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REVIEW ARTICLE

Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review

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Abstract

One of the major challenges faced in commercial production of lignocellulosic bioethanol is the inhibitory compounds generated during the thermo-chemical pre-treatment step of biomass. These inhibitory compounds are toxic to fermenting micro-organisms. The ethanol yield and productivity obtained during fermentation of lignocellulosic hydrolysates is decreased due to the presence of inhibiting compounds, such as weak acids, furans and phenolic compounds formed or released during thermo-chemical pre-treatment step such as acid and steam explosion. This review describes the application and/or effect of biological detoxification (removal of inhibitors before fermentation) or use of bioreduction capability of fermenting yeasts on the fermentability of the hydrolysates. Inhibition of yeast fermentation by the inhibitor compounds in the lignocellulosic hydrolysates can be reduced by treatment with enzymes such as the lignolytic enzymes, for example, laccase and micro-organisms such as *Trichoderma reesei, Coniochaeta ligniaria* NRRL30616, *Trametes versicolor*, *Pseudomonas putida* Fu1, *Candida guilliermondii*, and *Ureibacillus thermosphaericus*. Microbial and enzymatic detoxifications of lignocellulosic hydrolysate are mild and more specific in their action. The efficiency of enzymatic process is quite comparable to other physical and chemical methods. Adaptation of the fermentation yeasts to the lignocellulosic hydrolysate prior to fermentation is suggested as an alternative approach to detoxification. Increases in fermentation rate and ethanol yield by adapted micro-organisms to acid pre-treated lignocellulosic hydrolysates have been reported in some studies. Another approach to alleviate the inhibition problem is to use genetic engineering to introduce increased tolerance by *Saccharomyces cerevisiae*, for example, by overexpressing genes encoding enzymes for resistance against specific inhibitors and altering co-factor balance. Cloning of the laccase gene followed by heterologous expression in yeasts was shown to provide higher enzyme yields and permit production of laccases with desired properties for detoxification of lignocellulose hydrolysates. A combination of more inhibitor-tolerant yeast strains with efficient feed strategies such as fed-batch will likely improve lignocellulose-to-ethanol process robustness.

Keywords: Biological detoxification, fermentation inhibitors, lignocellulose hydrolysates, adaptation, genetic engineering, bioethanol

Introduction

Biofuels, such as bioethanol, have received increasing attention as an alternative to petroleum-based fuels due to the current dependency on supplies from other countries, and depletion of the oil reserves and the increased concentration of the greenhouse gas, CO_2 in the atmosphere (Farrell *et al.*, 2006; Kuhar *et al.*, 2008). Bioethanol is an important modern adjunct to petroleum because it is produced from renewable biomass, produces fewer emissions than fossil fuels, generates no net CO_2 and is compatible with current fuel distribution infrastructure (Olsson and Hahn-Hagerdal, 1996). The bioethanol used today is mainly produced from sugar cane (Brazil, South Africa) or corn starch (USA). In order to increase the amount of raw materials available, reduce fuel-food conflicts and make the production economically competitive with petroleum-based fuels; other cheaper raw materials must be used (Almeida *et al.*, 2009). Lignocellulose-derived-feedstocks are abundant and embody a poorly utilized renewable resource (Keating *et al.*, 2006; Taherzadeh and

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Karimi, 2007; Kuhar *et al.*, 2008). Examples of lignocellulosic biomass, includes wastes products from forestry industry, agricultural residues (sugarcane bagasse, corn stover, straw), and municipal solid waste, which can serve as alternative renewable resources for large-scale bioethanol production at a reasonable cost (Saha, 2003; Liu et al., 2005; Okuda *et al.*, 2008). The choice of raw material depends on location and availability among other factors. Several countries, including South Africa, are currently engaged in major research projects studying the utilization of lignocellulosic materials for production of bioethanol, a second generation biofuel.

The carbohydrates contained in lignocellulose are polymeric compounds such as cellulose and hemicelluloses, which are covered by lignin. Lignocellulosic materials are thus recalcitrant to hydrolysis (saccharification) and require several steps before they are converted to bioethanol which makes the process somewhat complex. The conversion of lignocellulosic biomass-to-ethanol is carried out in four main steps: thermo-chemical pre-treatment, enzymatic hydrolysis of the cellulose, a yeast-based fermentation of the resulting sugars and distillation. Pre-treatment is carried out under severe conditions to break and/or remove the lignin, depolymerize cellulose and hemicellulose and make the biomass more amenable to hydrolytic enzymes (Pu *et al.*, 2008; Sun and Cheng, 2002). The subsequent enzymatic hydrolysis of the cellulose and hemicellulose can be performed simultaneously with fermentation of the produced monomeric sugars at the same time and in the same vessel in what is called simultaneous saccharification and fermentation (SSF) or in separate vessels in what is called separate hydrolysis and fermentation (SHF). The overall ethanol yield for most lignocellulose biomass has been reported to be higher when using SSF which also reduces capital cost among other advantages over SHF (Wingren *et al.*, 2003; Soderstrom *et al.*, 2005; Ohgren *et al.*, 2006a; Linde *et al.*, 2008; Tomás-Pejó *et al.*, 2008).

