REVIEW ARTICLE

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 polymers 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 regarding 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

Bioethanol, biodelignification, biodetoxification, biorefinery, ligninolytic enzymes, lignocellulose, white-rot fungi

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 (FitzPatrick et al., 2010; Himmel et al., 2007; Martínez et al., 2009; Ragauskas et al., 2006). In 2011, the bioethanol production was worldwide more than 1011 L (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 inexpensive feedstocks for biofuels production (Berndes et al., 2001; Taherzadeh & Karimi, 2008).

Lignocellulose production is estimated to be more than 109 tons per year. It is the major renewable organic matter in nature (Reddy & Yang, 2005). The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. From the biochemical point of view, high amounts of sugars present in cellulose and hemicellulose can be chemically produced, using acid as the catalyst, or enzymatically hydrolysed and converted into biofuels by a fermentation process (Taherzadeh & Karimi, 2007a,b; Tomás-Pejo et al., 2008). In contrast, the third major component of lignocellulosic materials, lignin, does not consist of fermentable sugars and plays an important role providing a recalcitrant structure difficult to disrupt (Brett & 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 (Alvira et al., 2010; Klinke et al., 2004; Palmqvist & Hahn-Hägerdal, 2000a,b; Panagiotou & Olsson, 2007;
Biodelignification and biodetoxification methods

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Biological delignification

Hydrolysing lignocellulosic sugars enzymatically is advantageous compared to acid hydrolysis due to its higher conversion efficiency, lower process energy requirements and reduced formation of fermentation inhibitors (Taherzadeh & Karimi, 2007b; Tomáš-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 the 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., 2011b). 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 solubilization and/or degradation (wet oxidation, acid pretreatment and steam explosion) and cellulose decrystallization (ammonia fiber explosion) (Alvira et al., 2010; Tomáš-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 reduced 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 (Isroi et al., 2011; Martínez et al., 2005). 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 & 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 & Staples, 2002). However, in the presence of low molecular weight compounds that form stable radicals and act as redox mediators, laccases can also oxidize non-phenolic lignin units (Bourbonnais & Paice, 1990).

Ligninolytic peroxidases are high redox potential heme-peroxidases that require H2O2 as a 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 Mn3+ 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 H2O2 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 the 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 & Martínez, 2009).

Microbial delignification processes

The biological pretreatment where 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 hand, solid state fermentations are usually performed at a moisture content between 45 and 85% with an inoculum level of 1–10 mg/g substrate (dry weight), at pH 4–5 and temperatures between 15 and 40°C for 1–12 weeks, depending on the strain used (Canam et al., 2011; Itoh et al., 2003; Lee et al., 2007; Muñoz et al., 2007; Singh et al., 2008; Salvachúa et al., 2011; Wan & Li, 2010). The patterns of cell wall deconstruction by “white-rot” fungi vary among species and strains. Several fungi have been tested using different lignocellulosic feedstocks as a pretreatment method for bioethanol production, obtaining delignification efficiencies from 6% to 92% (Supplementary Table 1).

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

The positive effect observed in the fermentable sugars yield, together with the lower amount of inhibitory compounds formed during microbial delignification, could also result in an increase of the final ethanol production. In this context, Kuhar et al. (2008) pretreated wheat straw and mesquite by solid state fermentation with the isolated Basidiomycete fungus RCK-1, followed by acid hydrolysis and fermentation with Pichia stipitis, increasing by 33% and 10% the ethanol yield, and 80% and 57% the ethanol 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 with 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 Zymomonas 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 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 Panus tigrinus and Trametes versicolor, respectively, after 7 days 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 Ceriporiopsis subvermispora which consumes less than 6% of total sugars (Wan & Li, 2010), or the use of genetically modified microorganisms such as the cellobiose dehydrogenase-deficient T. versicolor strain (Canam et al., 2011), are promising options for 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, Irpex 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 a 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 sugar consumption, are main challenges to overcome. Exploring new microorganisms and improving process conditions are important aspects in order 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 reaction rates and delignification efficiency, reducing the process time 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 containing a single purified and concentrated enzyme (Kuila et al., 2011a,b; Lu et al., 2010; Moilanen et al., 2011). For 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°C, respectively) (Ibarra et al., 2006; Kuila et al., 2011a,b). The liquid:solid ratio and the enzyme loading at which the delignification is carried out are, however, two important factors (Kuila et al., 2011a,b; Mukhopadhyay et al., 2011). Delignification assays have been performed up to 2:1 liquid:solid ratio (Kuila et al., 2011a,b; Mukhopadhyay et al., 2011), but lower solid charges (20:1 or even less) are usually used (Moilanen et al., 2011; Palonen & Viikari, 2004; Qiu & Chen, 2012). On the other hand, enzymes can be added at low (0.03–10IU/g) or high (4000–80000 IU/g) loadings, depending on process optimization (Chen et al., 2012; Qiu & Chen, 2012).

