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# Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production

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## 1. Introduction

Lignocelluloses are the most abundant raw materials on Earth comprised of cellulose, hemicelluloses and lignin. After cellulose, hemicellulose is the principal fraction of the plant cell wall that could serve as a potential substrate for the production of value-added products under optimized conditions [Chandel & Singh, 2011; Chandel et al., 2010a; Hahn-Hagerdal et al., 2007; Saha, 2003]. Largely, the secondary cell wall of plants contains cellulose (40–80%), hemicellulose (10–40%), and lignin (5–25%). The carbohydrate fraction of the plant cell wall can be converted into fermentable monomeric sugars through acidic and/or enzymatic (hemicellulase/cellulase) reactions, which have been exploited to produce ethanol, xylitol, n-butanol and 2, 3-butanediol via microbial fermentation processes [Sun, 2009.; Chandel et al., 2010a; Carvalheiro et al., 2005; Saha, 2003]. Until now the pretreatment is unavoidable necessity, which has been examined and employed extensively in the past [Moiser et al., 2005, Taherzadeh & Karimi 2007; Kumar et al., 2009; Chandel et al., 2010b]. The acidic pretreatment of lignocellulosics hydrolyzes the hemicellulose fraction, enabling subsequent enzymatic digestion of the cellulose in fermentation reaction [Kumar et al., 2009; Chandel et al., 2007a; Chandel et al., 2007b; Chandel et al., 2007c]. However, the non-specificity of acidic treatment led to the formation of complex sugars and compounds inhibitory to the microorganisms for ethanol production [Parawira & Tekere, 2011].

The depolymerization of hemicellulose by chemical process yields xylose as the major fraction and arabinose, mannose, galactose, and glucose in smaller fractions in addition to potential microbial inhibitors [Chandel et al., 2010a; Gírio et al., 2010; Chandel et al., 2009; Chandel et al., 2007a]. These inhibitors can be divided into three major groups (Fig. 1), i.e. organic acids (acetic, formic and levulinic acids), ii. furan derivatives [furfural and 5-hydroxymethylfurfural (5-HMF)], iii. phenolic compounds [Chandel et al., 2010a; Chandel et al., 2007b; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000a], affecting overall cell physiology and often result in decreased viability, ethanol yield, and productivity [Chandel et al., 2007a; Palmqvist & Hahn-Hagerdal, 2000a]. The ethanologenic microorganisms have ability to degrade some of the inhibitors; however, the toxicity of hydrolysate was determined by the aggregate effect of compounds [Mussatto and Roberto, 2004; Zaldivar et al., 2001]. Progress has been made to achieve higher levels of sugars by diminishing the overall impact of fermentative inhibitors which in-turn improves the fermentability of lignocellulosic hydrolysates [Alriksson et al., 2011; Sun & Liu, 2011;

Parawira & Tekere, 2011]. The ion exchange resins, active charcoal, enzymatic detoxification using laccase, alkali treatments and overliming with calcium hydroxide are among selective detoxification strategies which have been investigated in the past [Jurado et al., 2009; Chandel et al., 2007a, b, c; Villarreal. et al., 2006]. Other strategies include changes in fermentation methodologies and metabolic engineering (incorporation of ligninase or laccase genes) have been introduced to overcome from the cell wall degrading inhibitors [Larsson et al., 2001]. Treatment with the soft-rot fungus *Trichoderma reesei* and other microorganisms to degrade inhibitors in a hemicellulase hydrolysate has also been proposed [Yu et al., 2011; Fonesca et al., 2011; Okuda et al., 2008; Tian et al., 2009; López et al., 2004]. This chapter aims to discuss the detoxification strategies which may assist to overcome the fermentation inhibitors of lignocellulosics. The biological pretreatment of lignocellulosic raw materials prior to their hydrolysis and the approaches of biotechnology routes making them resistant towards the inhibitors have also discussed.

