a feasible prospect.

Enzymatic delignification processes

The use of ligninolytic enzymes instead of microorganism populations is another feasible alternative for the delignification of lignocellulose. This strategy is substrate specific and offers the possibility to increase the reaction rates and the delignification efficiency, reducing the process from weeks to hours with no carbohydrate consumption (Vivekanand *et al*., 2008). Enzymatic delignification can be performed either by using a culture supernatant with different ligninolytic activities or with a prepared solution with a single purified and concentrated enzyme (Lu *et al*., 2010; Kuila *et al*., 2011a, 2011b; Moilanen *et al*., 2011). For the enzymatic delignification there is no need of nutrient supplementation and the optimal pH and temperature can have wider ranges (pH 3-8 and 25-80 ºC, respectively) (Ibarra *et al*., 2006; Kuila *et al*., 2011a, 2011b). The liquid:solid ratio and the enzyme loading at which the delignification is carried out are, however, two important factors (Kuila *et al*., 2011a, 2011b; Mukhopadhyay *et al*., 2011). Delignification assays have been performed up to 2:1 liquid:solid ratio (Kuila *et al*., 2011a, 2011b; Mukhopadhyay *et al*., 2011), but lower solid charges (20:1 or even less) are usually used (Moilanen *et al*., 2011; Qiu and Chen, 2012; Palonen and Viikari, 2004). On the other hand, enzymes can be added at low (0.03-10 IU/g) or high (4000-80000 IU/g) loadings, depending on process optimization (Qiu and Chen, 2012; Chen *et al*., 2012).

Among different ligninolytic enzymes, laccases, especially in the form of laccase-mediator system (LMS), have been widely used for different industrial applications, including bleaching and depitching in the paper industry, organic synthesis, polymer modification, and degradation and detoxification of recalcitrant environmental pollutants (Jurado *et al.*, 2011). In the recent years, the application of laccases for bioethanol production has gained considerable attention.

The modification or partial removal of lignin by laccases has been shown to be effective for improving enzymatic hydrolysis of different lignocellulosic materials (Chen *et*

al., 2012; Gutiérrez *et al.*, 2012; Kuila *et al.*, 2011a, 2011b; Li *et al.*, 2012; Lu *et al.*, 2010; Martín-Sampedro, *et al.*, 2012; Moilanen *et al.*, 2011; Mukhopadhyay *et al.*, 2011; Qiu and Chen, 2012; Palonen and Viikari, 2004; Yang *et al.*, 2011). In this sense, different strategies have been assayed, either using laccase enzyme alone or in combination with mediators (LMS) (Table 1). In the case of LMS, a suitable redox mediator must be stable in its oxidized and reduced states and both forms should not inhibit the catalytic activity (Morozova *et al*., 2007). These mediator compounds are simultaneously added with laccases, enhancing the enzyme action and broadening the range of targeted substrates. In a first step, the mediator is oxidized by laccase to an oxidized radical which, afterwards, is reduced to its initial form by the compound to be oxidized (Morozova *et al*., 2007).

Pleurotus sp. laccase was used by Mukhopadhyay *et al.* (2011) to treat a milled material from castor oil plant (*Ricinus communis*), obtaining an optimum delignification of 86% after 4 h. This treatment increased the saccharification performance almost 3 fold. Similar lignin loss (84-89%) was obtained after 8 h of treatment using the same laccase and milled Indian thorny bamboo (*Bambusa bambos*) (Kuila *et al.*, 2011a) or Spanish flag (Kuila *et al.*, 2011b). As a consequence, similar saccharification rates with reduced cellulase loading were obtained.

In the same way than microbial delignification, enzymatic delignification has been combined with other pretreatment technologies. Together with alkali pretreatments, laccase can enhance the saccharification yields although delignification does not significantly improve (Li *et al.*, 2012; Yang *et al.*, 2011). Li *et al.* (2012) showed that the porosity and surface area of corn straw increased significantly after combining 1.5% NaOH and *Trametes hirsuta* laccase, doubling the saccharification yield compared to alkaline treatment alone. Yang *et al.* (2011) observed the same effect after combining alkali and *Ganoderma lucidum*

laccase pretreatment on Indian colza (*Brassica campestris*) straw, obtaining 1.7 fold higher saccharification yields than with the alkaline treatment alone.

Laccase treatment on steam-exploded materials has also been described (Moilanen *et al.*, 2011; Qiu and Chen, 2012; Palonen and Viikari, 2004). Qiu and Chen (2012) reported the oxidation of lignin from steam-exploded wheat straw by *Sclerotium sp*. laccase, which resulted in the formation of micropores on the material and enhanced the accessibility of cellulose. On the other hand, Palonen and Viikari (2004) observed that *T. hirsuta* laccase enhanced 13% the saccharification yield of steam-exploded spruce by lignin modification. The authors described a reduction of the hydrophobicity of lignin and an eventual change of the polar characteristics of the surface to a negative charge by increasing the number of carboxylic groups. These modifications of pretreated fibers led to a reduction of the unproductive binding of cellulases, increasing, consequently, the saccharification process. Similar results during the enzymatic hydrolysis step were obtained by Moilanen *et al.* (2011) when acid steam-exploded spruce treated with *Cerrena unicolor* laccase was used as substrate; however, a contradictory effect was observed in acid steam-exploded giant reed treated with the same laccase. While laccase treatment improved the enzymatic hydrolysis of steam-exploded spruce by 12%, the same treatment reduced the hydrolysis yield of steam-exploded giant reed by 17%. This contradictory effect was attributed to an increase in the amount of cellulases that were non-specifically bound to the lignin or the strengthening in the lignin-carbohydrate complexes after laccase treatment in giant reed. Structural and compositional differences between softwood lignin and the lignin of annual plants can play an important role that could modulate laccase action, leading to opposite results.