Among different ligninolytic enzymes, laccases, especially in the form of a 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,b; Li et al., 2012; Lu et al., 2010; Martín-Sampedro, et al., 2012; Moilanen et al., 2011; Mukhopadhyay et al., 2011; Palonen & Viikari, 2004; Qiu & Chen, 2012; Yang et al., 2011a). In this sense, different strategies have been assayed, either using the 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 a castor oil plant (*Ricinus communis*), obtaining an optimum delignification of 86% after 4h. This treatment increased the saccharification performance almost 3-fold. Similar lignin loss (84–89%) was obtained after 8h 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 as 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., 2011a). 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. (2011a) 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; Palonen & Viikari, 2004; Qiu & Chen, 2012). Qiu & Chen (2012) reported the oxidation of lignin from steam-exploded wheat straw by *Sclerotium sp.* laccase, which resulted in the formation of micro pores on the material and enhanced the accessibility of cellulose. On the other hand, Palonen & Viikari (2004) observed that *T. hirsuta* laccase enhanced 13% the saccharification yield of steam-exploded spruce by lignin modification. The authors described a reduction in the hydrophobicity of lignin and an eventual change in 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 in the unproductive binding of cellulases increasing 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 laccace 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 eucalyptus wood and
Table 1. Use of laccases and LMS for enzymatic delignification.

| Highlights                  | Treatmenta                        | Raw material                        | Lignin lossb | Remarks                                                | Reference                   |
|-----------------------------|-----------------------------------|-------------------------------------|--------------|--------------------------------------------------------|-----------------------------|
| Improve enzymatic hydrolysis| Laccase (*Pleurotus* sp.)         | Thorny bamboo                       | 84% (8h)     | Lower cellulase loading for enzymatic hydrolysis       | Kuila et al., 2011a         |
|                             | Laccase (*Pleurotus* sp.)         | Spanish flag                        | 89% (8h)     | 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% (4h)     | 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., 2011a          |
|                             | 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 & 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 & Viikari, 2004     |
|                             | LMS (*T. versicolor-HBT*)         | Corn stover                         | n.r.         | Ensilage might provide channels to improve laccase accessibility | Chen et al., 2012           |
|                             | LMS (*T. villosa-HBT*)            | Eucalyptus and elephant grass       | 48% (eucalyptus) | 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 |
|                             | LMS (*P. sanguineus-VIO*)         | Extracted wheat straw and corn stover | 97% (24h)    | Less incubation time for laccase production by heterologous expression in *Pichia pastoris* | Lu et al., 2010             |

n.r. not reported.
aIn 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).
bTreatment time in brackets.
elephant grass. Consequently, the enzymatic treatment increased the glucose production by 61% and 12%; and the ethanol concentration by 4 and 2 g L\(^{-1}\) from both lignocellulosic materials fermented with \(S. \) cerevisiae Red Star. High delignification yield (up to 97%) was observed with \(Pycnoporus \) sanguineus laccase and violuric acid (VIO) as mediator on both wheat straw and corn stover pretreated by liquid hot water (Lu et al., 2010). In addition to lignin removal, the improvement of saccharification due to lignin modification by LMS has also been reported (Chen et al., 2012; Martín-Sampedro et al., 2012; Palonen & Viikari, 2004). In this context, Chen et al. (2012) described marked lignin modification on ensiled corn stover using \(T. \) versicolor laccase and HBT as mediator, which improved downstream cellulose hydrolysis about 7%. On the other hand, Martín-Sampedro et al. (2012) observed an increase in glucose yield in the hydrolysis step, from 24.7% to 27.1%, when steam-exploded eucalyptus was treated with \(Myceliophthora \) thermophila laccase in combination with HBT. By using N-hydroxy-N-phenylacetamide (NHA) as mediator, Palonen & Viikari (2004) increased the saccharification yield from 13% to 21% compared to the treatment with \(T. \) hirsuta laccase alone.