However, when lignocellulosic material is pre-treated using thermo-chemical methods such as steam explosion and dilute-acid, a hydrolysate rich in substances inhibitory to yeast and the enzymes is formed. These inhibitors are degradation by-products produced from the three main constituents of lignocellulosic material, that is, cellulose, hemicellulose, and lignin and reduce the ethanol productivity of the yeast and the final ethanol yield (Larsson *et al.*, 2000; Palmqvist and Hahn-Hagerdal, 2000a). Steam explosion and dilute-acid pre-treatment are commonly used in lignocellulosic biomass pre-treatment for enzymatic SSF because of their effectiveness and inexpensiveness (Saha, 2003). However, these methods are nonspecific and lead to the formation, in addition to sugars, of the inhibitory compounds (Palmqvist and Hahn-Hagerdal, 2000a; Hahn-Hagerdal *et al.*, 2001; Klinke *et al.*, 2004). The nature and concentrations of the final inhibitory compounds vary greatly with the amount of solids in the reactor, pre-treatment conditions applied (time, pH, temperature, concentration of chemicals, etc) and the raw material used (Palmqvist and Hahn-Hagerdal, 2000a, 2000b; Tengborg *et al.*, 2001; Villarreal *et al.*, 2006). For example, during steam pre-treatment the polymeric sugar chains are hydrolyzed to monomeric sugars at a high temperature in an acidic environment. Increasing the temperature, acid concentration or pre-treatment time will result in an increase in the amount of sugars being further degraded into by-products. Inhibitory compounds can be put into three major groups; furaldehydes, weak acids, and phenolics. Commonly found furaldehydes in the hydrolysate include furaldehyde 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF), weak acids include acetic acid, formic acid, and levulinic acid, and phenolic compounds such as vanillin, syringaldehyde, and conferyl aldehyde, all of which can negatively impact the fermentation process (Palmqvist and Hahn-Hagerdal, 2000b; Mussatto and Roberto., 2004).

During the thermo-chemical pre-treatment step using dilute-acid hydrolysis or steam explosion of biomass, inhibitors such as furfural and HMF are derived from the dehydration of pentoses and hexoses, respectively (Larsson *et al.*, 1999a; Palmqvist *et al.*, 2000b; Lewkowski, 2001). Aliphatic acids, such as acetic, formic, and levulinic acid are also formed by de-acetylation of hemicellulose or HMF breakdown. The acetyl groups present in the hemicellulose are released as acetic acid. Furfural and HMF reduce yeast enzymatic and biological activities, break down DNA, and inhibit protein and RNA synthesis (Modig *et al.*, 2002; Liu *et al.*, 2004). The mechanism(s) underlying inhibition are different and depend on the chemical structure of the inhibitor. For example, furfural inhibits glycolytic enzymes and aldehyde dehydrogenase activity, resulting in accumulation of acetaldehyde that would be responsible for the lag phase during growth of *Saccharomyces cerevisiae* in the presence of furans (Palmqvist *et al.*, 1999). Furfural (4 g/L) and HMF only partly contribute to the inhibition of the enzymes; the other by-products also contribute to the inhibition of enzymes when present at high concentrations (Taherzadeh *et al.*, 1999). Although formic acid has a low pKa of 3.75 and thus a lower concentration of undissociated molecules at the pH prevailing in fermentation, it is more toxic to the yeast due to its small size compared with acetic acid (pKa 4.75) and levulinic acid (pKa 4.66). The small size of formic acid molecule is thought to increase its mass transport through the cell wall, after entering the cytosol, the acid dissociates, lowering the pH and inhibiting cell growth. The organic acids inhibit the yeast when the concentration is so high that yeast cells start to die and they also partially deactivate enzymes (Maiorella *et al.*, 1983; Taherzadeh *et al.*, 1997; Larsson *et al.*, 1999b; Almeida *et al.*, 2007). Lignin is degraded to aromatic compounds such as low-molecular weight phenolics syringaldehyde, 4-hydroxybenzaldehyde and vanillin, which are also considered important inhibitors (Jonsson *et al.*, 1998; Cantarella *et al.*, 2004). The phenolic compounds are toxic to the yeast, although the mechanism is not fully known (Palmqvist *et al.*, 2000b). Furfural, HMF and acetic acid are the degradation products that are often present at easily detectable concentrations and are often used as a measure of the inhibiting effect of the pre-treatment liquid. However,

these compounds are probably not the most inhibitory compounds and it is, of course, the sum of all inhibitors present that determines ethanol productivity and yield. It is also possible that the presence of different inhibitory compounds may give rise to synergistic inhibitory effects. Most yeast, including industrial strains, are susceptible to the inhibitors associated with dilute-acid hydrolysis pre-treatment and steam pre-treatment, sometimes even at low concentrations (Palmqvist *et al*., 1999; Taherzadeh *et al*., 2000a; Martin and Johnsson, 2003; Liu *et al*., 2004). Some *S. cerevisiae* yeast strains may be relatively resistant to inhibitors, but detoxification may still be necessary in order to achieve maximum productivity in the fermentation process (Jonsson *et al*., 1998). In order to achieve a high final ethanol concentration and to reduce production costs, a high substrate loading and hence a high water-insoluble solids (WISs) without detoxification is crucial for the SSF process (Rudolf *et al*., 2005; Ohgren *et al*., 2006b, Ohgren *et al*., 2006c). As the dry matter content or WISs in SSF increase, the concentration of compounds inhibitory to yeast and enzymes also increases (Hoyer *et al*., 2008). The maximum concentration of WISs in SSF is restricted due to inhibition of the enzymes and the yeast, as well as mass transport problems caused by viscosity of the pre-treated material (Linde *et al*., 2007). However, the higher the concentration of WISs during SSF the less energy is needed in the subsequent distillation and evaporation steps and therefore lower production costs (Hoyer *et al*., 2008). The recovery of ethanol from the fermentation broth by distillation is one of the most energy-intensive steps in the lignocellulose-to-ethanol process. It is therefore important to solve the problems associated with a high concentration of WISs through biotechnological strategies to facilitate fermentation processes, many remediation treatments for removal of inhibitors prior to fermentation, including physical (evaporation), chemical (solvent extraction, activated charcoal adsorption and use of ion exchange resins, or overlime procedures), are often required to reduce these inhibitory compounds to noninhibitory levels (Parajo *et al*., 1996, Converti *et al*., 1999, Cruz *et al*., 1999, Zaldivar *et al*., 1999; Rodrigues *et al*., 2001; Yang and Wyman, 2008). Treatment with activated charcoal or anion exchange resins has been shown to effectively remove lignin-derived inhibitors and acetic acid, respectively, leading to hydrolysate that show a fermentation similar to that of an inhibitor-free model substrate. Anion exchange resins works most effectively when the hydrolysate is adjusted to a pH of 10 which requires significant quantities of base chemicals (Ranjan *et al*., 2009). Activated carbon application does not require pH adjustment and sugars have a relatively low affinity towards charcoal. However, activated carbon application is expensive since powdered activated charcoal cannot be regenerated and granules usually incurs a 10% loss during each thermal reactivation cycle. The effectiveness of a detoxification method depends both on the type of hemicellulose hydrolysate and on the species of micro-organism employed, because each type of hydrolysate has a different degree of toxicity, and each species of micro-organism has a different degree of tolerance to inhibitors (Larsson *et al*., 1999). However, most of these detoxification methods add high production costs, and complicate the biomass-to-ethanol process, and generate additional waste products (Zaldiver *et al*., 1999; Liu *et al*., 2005). Detoxification of the pre-treated hydrolysate by using different forms of alkali treatment on specific fermentation inhibitors was shown to lead to extensive loss of fermentable sugars (Persson *et al*., 2002). An alternative to physical and chemical detoxification is the use of micro-organisms and/or their enzymes. Biological detoxification, the focus of this review, offers an improvement compared to these physical and chemical methods because little waste is generated among other advantages.