Regarding laccases in the form of LMS, Gutiérrez *et al.* (2012) has recently described the ability of *Trametes villosa* laccase, in combination with 1-hydroxybenzotriazole (HBT) as synthetic mediator and an alkaline extraction, to remove lignin (30-50%) from both milled

292 eucalyptus wood and elephant grass. Consequently, the enzymatic treatment increased the
293 glucose production by 61% and 12%; and the ethanol concentration by 4 and 2 $g \cdot L^{-1}$ from
294 both lignocelullosic materials fermented with *S. cerevisiae* Red Star. High delignification
295 yield (up to 97%) was observed with *Pycnoporus sanguineus* laccase and violuric acid (VIO)
296 as mediator on both wheat straw and corn stover pretreated by liquid hot water (Lu *et al.*,
297 2010). In addition to lignin removal, the improvement of saccharification due to lignin
298 modification by LMS has also been reported (Chen *et al.*, 2012; Martín-Sampedro *et al.*,
299 2012; Palonen and Viikari, 2004). In this context, Chen *et al.* (2012) described a marked
300 lignin modification on ensiled corn stover using *T. versicolor* laccase and HBT as mediator,
301 which led to improve the downstream cellulose hydrolysis about 7%. On the other hand,
302 Martín-Sampedro *et al.* (2012) observed an increase of glucose yield in the hydrolysis step,
303 from 24.7% to 27.1%, when steam-exploded eucalyptus was treated with *Myceliophtora*
304 *thermophila* laccase in combination with HBT. By using N-hydroxy-N-phenylacetamide
305 (NHA) as mediator, Palonen and Viikari (2004) increased the saccharification yield from 13%
306 to 21% compared to the treatment with *T. hirsuta* laccase alone.
307 It can be inferred that the use of ligninolytic enzymes such as laccases or LMS for
308 biodelignification shows similar advantages than microorganisms in terms of improvements
309 on saccharification and fermentation, reducing very significantly the treatment time and
310 avoiding sugar consumption. Nevertheless, the cost of producing the enzyme and the use of
311 synthetic mediators still represent the main disadvantages that hinder their application in the
312 current bioethanol production process at large scales. To overcome these drawbacks, different
313 alternatives have been shown to reduce final production costs. For instance, the synthetic
314 mediators can be replaced by natural mediators derived from lignin (Martínez *et al.*, 2009)
315 and lignocellulosic feedstocks can be employed for the growth of enzyme-producing
316 microorganisms instead of the expensive conventional carbon sources (Jun *et al.*, 2011).

317 Moreover, an *in situ* enzyme production offers also the possibility of using the same raw
318 material that is going to be delignified, providing the optimal enzymatic activities for the
319 biodelignification step.
320

321 ## Biological detoxification

322 Although biological pretreatments show environmental advantages, these methods are
323 still non-viable for large-scale bioethanol production. In this context, physico-chemical
324 technologies such as hydrothermal processes have higher potential in the short term as cost-
325 effective methods at industrial scale. These pretreatments have been tested for ethanol
326 production at laboratory, pilot and demonstration scale with a wide range of raw materials,
327 including softwood (Cara *et al.*, 2006; Monavari *et al.*, 2010), hardwood (Oliva *et al.*, 2003)
328 and herbaceous crops (Ballesteros *et al.*, 2006). The main disadvantage of these pretreatments
329 is the formation of different inhibitory compounds, predominantly derived from lignin and
330 hemicellulose degradation, that can affect enzymatic hydrolysis as well as fermentation steps
331 (Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Klinke *et al.*, 2004; Panagiotou and Olsson,
332 2007). According to their nature, inhibitory compounds can be classified into furan
333 derivatives, weak acids or phenolic compounds. The most common furan derivatives are 2-
334 furaldehyde (furfural) and 5-hydroxymethylfurfural (HMF), which come from degradation of
335 sugars (pentoses and hexoses respectively) contained in cellulose and hemicellulose. Among
336 weak acids, acetic acid is originated from acetyl groups of hemicelluloses whereas formic
337 acid and levulinic acid come from further degradation of furfural and HMF. Finally, a wide
338 variety of phenolic compounds are released from lignin.
339 One possibility that has been typically performed to eliminate soluble inhibitory
340 compounds is filtering and washing the pretreated material. However, these processes involve

additional and expensive steps, waste of water and loss of soluble sugars. In this context, several detoxification processes have been developed to overcome these obstacles and reduce the inhibitory potential of pretreated materials. Some detoxification processes can also require additional equipments and generate other residual by-products. For that, they need to be adapted to the process configuration and evaluated according to the fermentation conditions.