It can be inferred that the use of ligninolytic enzymes such as laccases or LMS for biodelignification shows similar advantages than microorganisms in terms of improvements on saccharification and fermentation, reducing very significantly the treatment time and avoiding sugar consumption. Nevertheless, the cost of producing the enzyme and the use of synthetic mediators still represent the main disadvantages that hinder their application in the current bioethanol production process at large scales. To overcome these drawbacks, different alternatives have been shown to reduce final production costs. For instance, the synthetic mediators can be replaced by natural mediators derived from lignin (Martínez et al., 2009) and lignocellulosic feedstocks can be employed for the growth of enzyme-producing microorganisms instead of the expensive conventional carbon sources (Jun et al., 2011). Moreover, an in situ enzyme production also offers the possibility of using the same raw material that is going to be delignified, providing the optimal enzymatic activities for the biodelignification step.

**Biological detoxification**

Although biological pretreatments show environmental advantages, these methods are still non-viable for large-scale bioethanol production. In this context, physico-chemical technologies such as hydrothermal processes have higher potential in the short term as cost-effective methods at the industrial scale. These pretreatments have been tested for ethanol production at laboratory, pilot and demonstration scales with a wide range of raw materials, including softwood (Cara et al., 2006; Monavari et al., 2010), hardwood (Oliva et al., 2003) and herbaceous crops (Ballesteros et al., 2006). The main disadvantage of these pretreatments is the formation of different inhibitory compounds, predominantly derived from lignin and hemicellulose degradation, that can affect enzymatic hydrolysis as well as fermentation steps (Palmqvist & Hahn-Hägerdal, 2000a,b; Klinke et al., 2004; Panagiotou & Olsson, 2007). According to their nature, inhibitory compounds can be classified into furan derivatives, weak acids or phenolic compounds. The most common furan derivatives are 2-furaldehyde (furfural) and 5-hydroxymethylfurfural (HMF), which come from degradation of sugars (pentoses and hexoses, respectively) contained in cellulose and hemicellulose. Among weak acids, acetic acid originates from acetyl groups of hemicelluloses whereas formic acid and levulinic acid come from further degradation of furfural and HMF. Finally, a wide variety of phenolic compounds are released from lignin.

One possibility that has been typically performed to eliminate soluble inhibitory 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 equipment 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, over-liming, activated 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 & Tekere, 2011).

**Microbial detoxification processes**

Microbial detoxification involves the utilization of microorganisms to decrease the inhibitory effects 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 as for microbial delignification: optimal nutrient addition, pH (4–6), temperature (25–50°C), treatment time (12–144 h), inoculum size (1–10% (v/v) or 0.5–10 g/L (dry weight)) and microorganism strain (Fonseca et al., 2011; Larsson et al., 1999; López et al., 2004; Nichols et al., 2008, 2010; Okuda et al., 2008; Palmqvist et al., 1997; Yu et al., 2011a; Zhang et al., 2010).