Biological methods of detoxification involve use of micro-organisms and/or their enzymes that act on the specific toxic compounds present in the hydrolysates and change their composition or structure to less toxic ones. The advantages of biological detoxification over chemical or mechanical methods include mild reaction conditions, avoiding further use of toxic and corrosive chemicals, fewer side-reaction toxic products, and less energy demand. Biological abatement is unquestionably environment friendly; it generates little waste and can be performed directly in the fermentation vessel prior to fermentation or simultaneously with the fermentation (López *et al*., 2004). The disadvantages, however, include long incubation periods required with the micro-organisms and the loss of sugars (Yang and Wyman, 2008). Therefore, to economize microbial detoxification of lignocellulosic hydrolysates, there is a need to investigate other strategies such as adaptation and targeted genetic engineering to improve the tolerance of ethanol-producing micro-organisms.

Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates

Several biological methods can be used to overcome inhibitory effects of aliphatic acids, furaldehydes and phenolic compounds on yeast fermentation in pre-treated lignocellulose materials. Possible options include detoxification of the pre-treated material before fermentation by using enzymes such as laccases or use of the natural or targeted genetic engineered bioreduction capability of the fermenting micro-organism which will detoxify the medium during the fermentation. The ability to degrade inhibitors exists in *S. cerevisiae* and other micro-organisms and we only need to exploit or enhance this natural strategy to overcome inhibitors in lignocellulose biomass in some cases through adaptation and genetic engineering. The fermentation can be carried out in a process design such as fed-batch that will allow for the natural reduction capability of the micro-organisms to be exploited.

Microbial detoxification

There have been several attempts to treat lignocellulose hydrolysates using biological methods for ethanol production. A number of micro-organisms including yeasts, fungi, and bacteria naturally can detoxify inhibitory

Table 1. Summary of biological detoxification of acid pre-treated lignocellulose hydrolysates using micro-organisms.

Hydrolysates	Micro-organism	Conditions	Effects	Reference
Waste house wood	*Ureibacillus thermosphaericus*	50°C, 24 h	Degraded phenolic, furfural and HMF in hydrolysates	Okuda *et al.*, 2008
			Improved ethanol production comparable to control	Naoyuki *et al.*, 2008
Corn stover	*Coniochaeta ligniaria* C8	30°C, 24 h	Removed inhibitors in furans, phenolic and aromatic and aliphatic acids and improved xylose utilization	Nichols *et al.*, 2008
Willow	*Trichoderma reesei*	–	Removed acetic acid, furfural and benzoic acid derivatives	Palmqvist *et al.*, 1997
Corn stover	*Coniochaeta ligniaria* C8	30°C, 5 days	Effective removal of furfural and HMF	Lopez *et al.*, 2004
Spruce	*Trichoderma reesei*		Effective removal of furfural and HMF	Larsson *et al.*, 1999
	RUT C30 NRRL11460			
Hardwood-spent sulfite liquor	*Saccharomyces cerevisiae* mutant		Removed acetic acid selectively	Schneider, 1996
Spruce	Encapsulated *Saccharomyces cerevisiae*and furfural		Effective detoxification of HMF	Talebnia and Taherzadeh, 2006

HMF, 5-hydroxymethyl-2-furaldehyde.

compounds and they have been used to pre-detoxify lignocellulosic hydrolysates before fermentation with *S. cerevisiae* for ethanol production (Table 1). Okuda *et al.* (2008) investigated the biological detoxification of a waste house wood (WHW) hydrolysate by a thermophilic bacterium, *Ureibacillus thermosphaercus*. The improvement of ethanol production by this bacterial detoxification is comparable to that of calcium hydroxide treatment (over-liming). Chromatographic analysis confirmed that *U. thermosphaercus* degraded the furfural or HMF present in the synthetic hydrolysates, and the phenolic compounds present in the WHW hydrolysates. The bacterium grows rapidly and consumes less than 5% fermentable sugars.

A fungal isolate, *Coniochaeta ligniaria,* (NRRL30616), was reported to metabolize furfural, HMF, aromatic and aliphatic acids, and aldehydes present in corn stover hydrolysate (Nichols *et al.*, 2008). The *high*-pressure *liquid chromatography*-ultraviolet-tandem mass spectrometry (HPLC-UV-ms/MS) analysis showed that compounds representing all of the classes of inhibitory side-products were removed during the course of fungal growth and improved xylose utilization in subsequent ethanol fermentations. In a related study, López *et al.* (2004) isolated a number of micro-organisms, including five bacteria related to *Methylobacterium extorquens, Pseudomonas* sp., *Flavobacterium indologenes, Acinetobacter* sp., *Arthrobacter aurescens*, and one fungus, *C. ligniaria* C8 (NRRL30616). These micro-organisms were capable of depleting toxic compounds from defined mineral medium containing a mixture of ferulic acid, HMF, and furfural as carbon and energy sources. However, only *C. ligniaria* C8 (NRRL30616) was effective in removing furfural and HMF from corn stover hydrolysate.