Although different physical (evaporation, membrane separations) and chemical detoxification (neutralization, overliming, actived charcoal, ion exchange) processes have been described, biological methods that use either microorganisms or enzymes offer many advantages such as mild reaction conditions, no chemical addition, fewer side-reactions and low energy requirements (Parawira and Tekere, 2011).

Microbial detoxification processes

Microbial detoxification involves the utilization of microorganisms to decrease the inhibitory effect of the degradation compounds formed during physico-chemical pretreatments of lignocellulose. The factors that must be considered for an efficient microbial detoxification process are mostly the same than for microbial delignification: optimal nutrient addition, pH (4-6), temperature (25-50 ºC), treatment time (12-144 h), inoculum size (1-10% (v/v) or 0.5-10 g/L (dry weight)) and microorganism strain (Palmqvist *et al*., 1997; Larsson *et al*., 1999; López *et al*., 2004; Nichols *et al*., 2008, 2010; Okuda *et al*., 2008; Zhang *et al*., 2010; Fonseca *et al*., 2011; Yu *et al*., 2011).

One possible strategy for microbial detoxification is to carry out an additional step using fungi, bacteria or yeast prior to ethanol fermentation. Among different microorganisms, fungi such as *T. reesei* or *Coniochaeta ligniaria* have been the most studied for this purpose. Palmqvist *et al.* (1997) observed a considerable removal of phenols, furans, and weak acids after growing *T. reesei* on the hydrolysate obtained from acid steam-exploded willow,

366 improving both the ethanol productivity and yield when using *S. cerevisiae* as fermenting
367 microorganism. *T. reesei* was also used by Larsson *et al.* (1999) for improving the
368 fermentability of diluted-acid hydrolysate of spruce, observing a removal of furans and a
369 small percentage of phenols without affecting the concentration of weak acids. López *et al.*
370 (2004) isolated a new fungus *C. ligniaria* NRRL30616 with the ability to metabolize furfural
371 and HMF as well as aromatic and aliphatic acids and aldehydes. This strain was further used
372 by Nichols *et al*. (2008, 2010) in dilute-acid hydrolysates from corn stover, alfalfa stems, reed
373 canarygrass and switchgrass, favoring xylose utilization by *Saccharomyces* sp. LNH-ST
374 (Nichols *et al.*, 2008) and reducing the lag phase of *S. cerevisiae* D5a (Nichols *et al.*, 2010) in
375 the subsequent ethanol fermentations. The fungus strain *Amorphotheca resinae* ZN1 was also
376 able to degrade all kinds of inhibitory compounds present in steam-exploded corn stover and
377 dilute-acid pretreated corn stover, rice straw, wheat straw and rape straw (Zhang *et al.*, 2010).
378 *Aspergillus nidulans* FLZ10 produced a complete degradation of furfural and HMF and a
379 partial removal of formic acid and acetic acid when was used on steam-exploded corn stover
380 (Yu *et al.*, 2011).

381 Another interesting feature that may be exploited is that some fungi can produce
382 hydrolytic enzymes while detoxification takes place. Palmqvist *et al*. (1997) reported a
383 cellulase activity of 0.2 $FPU \cdot mL^{-1}$ after the detoxification of willow hydrolysate with *T.*
384 *reesei*. This activity was enhanced by addition of solid pretreated willow as cellulose source
385 to 0.6 $FPU \cdot mL^{-1}$. Using *A. nidulans* FLZ10 it was obtained an activity of 0.2 $FPU \cdot mL^{-1}$
386 without cellulose addition and 0.5 $FPU \cdot mL^{-1}$ when cellulose was added into the broth (Yu *et*
387 *al*., 2011).

388 Bacteria and yeasts have also been used for detoxification purposes in a lesser extent.
389 The thermophilic bacterium *Ureibacillus thermophaercus* was used by Okuda *et al.* (2008)
390 which removed furfural and HMF from a synthetic hydrolysate, and the phenolic compounds