## 2. Inhibitors profile and lignocellulose hydrolysates

Thermochemical degradation of hemicellulose liberates majority of sugar monomers i.e. xylose, mannose, acetic acid, galactose, and glucose, in conjunction with number of inhibitors toxic to the fermenting microorganism (Fig. 1, 2). These inhibitors include furans (furfurals and 5-Hydroxy methyl furfural (5-HMF)), phenolics, weak acids (acetic acid, levulinic acid, formic acid etc.), raw material extractives (acidic resins, tannic and terpene acids), and heavy metal ions (iron, chromium, nickel and copper) (Fig. 1). Among inhibitors Hibbert's ketones have also been noticed in the acid hydrolysates of pine and spruce [Klinke et al., 2004; Clarck & Mackie, 1984]. Table 1 summarizes the inhibitors profile derived from variety of lignocellulosic materials.

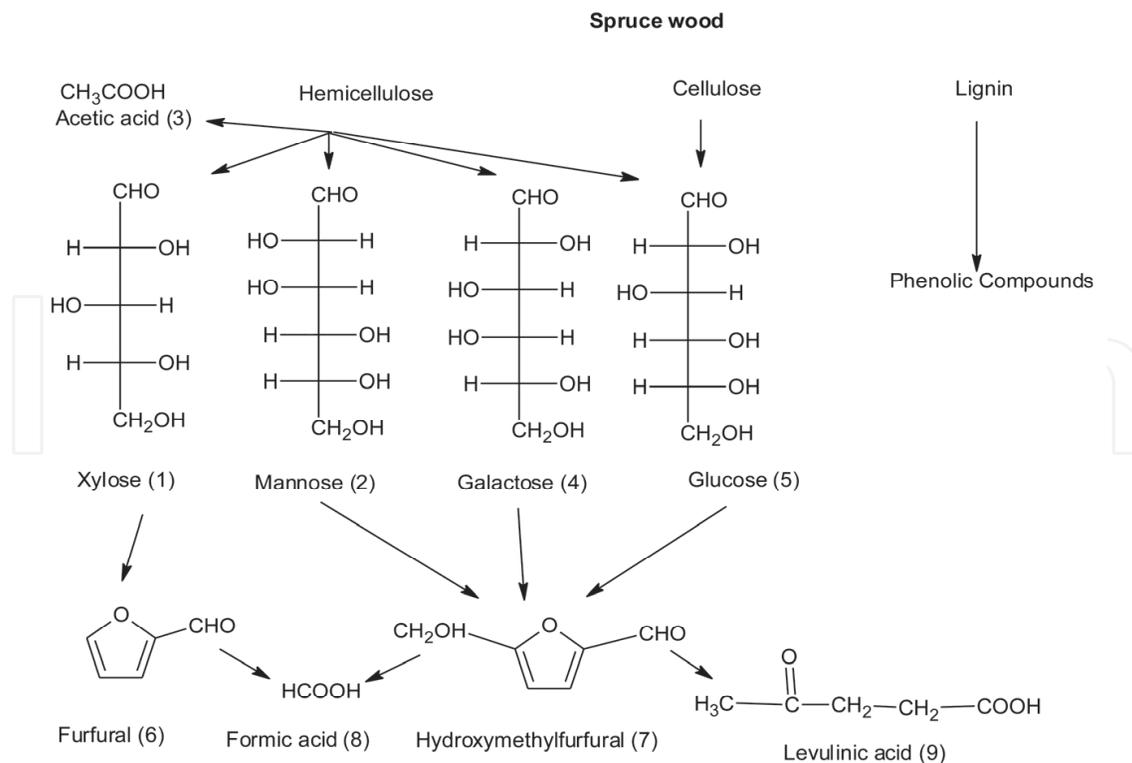


Fig. 1. Structural profile of lignocellulose derived fermentation inhibitors [Source: Palmqvist & Hahn-Hagerdal, 2000b]