The filamentous soft-rot fungus, *Trichoderma reesei*, has been reported to degrade inhibitors of a willow hemicellulose hydrolysate (Palmqvist *et al.*, 1997). The cellulase-producing fungus, *T. reesei*, decreased the absorbance of the hydrolysate at 280 nm, which reflected the decrease in concentrations of furans. Acetic acid, furfural, and benzoic acid derivatives were also removed from the hydrolysate by the treatment with *T. reesei*. This *T. reesei* treatment improved ethanol productivity threefold and ethanol yield fourfold. However, the growth rates of these fungi were low and they assimilated sugars from hydrolysates. In another study by Larsson *et al.* (1999), the detoxification of dilute-acid hydrolysate of spruce by *T. reesei* was the most efficient one compared to anion exchange, over-liming, and treatment with laccase enzyme. But again, this organism consumed significant amounts of fermentable sugars (35%).

Recently, Hou-Rui *et al.* (2009) reported successful detoxification of sugarcane bagasse hydrolysate by the novel yeast isolates, *Issatchenkia occidentalis* (Lj-3, CCTCC M 2006097) and *Iris orienalis* (S-7, CCTCC M 200060098). HPLC elution curve of UV-absorption compounds represented by acetic acid, furfural, and guaiacol (toxic compounds found in the hemicellulosic hydrolysate) showed that several chromatographic peaks were evidently diminished after the yeast detoxification. Fermentation results of the biodetoxified hydrolysate showed an increase in xylitol productivity and yield in comparison to that of non-detoxified hydrolysate.

In another study, Schneider (1996) reported successful selective removal of acetic acid from hardwood-spent sulphite liquor using a *S. cerevisiae* mutant that grew on acetic acid but not on D-xylose, D-glucose, D-mannose, or D-fructose. The acetic acid-detoxification was achieved by aerobic fermentation of the lignocellulosic hydrolysate using the *S. cerevisiae* mutant. After 24 h aerobic growth, the acetic acid concentration in the hardwood liquor was reduced to levels that no longer inhibited ethanol fermentation. This was evidenced by the ability of several D-xylose fermenting yeasts' abilities to utilize essentially all of the sugars and produce 73% theoretical ethanol yield within 24 h from acetic acid-depleted liquor.

Dilute-acid lignocellulosic (spruce) hydrolysate was successfully detoxified in situ and fermented to ethanol by encapsulated *S. cerevisiae* at dilution rates up to 0.5 per hour in continuous cultivation (Talebnia and Taherzadeh, 2006). The hydrolysate was so toxic that freely suspended

yeast cells could ferment it continuously only up to dilution rate of 0.1 per hour, where the cells lost 75% of their viability measured by colony forming unit. However, encapsulation protected the cells against the inhibitors present in the hydrolysate and thus increased their capacity for *in situ* detoxification of the hydrolysate. The encapsulated yeast cells produced an ethanol yield of 0.44 g/g and HMF present in the hydrolysate was converted by 48–71%. Furfural was converted completely at dilution rates of 0.1 and 0.2 per hour, and partially at the higher dilution rates. Furfural and HMF are believed to be reduced to alcohols by alcohol dehydrogenase (Taherzadeh *et al.*, 1999). However, the use of encapsulated cells can be hampered by the gradual deactivation of yeast cells and the high cost.

Various other bacteria and yeast have been reported to partially transform furfural to furfuryl alcohol or furoic acid (Boopathy *et al.*, 1993; Gutiérrez *et al.*, 2002, 2006). Strains capable of aerobic furfural degradation include *Pichia putida* strain Fu1, (Koenig and Andreesen, 1989), *Aspergillus ascendens* and *Escherichia coli* mutants such as NAR30 (Wang *et al.*, 1994). Anaerobic degradation of furfural to acetate, CO_2 and/or methane by *Desulfovibrio furfuralis* was reported by Boopathy and Daniels, (1991). This sulphate-reducing bacterium was isolated from a continuous anaerobic digester used for treatment of furfural-containing wastewater. This *D. furfuralis* isolate could use furfural, furfuryl alcohol and 2-furoic acid as sole carbon and energy source in a defined mineral sulphate medium. Folkerts *et al.* (1989) reported a furfural-degrading, sulphate-reducing bacterium, *D. furfuralis,* which grew on furfural as a sole carbon source with sulphate as an electron acceptor. In addition, the ethanologenic bacteria *E. coli* strains KO11 and LYO1, and *Klebsiella oxytoca* strain P2, were also found to completely transform furfural into furfuryl alcohol (Gutiérrez *et al.*, 2002; 2006). These micro-organisms use furfural reductase to reductively detoxify furfural to less toxic furfuryl alcohol, which suggests a beneficial role for this enzyme in mitigating furfural toxicity encountered during ethanol production from lignocellulosic biomass.

Application of enzymes in detoxification

Another approach to detoxify lignocellulose hydrolysates is the enzymatic detoxification. One of the advantages of using enzymes is that higher temperatures can be used for efficient detoxification. These temperatures are not necessarily optimal for the growth of the micro-organisms. Secondly, the crude and/or pure enzyme preparations have high catalytic efficiencies, and thus the detoxification can be achieved faster by enzymes than by microbial cultures. Utility cost of enzymatic detoxification is low compared to other methods because it is usually conducted at mild conditions (pH 5, mesophilic temperature). Disadvantages of using enzymes are (i) the prolonged incubation needed for detoxification; (ii) high enzyme production costs. The commercial enzymes are still expensive despite strides made to reduce enzyme cost through modern biotechnology.