391 from diluted acid waste house wood. López *et al.* (2004) isolated five bacteria related to
392 *Methylobacterium extorquens*, *Pseudomonas sp*., *Flavobacterium indologenes*, *Acinetobacter*
393 *sp*., and *Arthrobacter aurescens* capable of depleting ferulic acid, HMF and furfural from a
394 defined mineral medium. An example of a microbial detoxification step by yeast prior to
395 fermentation was reported by Fonseca *et al.* (2011), who described the capacity of
396 *Issatchenkia occidentalis* CCTCC M 206097 for removing syringaldehyde, ferulic acid,
397 furfural and HMF from hemicellulosic hydrolysate of sugarcane bagasse.
398 According to previous reported data, separated microbial detoxification has been
399 usually performed in the liquid fraction or hydrolysates. The use of the whole pretreated
400 material (slurry), however, offers different advantages instead: 1) there is no need of
401 equipment to separate the liquid and solid fractions, therefore the processing time and costs
402 are reduced; 2) sugar loss during the washing of the material is avoided, which, in turn,
403 decreases the wastewater generated; and 3) in the case of using an enzyme-producing
404 microorganism for biodetoxification, the presence of cellulose enhances the production of
405 hydrolytic enzymes, decreasing the doses of extra cellulase addition for the enzymatic
406 saccharification.
407 *In situ* microbial detoxification can also be performed due to the natural ability of
408 diverse fermenting microorganisms to remove some inhibitory compounds. For instance, most
409 of *S. cerevisiae* strains can convert furan derivatives into less inhibitory compounds
410 (Schneider, 1996; Palmqvist and Hahn-Hägerdal, 2000a; Thomsen *et al.*, 2009; Ferreira *et al.*,
411 2011): furfural can be reduced to furfuryl alcohol and HMF to 2,5-HMF alcohol (Liu *et al.*,
412 2005). *S. cerevisiae* also possesses the capacity to metabolize some phenolic compounds due
413 to the presence of a phenylacrylic acid descarboxylase that catalyses a decarboxylation step
414 by which aromatic carboxylic acids are converted to the corresponding vinyl derivatives
415 (Goodey and Tubbs, 1982). Schneider (1996) reported the selective removal of acetic acid

from hardwood-spent sulfite liquor using the mutant yeast *S. cerevisiae* YGSCD 308.3, which led to the bioconversion of all sugars to ethanol. Thomsen *et al.* (2009) described the capacity of *S. cerevisiae* for detoxifying hydrolysates from hydrothermal pretreated wheat straw by degradation of furfural and phenolic aldehydes. In addition to *S. cerevisiae*, the ability to remove different inhibitory compounds has also been described in *P. stipitis* strains, such as *P. stipitis* CBS5773, which removed furfural and HMF when growing in an acid hydrolysate from silver wattle (Ferreira *et al.*, 2011).

Taking advantages of the inherent ability of some microorganisms to reduce the inhibitors and/or their natural tolerance toward these compounds, the better fermentability of the lignocellulosic broths could be induced by different strategies (Supplementary Table 2):

- *Co-culture*.

  Free-living microorganisms interact by competing or helping each other (consortia). In the latter case, consortia are interactive groupings of microorganisms, ranging from defined dual species communities to undefined multispecies aggregations (Zuroff and Curtis, 2012). This ability to grow simultaneously in the same media can be exploited in the bioconversion of glucose and other sugars into ethanol. However, the main drawback of utilizing uncharacterized or defined consortia for biofuel production is the high complexity when producing a defined product (Zuroff and Curtis, 2012). With the aim of improving bioethanol production, a co-culture of *A. nidulans* FLZ10 together with *S. cerevisiae* was employed by Yu *et al.* (2011) to simultaneously detoxify and ferment steam-exploded corn stover. The final ethanol production increased more than 3-fold by using both microorganism, reaching a concentration of 34 $g \cdot L^{-1}$. Furthermore, due to the capacity of *A. nidulans* FLZ10 to produce hydrolytic enzymes, saccharification was improved; solubilising the 95% of the total input glucose.

- *Evolutionary engineering or adaptation.*

Based on the tolerance of several fermenting microorganisms to some inhibitory compounds, adaptation by the constant exposure of the microorganism to sublethal inhibitory concentrations could increase the detoxification rates and improve fermentation yields. In this context, different microorganisms have been evolved to overcome the inhibition and improve their fermentation abilities in several pretreated materials. Thus, Liu *et al.* (2005) developed new evolved strains (*S. cerevisiae* 307-12H60 and 307-12H120 and *P. stipitis* 307 10H60) that showed more tolerance to furfural and HMF, after several cultures in synthetic media containing increasing concentration of inhibitors. Such strains grew and metabolized glucose with faster rates than the control strain. Tian et al., (2010) used the evolved *S. cerevisiae* Y5 strain, showing good inhibitor tolerance and the capacity of metabolizing furans, while maintaining high ethanol productivity. On the other hand, Stoutenburg *et al.* (2011) developed several strains from the parental *P. stipitis* after its adaptation on wood hydrolysate. The resulting yeast variants were able to produce 75% more ethanol in comparison to the wild type. In the same way, Yang *et al.* (2011) also used an evolved *P. stipitis* strain to ferment enzymatic hydrolysate from steam-exploded corn stalk, obtaining more than 92% of the theoretical ethanol yield.

Evolutionary engineering has also been investigated with xylose-fermenting yeasts. In this context, Martín *et al.* (2007) obtained an evolved xylose-utilizing *S. cerevisiae* strain by its cultivation for 353 h in increasing inhibitory concentrations of sugarcane bagasse hydrolysates. Compared to the parental strain, the evolved microorganism showed better furfural conversion rate, ethanol yield (from 0.18 $g \cdot g^{-1}$ to 0.38 $g \cdot g^{-1}$) and productivity (from 1.2 $g \cdot L^{-1} \cdot h^{-1}$ to 2.6 $g \cdot L^{-1} \cdot h^{-1}$), using bagasse hydrolysates as fermentation broth. With a similar strategy, Tomás-Pejó *et al.* (2010) improved the

xylose-fermenting *S. cerevisiae* F12 for bioethanol production, allowing the growth of the microorganism at higher substrate loadings. After the adaptation, the evolved strain was more tolerant to the inhibitory compounds present in the liquid fraction obtained from steam-pretreated wheat straw, observing an improvement of 65% and 20% in xylose consumption and final ethanol concentration, respectively, compared to the parental strain.