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 mostly studied for this purpose. Palmqvist et al. (1997) observed considerable removal of phenols, furans and weak acids after growing \(T. \) reesei on the hydrolysate obtained from acid steam-exploded willow, improving both ethanol productivity and yield when using \(S. \) cerevisiae as the fermenting microorganism. \(T. \) reesei was also used by Larsson et al. (1999) for improving the fermentability of diluted-acid hydrolysate of spruce, observing a removal of furans and a small percentage of phenols without affecting the concentration of weak acids. López et al. (2004) isolated a new fungus \(C. \) ligniaria NRRL30616 with the ability to metabolize furfural and HMF as well as aromatic and aliphatic...
acids and aldehydes. This strain was further used by Nichols et al. (2008, 2010) in dilute-acid hydrolysates from corn stover, alfalfa stems, reed canary grass and switch grass, favoring xylose utilization by *Saccharomyces* sp. LNHH-ST (Nichols et al., 2008) and reducing the lag phase of *S. cerevisiae* D5a (Nichols et al., 2010) in the subsequent ethanol fermentations. The fungus strain *Amorphotheca resinae* ZNI was also able to degrade all kinds of inhibitory compounds present in steam-exploded corn stover and dilute-acid pretreated corn stover, rice straw, wheat straw and rape straw (Zhang et al., 2010). *Aspergillus nidulans* FLZ10 completely degraded furfural and HMF and partially removed formic acid and acetic acid when used on steam-exploded corn stover (Yu et al., 2011a).

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

Bacteria and yeasts have also been used for detoxification purposes to a lesser extent. The thermophilic bacterium *Ureibacillus thermophaercus* was used by Okuda et al. (2008) which removed furfural and HMF from a synthetic hydrolysate, and the phenolic compounds from diluted acid waste house wood. López et al. (2004) isolated five bacteria related to *Methyllobacterium extorquens, Pseudomonas* sp., *Flavobacterium indologenes, Acinetobacter* sp., and *Arthrobacter aurescens* capable of depleting ferulic acid, HMF and furfural from a defined mineral medium. An example of a microbial detoxification step by yeast, prior to fermentation, was reported by Fonseca et al. (2011), who described the capacity of *Issatchenkia orientalis* CCTCC M 206097 for removing syringaldehyde, ferulic acid, furfural and HMF from hemicellulosic hydrolysate of sugarcane bagasse.

According to previous reported data, separated microbial detoxification has been usually performed in the liquid fraction or hydrolysates. The use of the whole pretreated material (slurry), however, offers different advantages instead: (1) there is no need of equipment to separate the liquid and solid fractions, therefore the processing time and costs are reduced; (2) sugar loss during the washing of the material is avoided, which, in turn, decreases the wastewater generated; and (3) in the case of using an enzyme-producing microorganism for biodetoxification, the presence of cellulose enhances the production of hydrolytic enzymes, decreasing the doses of extra cellulase addition for the enzymatic saccharification.

*In situ* microbial detoxification can also be performed due to the natural ability of diverse fermenting microorganisms to remove some inhibitory compounds. For instance, most of *S. cerevisiae* strains can convert furan derivatives into less inhibitory compounds (Ferreira et al., 2011; Palmqvist & Hahn-Hägerdal, 2000a; Schneider, 1996; Thomsen et al., 2009): furfural can be reduced to furfuryl alcohol and HMF to 2,5-HMF alcohol (Liu et al., 2005). *S. cerevisiae* also possesses the capacity to metabolize some phenolic compounds due to the presence of a phenylacrylic acid decarboxylase that catalyses a decarboxylation step by which aromatic carboxylic acids are converted to the corresponding vinyl derivatives (Goodey & 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 materials 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 & 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 & 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. (2011a) 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 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, exhibiting good inhibitor tolerance and the capacity of
metabolizing furans, while maintaining high ethanol volumetric productivity. On the other hand, Stoutenburg et al. (2011) developed several strains from the parental P. stipitis following 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. (2011b) 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, Martin 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 rates, ethanol yield (from 0.18 g g\(^{-1}\) to 0.38 g g\(^{-1}\)) and ethanol volumetric productivity (from 1.2 g L\(^{-1}\) h\(^{-1}\) to 2.6 g L\(^{-1}\) h\(^{-1}\)), using bagasse hydrolysates as fermentation broth. With a similar strategy, Tomás-Pejo et al. (2010) improved the xylose-fermenting S. cerevisiae F12 for bioethanol production, permitting 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, enhanced sugar consumption and shortened overall fermentation times.