In addition to plants, a number of fungi particularly the basidiomycetous white-rot fungi produce laccases and peroxidases. Laccases (benzenediol: oxygen oxireductases EC 1.10.3.2) are multicopper enzymes capable of oxidizing phenols and aromatic amines, reducing molecular oxygen to water (Couto and Toca-Herrera, 2007; Bleve *et al.*, 2008). For biological detoxification of lignocellulosic hydrolysates, Jonsson *et al.* (1998) reported that some of the phenolic compounds in a willow hemicellulose hydrolysate from willow pretreated with steam and SO_2 were degraded by laccase and lignin peroxidase from the white-rot basidiomycete fungus *Trametes versicolor*. Ethanol productivity was subsequently increased by two to three times. The laccase treatment led to selective and virtually complete removal of phenolic monomers and phenolic acids. The absorbance at 280 nm, indicative of the presence aromatic compounds, did not decrease, whereas an increase in absorbance for the large-size material and a decrease for the small-size material were observed for all wavelengths tested. The detoxifying mechanism was therefore suggested to be oxidative polymerization of low-molecular weight phenolic compounds.

Enzymatic treatment of dilute-acid spruce hydrolysate with the phenoloxidase/laccase from *T. versicolor* was reported to be the most efficient detoxification method that specifically removed phenolic compounds (Larsson *et al.*, 1999a). Detoxification of sugarcane bagasse hydrolysate by laccase improved ethanol production by *Candida shehatae* NCIM 3501. The ethanol yield achieved after laccase treatment was comparable to the one detoxificated by using ion exchange and activated carbon (Chandel *et al.*, 2007). The laccase treatment of acid hydrolysates of sugarcane bagasse brought about a 77.5% reduction of total phenolics without affecting furans and acetic acid content. Of the various methods of detoxification tested, laccase treatment brought about a negligible loss of total sugars and maximum removal of 4-hydroxyacetophenone, 4-hydroxy benzoic acid, vanillic acid, ρ-coumaric acid, syringic acid, and ferulic acid present in the acid hydrolysate. The effects of enzymes on detoxification of lignocellulosic hydrolysates are summarized in Table 2.

Martin *et al.* (2002) compared the effect of laccase treatment and over-liming, on the composition and fermentability of enzymatic hydrolysates of sugarcane bagasse by the genetically engineered xylose-utilizing *S. cerevisiae* strains. They reported that approximately 80% of the phenolic compounds were specifically removed by the laccase treatment. Over-liming partially removed phenolic compounds, but also acetic acid, furfural and HMF. Sugars were marginally affected by the detoxification treatments. Both treatments resulted in improved ethanol yield and productivity. The results showed that removal of inhibitors was necessary for achieving good fermentation performance even with recombinant xylose-utilizing yeasts.

Table 2. Summary of biological detoxification of acid pre-treated lignocellulose hydrolysates using enzymes.

Hydrolysates	Enzymes	Effects	Reference
Willow	Laccase from *Trametes versicolor*	Selective and complete removal of phenolic monomers, increased ethanol productivity	Jonsson *et al.*, 1998
Spruce	Laccase from *Trametes versicolor*	Specific removal of phenolic compounds, increased ethanol yield	Larsson *et al.*, 1999
Sugarcane bagasse-	Laccase from *Cyathusc stercoreus*	Selective removal of phenolic compounds Caused negligible loss in total sugars Increased ethanol yield	Chandel *et al.*, 2007
Sugarcane bagasse	Phenoloxidase laccase from *Coriolus (Trametes) versicolor*	Removed about 80% of phenolic compounds Improved ethanol productivity	Martin *et al.*, 2002

The success of laccases to the above-mentioned processes requires production of large quantities of enzyme at low cost. A number of different yeasts have been engineered to produce laccase in large quantities to try to reduce the cost of laccase enzymes (Couto and Toca-Herrera, 2007). Heterologous production of laccases in fungal hosts that are capable of producing high amounts of extracellular enzymes can overcome the problem often encountered with industrial laccases production by the native hosts.

Adaption of micro-organisms to toxic hydrolysate

Adaptation (evolutionary engineering) of the fermentation micro-organisms to the lignocellulosic hydrolysate has been suggested as an alternative detoxification approach. Although chemical, enzymatic, and microbial detoxification improves the fermentability of hydrolysates, it is desirable to develop adaptive ethanol-producing micro-organisms that require minimal or no detoxification treatment. These adapted organisms not only reduce the detoxification cost, but also avoid loss of fermentable sugars (Keller *et al.*, 1998; Rivard *et al.*, 1996; Martín *et al.*, 2007).

S. cerevisiae, the work-horse in ethanol production, has an innate tolerance to some of the inhibitory compounds in lignocellulose hydrolysates such as furan and phenolics due to its ability to convert them to less harmful ones (Olsson and Hahn-Hagerdal, 1993; Hahn-Hagerdal *et al.*, 1994; Almeida *et al.*, 2007). HMF is reduced to 2,5-bis-hydroxymethylfuran (HMF alcohol) and furfural to furfuryl alcohol under aerobic and anaerobic conditions (Liu *et al.*, 2005). Industrial polyploid yeast strains appear to be more resistant than laboratory strains in lignocellulose hydrolysates. *S. cerevisiae* also has the natural ability to metabolize some phenolic compounds present in the lignocellulose hydrolysate probably due to the presence of phenylacrylic acid decarboxylase (Pad1p; Klinke *et al.*, 2003). Based on this innate capability, yeast strains can be adapted by constant exposure to sublethal inhibitor concentrations in hydrolysates in fed-batch or continuous cultivation. The organism may furthermore obtain an enhanced conversion capacity by short- and long-term adaptation (Almeida *et al.*, 2009). Adaptation has been shown to increase the ability of a broad range of yeast strains to grow in lignocellulosic hydrolysates, resulting in increased fermentation rates and ethanol yields (Table 3). A robust tolerant yeast is required to maintain a high ethanol yield with increased substrate loadings in SSF and to reduce the amount of yeast and enzymes used.