Adaptation can be performed either in batch or continuous culture. In this context, Koppram *et al*. (2012) obtained different evolved xylose-fermenting strains from the parental *S. cerevisiae* TMB3400 using both operational modes. All generated strains showed higher tolerance to the inhibitors present in the spruce hydrolysate with higher detoxification rates for HMF and furfural, enhancing sugars consumption and shortening the overall fermentation time.

- *Genetic engineering.*

Genetic modification offers the possibility to introduce a particular characteristic that is not present naturally in a certain microorganism. The improvement of yeast tolerance to inhibitory compounds has been achieved by overexpressing homologous or heterologous genes encoding enzymes as well as by random mutagenesis. Some authors have reported improved yeast detoxification rates of furfural and HMF by overexpression of different genes such as reductase and dehydrogenase encoding genes. Petersson *et al.* (2006) attributed to an NADPH-dependent alcohol dehydrogenase enzyme (ADH6p) the reduction of furfural and HMF in *S. cerevisiae*. In this context, the overexpression of the corresponding ADH6p gene led to a strain with at least four times higher specific uptake rate of HMF. This strain was further used by Almeida *et al.* (2008) for the fermentation of a spruce hydrolysate, improving the ethanol productivity. In the same way, the overexpression of ZWF1 gene from the

pentose phosphate pathway (PPP) in *S. cerevisiae* has also improved the tolerance of yeast towards furan derivatives (Gorsich *et al.*, 2006). This tolerance is most probably explained due to an increase in the intracellular levels of NADPH by the prevalence of PPP against other pathways.

The design of a genetically engineered *S. cerevisiae* strain resistant to phenolic compounds has also been studied. Larsson *et al.* (2001a) reported that the overexpression of *S. cerevisiae* Pad1p gene that encodes a phenylacrylic acid descarboxylase, resulted in improved tolerance to phenylacrylic acids. The engineered *S. cerevisiae* strains were cultivated in a synthetic basal medium supplemented with ferulic acid and cinnamic acid as well as in a spruce hydrolysate. Compared to the parental strain, those recombinants which overexpressed Pad1p protein had the capacity to transform both acids at higher rates, showing faster cell growth and higher ethanol production rate. On the other hand, the heterologous laccase expression in *S. cerevisiae* has also been explored to increase the reduction of phenolic compounds. Larsson *et al.* (2001b) designed a recombinant *S. cerevisiae* strain carrying the laccase gene from the white-rot fungus *T. versicolor* and overexpressing the homologous t-SNARE Sso2p, a membrane protein involved in the protein secretion machinery. This strain showed higher laccase activity than the *S. cerevisiae* carrying the laccase gene only and had the ability to convert coniferyl aldehyde at a faster rate. In addition, this transformant was able to ferment a dilute-acid spruce hydrolysate, showing higher ethanol productivity compared to control.

- *Others.*

Besides co-culture and evolutionary or genetic engineering modifications, different alternative approaches have been developed to increase the intrinsic tolerance or the inherent detoxification capacity of some strains. These approaches are cell retention,

encapsulation and flocculation. In the first case, the fermenting microorganism is maintained at high cell density by recirculation, being able to transform higher amount of inhibitory compounds. Using cell recirculation by cross-flow filtration, Brandberg *et al.* (2005) enhanced the conversion of furan derivatives, increasing the sugar conversion rate of *S. cerevisiae* up to 99%. Furthermore, the ethanol and biomass concentration were also increased.

In the case of encapsulation, cells are retained in alginate with a high local cell density inside the capsule. This situation forces the cells close to the membrane to convert inhibitors, letting cells in the core experience a lower level of degradation compounds and ensuring the survival of the population. Encapsulated *S. cerevisiae* cells fermented a dilute-acid spruce hydrolysate successfully, obtaining an ethanol yield of 0.44 $g \cdot g^{-1}$ and keeping more than 75% of cell viability (Talebnia and Taherzadeh, 2006). In a recent study, Westman *et al.* (2012) have shown that in spite of the favourable effect on furans reduction, encapsulation does not aid in the protection against carboxylic acids, showing that the protective effect from encapsulation is specific to some inhibitors. Furthermore, the main disadvantage of encapsulation is the gradual cell deactivation and the increased final cost in bioethanol production.

Finally, in the case of using a flocculation strategy, similar effects to encapsulation could be found as cells protect each other by forming aggregates. Hence, a flocculating *S. cerevisiae* improved the fermentability of a dilute-acid spruce hydrolysate by depleting furfural and HMF, reaching similar yield than using encapsulated cells (0.44 $g \cdot g^{-1}$) (Purwadi *et al.*, 2007).