**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 rates 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 the 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 the 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, the recombinants which overexpressed the Pad1p protein had the capacity to transform both acids at higher rates, showing faster cell growth and higher ethanol production rates. 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 strain 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 the 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 amounts 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 g\(^{-1}\) and maintaining higher than 75% of cell viability (Talebnia & Taherzadeh, 2006). In a recent study, Westman et al. (2012) have shown that in spite of the favourable effect on furan 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 during 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 flocculent S. cerevisiae strain improved the fermentability of a dilute-acid spruce hydrolysate by depleting furfural and HMF, reaching similar
yields to using encapsulated cells (0.44 g g\(^{-1}\)) (Purwadi et al., 2007).

Comparing separated and in situ microbial detoxification processes, the latter strategy can be advantageous as there is no glucose consumption by other microorganisms (regardless co-culture strategy) and, indeed, all sugars can potentially be converted into ethanol. Furthermore, as the detoxifying and fermenting microorganisms are the same, there is better process integration, this 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°C) 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 stercoratus). These enzymes, which act selectively on phenolic compounds generating unstable phenoxy-radicals that polymerizes into less toxic aromatic compounds (Alvira et al., 2013; Kolb et al., 2012), have been studied on different pretreated materials (Table 2). Jönsson et al. (1998) explored laccase and peroxidase enzymes from T. versicolor to detoxify the liquid fraction from acid steam-exploded willow, observing higher glucose consumption rates, ethanol volumetric productivity and ethanol yield using S. cerevisiae as the 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. stercoratus 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 fermentability were studied by using a recombinant xylose-utilizing S. cerevisiae strain, resulting in improved ethanol yield and ethanol volumetric productivity with both treatments.

As observed during the enzymatic delignification by laccases, a contradictory effect in sugar recovery has also been observed after a detoxification step by these enzymes. In addition to the mechanisms explained before (increase in the unproductive binding and the strengthening of lignin-carbohydrate complexes), the resulting oligomers from the oxidative polymerization after laccase treatment can be less toxic to the yeast than simple phenolic compounds, but they can nevertheless exert greater inhibition on hydrolytic enzymes (Tejirian & Xu, 2011). Jurado et al. (2009) used laccases from C. rigidia and T. villosa to detoxify enzymatic hydrolysates from both acid and non-acid steam-exploded wheat straw. While the fermentability of both laccase-treated materials was improved, a lower sugar recovery was observed during the saccharification step of detoxified samples. The same phenomenon was observed by Moreno et al. (2012) who used laccases from P. cinnabarinus and T. villosa to detoxify the whole slurry from acid-exploded wheat straw. Lower glucose recovery was observed when laccase treatments were carried out before enzymatic hydrolysis, suggesting a negative

| Treatment | Raw material | Remarks | Reference |
|-----------|--------------|---------|-----------|
| Laccase and lignin peroxidise (T. versicolor) | SO₂-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 | Martin et al., 2002 |
| Laccase (C. stercoratus) | Acid hydrolysate of sugarcane bagasse | High biomass production with high ethanol yields | Chandel et al., 2007 |
| Laccase (C. rigidia 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 |
effect of laccases on the saccharification step. However, both laccases enhanced the performance of the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875, enabling the fermentation of inhibitory broths at higher substrate consistencies and increasing the ethanol concentrations and ethanol volumetric productivities. Moreover, when comparing the fermentation performance of *K. marxianus* with an industrial *S. cerevisiae* strain, similar ethanol concentrations and yields were obtained (Moreno et al., 2013). By contrast, Kalyani et al. (2012) isolated and characterized a new laccase from *C. perennis*, which detoxified phenolic compounds in acid-pretreated rice straw while the saccharification yields were enhanced.

Enzymatic detoxification can reduce reaction times and increase catalytic efficiency compared to microbial detoxification. Nevertheless, this process also presents 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 costs 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 produce sugars, alcohols, esters, carboxylic acids and aromatic chemicals. To reduce the recalcitrant structure and fractionate the main components of lignocellulose, pretreatment is a key step to guarantee the efficient use of these feedstocks and has an important contribution in the overall cost. As 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, 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 as saccharification and fermentation, leading to better process integration and avoiding the necessity of 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 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 implies further investments and developments from laboratory and pilot scale that support this hypothesis and convert them into real applications in 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 in order 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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Supplementary material available online

Supplementary Tables 1 and 2