A problem which normally occurs in SSF when inhibitor concentration is high is a long lag phase during fermentation due to change from cultivation to fermenting conditions. The long lag phase increases the total time required for SSF and increases the capital cost (Linde, 2007). Attempts have been made to overcome this problem by adapting the yeast to the inhibitors by cultivating the yeast in hydrolysate. For a sustainable and cost-effective biomass-to-ethanol industrial process, it is important to develop more tolerant yeast strains that can detoxify the inhibitors *in situ* and produce bioethanol (Liu, 2006). Adaptation of yeast was shown to be important in SSF experiments by Alkasrawi *et al.* (2006). The adapted yeast had higher fermentation rates in spruce hydrolysate than the unadapted parent in pure glucose medium. This was partly due to the higher conversion of HMF by the adapted yeast. Keller *et al.* (1998) also demonstrated that *S. cerevisiae* D_5A and K-1 strains could adapt and grow in Douglas fir acid hydrolysate (15% solids) with a reduced lag phase and a quadrupled ethanol concentration. At 15% total solids concentration, which is near the minimum solids concentration for economic ethanol production, the wild type produced no ethanol even in the presence of 2% (20 g/L) sugar concentration from the beginning. The ethanol volumetric productivity of the adapted strains at 15% solids was about 0.41 g/L/h over the first 24 h. Similar positive results from using adapted yeast cultivated on lignocellulose hydrolysate have been reported by Rudolf *et al.* (2005), Sassner *et al.* (2006), Alkasrawi *et al.* (2006), Martín *et al.* (2007) and Linde *et al.* (2007). Modig *et al.* (2008) proposed that induction of HMF and furfural reduction activities might play an important role for yeast adaption to the hydrolysate.

Long-term adaptation have also been reported by Liu *et al.* (2005) and Heer and Sauer, (2008), as summarized in Table 3. Heer and Sauer (2008) developed *S. cerevisiae* TMB3400 strain with increased tolerance towards furfural and HMF by growing the yeast in basal medium containing increasing furfural concentration for over 300 generations. The best adapted strain showed a lag phase of 17 h compared to the 90 h for the parental strain in media supplemented with 17 mM furfural.

Similar results were obtained with adaptation of a recombinant xylose-utilizing *S. cerevisiae* strain to a

Table 3. Strain improvement by adaptation or evolutionary engineering.

Yeast	Hydrolysate	Effects	Reference
Saccharomyces cerevisiae TMB 3001	Sugarcane acid hydrolysate	Increased tolerance against furaldehydes, phenolic compounds and aliphatic acids; improved ethanol production	Martin *et al.*, 2007
Pichia stipitis	Corncob acid hydrolysate	High fermentation rate	Amartey and Jeffries, 1996
Pichia stipitis CBS 5776	Red oak acid hydrolysate	Improved ethanol yield	Olsson and Hahn-Hagerdal, 1996
Pichia stipitis NRRL Y-7124	Basal media with HMF	Enhanced ability to reduce HMF to 2,5-bis-hydroxymethylfuran	Liu *et al.*, 2005
Saccharomyces cerevisiae NRRL Y-12632	Basal media with furfural	Enhanced conversion of furfural to furfuryl alcohol	
TMB3400	Basal medium with furfural	Shortened the lag phase from 90 h to 17 h Improved viability in the presence of furfuryl	Heer and Sauer, 2008
Saccharomyces cerevisiae D5A *Saccharomyces cerevisiae* K-1	Dilute-acid Douglas fir hydrolysate	Shortened lag phase, quadrupled ethanol concentration, productivity was 0.41 g/L/h in the first 24 h in simultaneous saccharification and fermentation	Keller *et al.*, 1998

HMF, 5-hydroxymethyl-2-furaldehyde.

Table 4. Strain improvement by targeted genetic engineering.

Gene-enzyme	Origin	Identification	Experimental conditions	Strain improvement	Reference
ADH6	*Saccharomyces cerevisiae*	Microarray analysis	Pulse addition of 1.5 g/L HMF in (an)aerobic batch cultivation in mineral medium	Four times higher specific uptake rate of HMF	Petersson *et al.*, 2006
			Batch fermentation of spruce hydrolysate	Four times higher specific uptake rate of HMF and 20% higher specific ethanol productivity	Almeida *et al.*, 2008a
MUT-ADH1	*Saccharomyces cerevisiae*	Protein purification, MS, and gene isolation	Semi-aerobic growth in mineral medium supplemented with 20 mM HMF	Reduction of lag phase and two times faster HMF conversion	Laadan *et al.*, 2008
	TMB3000 strain		Batch fermentation of spruce hydrolysate	Four times higher specific uptake rate of HMF and 18% higher specific ethanol productivity	Almeida *et al.*, 2008a
ADH7	*Saccharomyces cerevisiae*	Microarray analysis	Growth in minimal medium supplemented with 40 mM HMF	Exit lag phase after 94 h While the control failed to grow even after 156 h	Larroy, 2002; Liu *et al.*, 2008; Petersson *et al.*, 2006
XYL1	*Pichia stipitis*	Protein purification	Growth in minimal medium	Increased *in vivo* HMF	Almeida et al., 2008b
ZWF1	*Saccharomyces cerevisiae*	Screening of a *Saccharomyces cerevisiae* gene disruption library	Growth in SD-complete supplemented with 50 mM furfural	Allowed growth at this furfural concentration, which was lethal for control strain	Gorsich *et al.*, 2006
FFR	*Escherichia coli* LYO1	Protein purification	Data not available		Gutiérrez *et al.*, 2006