Comparing between separated and *in situ* microbial detoxification processes, the latter strategy can be advantageous as there is no glucose consumption by other microorganism

(regardless co-culture strategy) and, indeed, all sugars can be potentially converted into ethanol. Furthermore, as the detoxifying and fermenting microorganism are the same, there is a better process integration that decreases the overall costs by avoiding extra equipment.

Enzymatic detoxification processes

Enzymatic detoxification is one of the main biotechnological methods used to diminish the inhibitory compounds of fermentation broths. Using enzymes for detoxification, sugar consumption by microorganism is avoided and the process can be carried out at optimal conditions of pH (3-8) and temperature (25-80 ºC) for enzymes. The most common enzymes used for enzymatic detoxification are laccases and peroxidases, which derive from diverse white rot fungi (*T. versicolor, T. villosa, Coriolopsis rigida, P. cinnabarinus, Coltricia perennis, Cyathus stercoreus*). These enzymes, which act selectively on phenolic compounds generating unstable phenoxy-radicals that polymerizes into less toxic aromatic compounds (Kolb *et al.*, 2012; Alvira *et al*., 2013), have been studied on different pretreated materials (Table 2). Jönsson *et al.* (1998) explored laccase and peroxydase enzymes from *T. versicolor* to detoxify the liquid fraction from acid steam-exploded willow, observing higher glucose consumption rates, ethanol volumetric productivities and ethanol yields using *S. cerevisiae* as fermenting microorganism. In the same way, Larsson *et al.* (1999) described similar results together with a higher yeast growth on the liquid fraction from acid steam-exploded spruce treated with *T. versicolor* laccase. Acid hydrolysate from sugarcane bagasse was also detoxified by laccase from *C. stercoreus* and fermented with *Candida shehatae* resulting in ethanol yields comparable to the one detoxified by activated carbon (Chandel *et al.*, 2007). On the other hand, Martín *et al.* (2002) compared *T. versicolor* laccase and overliming to detoxify an enzymatic hydrolysate from steam-exploded sugarcane bagasse. Their effects on

565 the fermentability were studied by using a recombinant xylose-utilizing *S. cerevisiae* strain,
566 resulting in improved ethanol yield and productivity with both treatments.

567 As observed during the enzymatic delignification by laccases, a contradictory effect in
568 the sugar recovery has also been observed after a detoxification step by these enzymes. In
569 addition to the mechanisms explained before (increase in the unproductive binding and the
570 strengthening of lignin-carbohydrate complexes), the resulting oligomers from the oxidative
571 polymerization after laccase treatment can be less toxic to the yeast than simple phenolic
572 compounds, but they can nevertheless exert greater inhibition on hydrolytic enzymes (Tejirian
573 and Xu, 2011). Jurado *et al.* (2009) used laccases from *C. rigida* and *T. villosa* to detoxify
574 enzymatic hydrolysates from both acid and non-acid steam-exploded wheat straw. While the
575 fermentability of both laccase-treated materials was improved, a lower sugar recovery was
576 observed during the saccharification step of detoxified samples. The same phenomenon was
577 observed by Moreno *et al.* (2012) who used laccases from *P. cinnabarinus* and *T. villosa* to
578 detoxify the whole slurry from steam-exploded wheat straw. Lower glucose recovery was
579 observed when laccase treatments were carried out before enzymatic hydrolysis, suggesting a
580 negative effect of laccases on saccharification step. However, both laccases enhanced the
581 performance of the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875, enabling
582 the fermentation of inhibitory broths at higher substrate consistencies and increasing the
583 ethanol concentrations and productivities. Moreover, when comparing the fermentation
584 performance of *K. marxianus* with an industrial *S. cerevisiae* strain, similar ethanol
585 concentrations and yields were obtained (Moreno *et al*., 2013). By contrast, Kalyani *et al.*
586 (2012) isolated and characterized a new laccase from *C. perennis*, which detoxified phenolic
587 compounds in acid-pretreated rice straw while the saccharification yields were enhanced.

588 Enzymatic detoxification can reduce reaction time and increase catalytic efficiency
589 compared to microbial detoxification. Nevertheless, this process also present some

disadvantages such as the high enzyme production cost and their limitation to remove all different kinds of inhibitory compounds. To solve these drawbacks, ligninolytic enzymes could be combined with the use of robust fermenting microorganisms developed by evolutionary and genetic engineering techniques. Moreover, these enzymes can be cloned into cellulase-producing microorganisms or be used as immobilized or co-immobilized enzymes, allowing a reduction of the production cost as well as an enzyme recycling, respectively.

Integrating biodelignification and biodetoxification processes in future biorefineries

Taking into account the environmental, social and economic pillars of sustainability, future biorefineries have to be able to produce not only high-volume and low-cost biofuels but also high-value compounds with minimal downstream wastes. With this purpose, in addition to bioethanol, lignocellulosic biomass offers the possibility to provide sugars, alcohols, esters, carboxylic acids and aromatic chemicals. To reduce the recalcitrant structure and fractionate the main components of lignocellulose, the pretreatment is a key step to guarantee the efficient use of these feedstocks and has an important contribution in the overall cost. As it was discussed above, diverse microorganisms and ligninolytic enzymes have the potential to be used as single pretreatment methods or to work in combination with other cost-effective physico-chemical technologies. After a biodelignification or a biodetoxification step, the pretreated material retains fewer inhibitory compounds, saving the freshwater required for washing the material and, consequently, decreasing wastewater. In addition, the lower inhibitory profiles allow the fermenting microorganism to work under higher substrate consistencies at higher rates, reducing the fermentation time and increasing the final ethanol concentration, which decreases the distillation and evaporation costs.

In a biorefinery concept, enzymes can offer greater application potentials than microorganisms because they encourage the production of value-added compounds in more efficient processes without consuming sugars or lignin. Moreover, the enzymatic delignification and detoxification can also be carried out in the same vessel than saccharification and fermentation, leading a better process integration and avoiding the necessity of any extra equipment.

To promote the right utilization of lignocellulosic materials, biodelignification and biodetoxification can be combined with different physico-chemical technologies for enhancing global yields. In this context, a wide variety of laccases have been successfully employed in both delignification and detoxification processes, boosting the saccharification and fermentation steps, although there are still no reported data showing a simultaneous enzymatic delignification and detoxification. The combination of different laccases that have already been evaluated for these bioprocesses or the isolation of new ligninolytic activities could help to improve the environmental and economical aspects of lignocellulosic bioethanol production. However, the use of these strategies imply further investments and developments from laboratory and pilot scales that support these hypothesis and convert them into real applications in the commercial facilities.

Conclusion

In order to provide different forms of energy and products in environmentally friendly frames, future biorefineries using lignocellulosic biomass as feedstock represent an alternative to the present industry based on fossil fuels. In this context, biodelignification and/or biodetoxification processes must be taken into account for lignocellulosic bioethanol production. The reduction in lignin content by biodelignification has been shown to enhance

the saccharification of different lignocellulosic materials, increasing final ethanol production. However, long reaction times of microbial delignification make the current process inappropriate for industrial application. In this context, the use of purified ligninolytic enzymes could provide an effective alternative, reducing the process from days to hours. In contrast, enzyme purification and/or the addition of extra compounds increase final costs and may avoid the benefits.

Although other physico-chemical pretreatments have been established to be cost-effective at large scale, the pretreated materials obtained contain several inhibitory compounds with different nature that hamper their fermentability. Among different approaches to overcome these inhibitors, microbial or enzymatic detoxification processes represent some advantages because they are performed under mild reaction conditions, require less energy and reduce byproducts. These biodetoxification methods can be carried out by a separated step or *in situ*, being the latter strategy much more attractive.

Developing new strategies for biodelignification and biodetoxification is imperative to avoid the bottlenecks in both biological processes. The use of low-cost materials for an *in situ* enzyme production or the generation of more tolerant fermenting microorganisms offer potential possibilities to reduce final biofuel production costs and make the overall process more sustainable.

Declaration of interest

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958

Table 1. Use of laccases and LMS for enzymatic delignification

Highlights	Treatment[a]	Raw Material	Lignin loss[b]	Remarks	Reference
Improve enzymatic hydrolysis	Laccase (*Pleurotus* sp.)	Thorny bamboo	84% (8 h)	Lower cellulase loading for enzymatic hydrolysis	Kuila *et al.*, 2011a
	Laccase (*Pleurotus* sp.)	Spanish flag	89% (8 h)	Better accessibility of cellulases by destroying ordered structures	Kuila *et al.*, 2011b
	Laccase (*C. unicolor*)	Steam exploded spruce	Lignin modification	Contradictory results using steam pretreated giant reed	Moilanen *et al*., 2011
	Laccase *(Pleurotus* sp.)	Castor oil plant	86% (4 h)	SEM images showed a clear degradation of surface tissues after the enzymatic delignification	Mukhopadhyay *et al.*, 2011
	Laccase (*G. lucidum*)	Indian colza straw	Lignin modification	Higher number and density of holes with greater width and depth than with alkali pretreatment alone	Yang *et al*., 2011
	Laccase (*T. hirsuta*)	Corn straw	Lignin modification	Not only porosity but also the available surface area is increased by combining alkali and laccase pretreatment	Li *et al*., 2012
	Laccase (*Sclerotium* sp.)	Steam-exploded wheat straw	n.r.	The compact wrap of lignin-carbohydrate complexes was reduced after phenol oxidation	Qiu and Chen, 2012
	LMS (*T. hirsute*-NHA)	Steam pretreated softwood	n.r.	Laccase treatment change hydrofobicity of lignin and the surface charge decreasing the unspecific adsorption of cellulases	Palonen and Viikari, 2004
	LMS (*T. versicolor*-HBT)	Corn stover	n.r.	Ensilage might provide	Chen *et al.*, 2012

				channels to improve laccase accesibility	
	LMS (*T. villosa*-HBT)	Eucalyptus and elephant grass	48% (eucalyptus) 32% (elephant grass)	A significant decrease of both aromatic and aliphatic lignin with high presence of oxidized S units in the residual lignin	Gutiérrez *et al.*, 2012
	LMS (*M. thermophile*-HBT)	Eucalyptus chips	Lignin modification	Similar results obtained with xylanase treatment	Martín-Sampedro *et al.*, 2012
High delignification	LMS (*P. sanguineus*-VIO)	Extracted wheat straw and corn stover	97% (24 h)	Less incubation time for laccase production by heterologous expression in *Pichia pastoris*	Lu *et al.*, 2010