ADH6, alcohol dehydrogenase 6; ADH7, alcohol dehydrogenase 7; HMF, 5-hydroxymethyl-2-furaldehyde; FFR, furfural reductase; MS, mass spectrometry; MUT-ADH1, mutated alcohol dehydrogenase 1; SD, synthetic defined *medium*; XYL1, xylose reductase; ZWF1, glucose-6-phosphate dehydrogenase.

sugarcane bagasse hydrolysate containing high concentrations of fermentation inhibitors (Martín *et al.*, 2007). The yeast was cultivated for 353 h using medium with increasing concentrations of inhibitors, including phenolic compounds, furaldehydes and aliphatic acids. The performance of the adapted strain was compared with the parental strain with respect to its ability to ferment three bagasse hydrolysate containing different

inhibitor concentrations. The adapted strain showed a better ethanol yield and furfural conversion rate than the parental strain in the two bagasse hydrolysates containing higher levels of inhibitors (50% hydrolysate).

The ethanologenic yeast *S. cerevisiae* demonstrated a dose-dependent inhibition by the inhibitors and the potential to convert furfural and HMF into less toxic compounds, furfural alcohol and 2,5-bis-hydroxymethylfuran (also called furan-2,5-dimethanol), respectively (Liu *et al.*, 2005).

Genetic and metabolic engineering

More tolerant yeast strains for SSF than those available today, may be developed by genetic engineering, for example, overexpressing genes encoding enzymes for resistance against specific inhibitors, and altering the co-factor balance in the yeast cell (Almeida *et al.*, 2007). Engineering can be used in different dimensions for detoxification or to overcome toxicity during fermentation. The reduction of HMF by strains of *S. cerevisiae* has been found to be both NADPH- and NADH-coupled, the ratio of the activities being strain-dependent (Wahlbom and Hahn-Hagerdal, 2002; Nilsson *et al.*, 2005). Tolerance to furfural-induced stress was found to be associated with the pentose phosphate pathway genes ZWF1, GND1, RPE1 and TKL1 in *S. cerevisiae* (Gorsich *et al.*, 2006). Several attempts have been made to genetically modify *S. cerevisiae* by overexpressing yeast reductase and dehydrogenase, and the pentose phosphate pathway genes in order to detoxify inhibitors in acid pre-treated lignocellulose hydrolysates (recently well reviewed by Almeida *et al.*, 2009, and therefore will not be discussed in detail in the present review). Improvement of yeast tolerance to lignocellulosic hydrolysates has been achieved by overexpressing homologous and heterologous reductase and dehydrogenase genes encoding enzymes that confer resistance towards specific inhibitors, summarized in Table 4 (adapted from Almeida *et al.*, 2009).

Table 5. Summary of heterologous expression of laccases in *Saccharomyces cerevisiae.*

Laccase	Origin	Host	Comments	Reference
PO1	*Coriolus hirsutus*	*Saccharomyces cerevisiae*	Active laccase secreted in the medium	Kojima *et al.*, 1990
PO2	*Coriolus hirsutus*	*Saccharomyces cerevisiae*	Active laccase secreted in the medium	
LCC1	*Trametes versicolor*	*Saccharomyces cerevisiae*	Undetectable laccase activity in the medium	Cassland and Jönsson, 1999
LCC2	*Trametes versicolor*	*Saccharomyces cerevisiae*	Active laccase secreted in the medium	Cassland and Jöhnsson, 1999
LCC2	*Trametes versicolor*	*Saccharomyces cerevisiae*	Laccase activity enhanced twofold, and production of ethanol in the presence of coniferyl aldehyde Transformant converted coniferyl at a faster rate than control	Larsson *et al.*, 2001a
LAC2	*Loblolly pine*	*Saccharomyces cerevisiae*	No laccase activity detected	Sato *et al.*, 2001
MtL	*Myceliophthora thermophyla*	*Saccharomyces cerevisiae*	Laccase secreted activity of 0.66 U/l (ABTS) Total activity was enhanced 170-fold by directed evolution	Bulter *et al.*, 2003
LAC1	*Trametes* sp. *strain C30*	*Saccharomyces cerevisiae*	Activity barely detected in the medium	Klonowaska *et al.*, 2005
LAC2	*Trametes* sp. *strain C30*	*Saccharomyces cerevisiae*	Undetectable laccase activity in the medium	
LAC3	*Trametes* sp. *strain C30*	*Saccharomyces cerevisiae*	Laccase secreted activity of 0.5 U/mL (SGZ)	
Ery3	*Pleurotus eryngii*	*Saccharomyces cerevisiae*	Laccase yield of 139 mU/mL was obtained when yeast cells were immobilized, a 1.6-fold increase compared with free yeast cells	Bleve *et al.*, 2008
LAC1	*Melanocaepus Albomyces*	*Saccharomyces cerevisiae*	Laccase production was increased modified cDNA	Kiiskinen and Saloheimo, 2004
POXC	*Pleurotus ostreatus*	*Saccharomyces cerevisiae*	Active laccase secreted in the medium, recombinant POXC transformants had higher secreted activity	Piscitelli *et al.*, 2005
POXA1b	*Pleurotus ostreatus*	*Saccharomyces cerevisiae*	Recombinant POXA1b	

ABTS - 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid], SGZ - syringaldazine

Laccases, versatile enzymes, have received much attention during the past decades due to their broad substrate specificity and their ability to use molecular oxygen as the final electron acceptor instead of hydrogen peroxide. The use of laccases in the detoxification of lignocellulose hydrolysates has been discussed and summarized in Table 2. An alternative approach would be to genetically engineer *S. cerevisiae* for production of active laccase. This would allow detoxification and ethanol fermentation to proceed simultaneously in one step, thus eliminating the detoxification step and reducing the cost of laccase (Larsson *et al.*, 2001a). Laccase genes have been expressed in the *S. cerevisiae* in multiple studies (Kojima *et al.*, 1990; Cassland and Johnsson, 1999; Hoshida *et al.*, 2001, 2005; Larsson *et al.*, 2001a,b; Bulter *et al.*, 2003; Kiiskinen and Saloheimo, 2004; Necochea *et al.*, 2005; Piscitelli *et al.*, 2005) as summarized in Table 5.