[a] In brackets is indicated the microorganisms which was the source of the enzyme. In the case of LMS it is also indicated the mediator used: NHA (N-hydroxy-N-phenylacetamide), HBT (1-hydroxybenzotriazole) or VIO (violuric acid)
[b] Treatment time in brackets
n.r. not reported

Table 2. Use of ligninolytic enzymes for phenol removal in different pretreated materials

Treatment	Raw Material	Remarks	Reference
Laccase and lignin peroxidise (*T. versicolor*)	SO_2-steam exploded willow	Enzymatic treatment increased large-size materials and decreased the small-size materials, suggesting a polymerization mechanism	Jönsson *et al.*, 1998
Laccase (*T. versicolor*)	Diluted-acid hydrolysate of spruce	Ethanol yield comparable with that obtained after detoxification with the anion exchange chromatography at pH 10	Larsson *et al.*, 1999
Laccase (*T. versicolor*)	Steam exploded sugarcane bagasse	Similar yields and productivities than the obtained with overliming	Martín *et al.*, 2002
Laccase (*C. stercoreus*)	Acid hydrolysate of sugarcane bagasse	High biomass production with high ethanol yields	Chandel *et al.*, 2007
Laccase (*C. rigida* and *T. villosa*)	Steam exploded wheat straw impregnated with water or acid	Better sugar recovery when performing enzymatic treatment after saccharification	Jurado *et al.*, 2009
Laccase (*C. perennis* SKU0322)	Acid pretreated rice straw	Enzyme tolerant to extreme conditions (low pH or high temperature)	Kalyani *et al.*, 2012
Laccase (*T. versicolor*)	Liquid hot water pretreated wheat straw	Selective action on different substituted phenols	Kolb *et al.*, 2012
Laccase (*T. villosa* and *P. cinnabarinus*)	Steam exploded wheat straw	Allow working at higher substrate consistencies	Moreno *et al.*, 2012; 2013
Laccase (*P. cinnabarinus*)	Steam exploded wheat straw	Improve volumetric productivity in samples with high dry matter consistencies	Alvira *et al.*, 2013

Figure 1

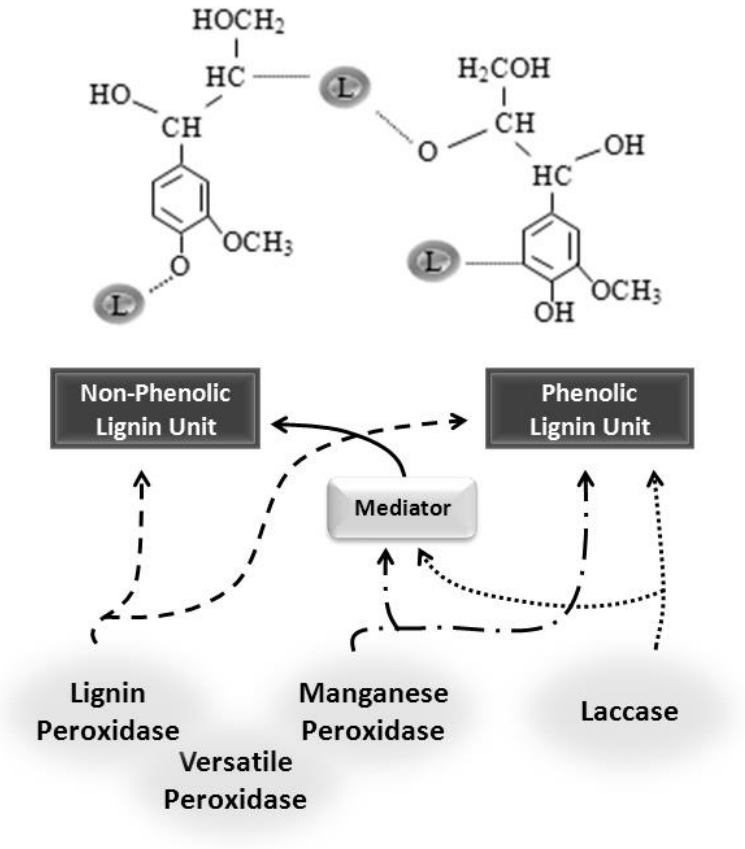

Figure 1. Ligninolytic enzymes and their selectively action on lignin components. While lignin peroxidase can directly oxidize both phenolic and non-phenolic compounds, manganase peroxidase and laccase can only act on phenolic compounds. However, in a secondary pathway, these enzymes can oxidize non-phenolic compounds indirectly by the action of a mediator (more details about mediators for laccases and manganese peroxidases are described in the text). Finally, the catalytic mechanism of the versatile peroxidase can either be similar to lignin or manganese peroxidase. L-containing circles represent the remaining lignin polymer.