One example is a laccase gene from the white-rot fungus *T. versicolor* which was expressed in *S. cerevisiae* to increase its resistance to phenolic inhibitors present in lignocellulose hydrolysates. The laccase-producing transformant had the ability to convert coniferyl aldehyde at a very fast rate, which enabled faster growth and ethanol formation (Larsson *et al.*, 2001a). The laccase-producing strain was also able to ferment a dilute-acid spruce hydrolysate at a faster rate than the control. A decrease in the content of low-molecular-mass aromatic compounds, accompanied by an increase in the content of high-molecular-mass compounds, was observed during fermentation with the laccase-expressing strain, demonstrating that laccase was active even at the very low levels of oxygen supplied. These results demonstrated the advantage of using recombinant laccase-producing yeast strains for producing bioethanol from lignocellulose. Heterologous expression in *S. cerevisiae* was also reported for laccase by Kiiskinen and Saloheimo (2004). Laccase is difficult to express in yeasts and has been obtained in low amounts in many of the work summarized in Table 5. Therefore, it is highly desirable to increase the activity of laccase produced by *S. cerevisiae* strains, for example, through enhancement of secretion by overexpression of the homologous t-SNARE Sso2p, a protein involved in the secretion machinery (Larsson *et al.*, 2001b). Most of the laccases expressed in *S. cerevisiae* have not been purified or characterized. There is also the need to optimize the culture conditions for those recombinant laccase-expressing yeasts to improve expression as reported by Cassland and Jonsson (1999), who improved laccase production by decreasing the cultivation temperature. Finding the bottlenecks of a specific expression system requires consideration of many possibilities. In addition, directed evolution studies for functional tailoring can be performed with the cloned laccase genes in *S. cerevisiae* in the future (Bulter *et al.*, 2003).

Larsson *et al.* (2001b) proposed that effective overexpression of *S. cerevisiae* Pad1p resulted in improved resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and anaerobic conditions. Pad1p catalyses a decarboxylation step by which aromatic carboxylic acids are converted to the corresponding vinyl derivatives. Pad1p-overexpressing *S. cerevisiae* was cultivated in basal medium supplemented with ferulic acid and cinnamic acid as well as in the spruce hydrolysate. Recombinants overexpressing Pad1p had the ability to convert ferulic and cinnamic acid at a faster rate, resulting in faster cell growth and a 24–29% higher ethanol production rate than the control.

Future research

A lot of research still needs to be carried out on the development and optimization of microbial and enzymatic detoxification of lignocellulosic hydrolysate. Much of the research has been carried out on the laboratory-scale and there is little work in pilot-scale and full-scale investigations on the use of enzymes to detoxify lignocellulosic hydrolysate before fermentable sugar conversion to chemicals or ethanol. Pilot-scale and full-scale research would facilitate better evaluation of the technology, its constraints and opportunities. The cost of the enzymes is of prime importance in order to realize the full potential of detoxification of lignocellulosic hydrolysates. The enzymes that are presently being investigated are still expensive because of their cost of production. However, this should not thwart the efforts to carry out more extensive research to identify the most promising enzymes and determine the optimal conditions for their application. In fact, the results of such research should provide the incentive for commercial development to finally produce the enzymes economically on a large-scale. The costs are expected to decrease as technology and techniques advance and as cheaper growth substrates are explored for cultivating the parent micro-organisms. Enzymes to be used in detoxifying lignocellulosic hydrolysates do not have to be highly purified.

Good results have been presented from adapted yeast strains and there is a need to carry out investigations to determine how long the improved characters derived from adaptation remain after selective pressures have been lifted. The biological detoxification step may increase the overall process cost due to the capital cost, but strain improvement by genetic engineering could be more costly. There is need for techno-economic analysis of biological detoxification and targeted genetic engineering strategies to come up with a proper conclusion.

Conclusions

Efforts to utilize renewable lignocellulosic resources for the production of biofuels such as bioethanol have resulted in considerable progress. Lignocellulosic ethanol is obtained by pre-treating the biomass to liberate cellulose and hemicellulose which are then hydrolyzed by enzymes to monomeric sugars. The sugar is then fermented to ethanol by yeast, mainly *S. cerevisiae* strains.

Several challenges must be met to make the cost of lignocellulosic ethanol competitive with that of petrol. One of the major challenges faced in commercial production of lignocellulosic bioethanol is the inhibitory compounds generated during the thermo-chemical pre-treatment step of biomass. These inhibitory compounds are toxic to the hydrolytic enzymes and the fermenting micro-organisms. Improved ethanol yields are expected if biotechnological strategies are employed to overcome inhibition of yeast fermentation of lignocellulose waste hydrolysates. From the data found in the literature, it can be concluded that detoxification using either micro-organisms or laccase enzymes can be used to improve ethanol yield and production from lignocellulose hydrolysates. Adaptation and genetic engineering of *S. cerevisiae* strains are also promising strategies for improved bioethanol production at a competitive cost. A number of authors have reported significant reduction in toxic compounds, which improved efficiency of bioethanol production when lignocellulosic hydrolysates were detoxified using these biological methods. Enzymes act on specific substances present in lignocellulosic hydrolysates, and therefore, make the hydrolysates more amenable to be easily converted to valuable products. However, the detoxification of lignocellulosic hydrolysates requires not only micro-organisms and their enzymes, but also optimization of the conditions. Novel *S. cerevisiae* strains with an improved ability to ferment lignocellulose hydrolysates have been obtained by the heterologous expression of laccase.

Declaration of interest

The authors declare no conflict of interest.

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