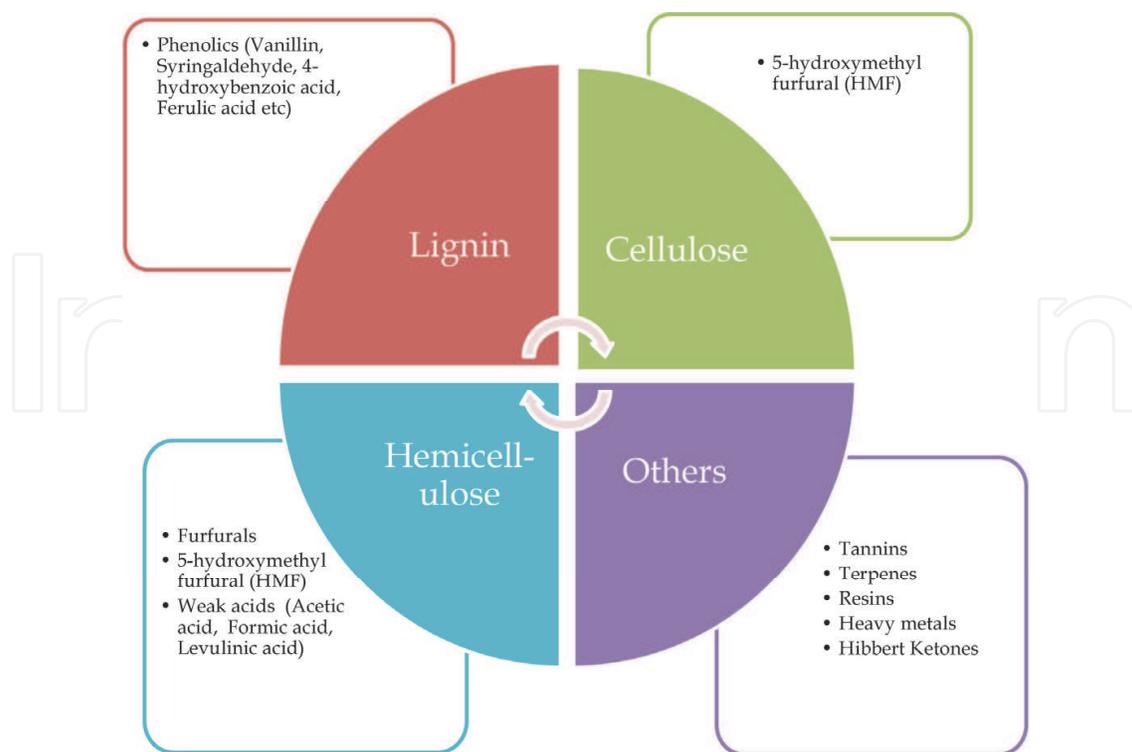


Fig. 2. Plant cell wall derived inhibitors. Cell wall component yields a variety of inhibitors in addition to sugar monomers after acid hydrolysis of lignocellulosic biomass at high temperature. Hemicellulose and cellulose breakdown releases weak acids, furans (5-HMF, furfurals) and 5-HMF respectively. Specialized plant cell wall components yield terpenes, tannins, Hibbert Ketones etc. A variety of phenolic monomers (syringaldehyde, vanillin, ferulic acid etc.) are generated from lignin in conjunction with hemicelluloses and cellulose-derived inhibitors.

Degradation of xylose at higher temperature and pressure reveal furfurals inhibitor. 5-(Hydroxymethyl) fufural (5-HMF) forms during hexose degradation, however the concentration of HMF in acid hydrolysates tends to low due to limited degradation of hexose in acidic saccharification. Among inhibitors most phenolics are being generated from lignin breakdown. These inhibitors include three forms of organic compounds such as acid, ketone and aldehyde (i.e. example, syringaldehyde, syringic acid and syringone, vanillic acid, vanillone, and vanildehyde) [Parawira & Tekere, 2011; Mussatto & Roberto, 2004; Palmqvist & Hahn-Hagerdal, 2000a, b]. 4-Hydroxy benzoic acid, ferulic acid and guaiacol are among most common lignin derived inhibitors observed in lignocellulose acid hydrolysates [Klinke et al., 2004]. Raw material extractives are generated due to the presence of tannic acid, terpenes and other kind of ploymers present in plant cell walls [Mussatto & Roberto, 2004]. The heavy metal ions could form due to the corrosion of reaction vessel used for hydrolysis reactions or other chemical moieties [Mussatto & Roberto, 2004; Parajo et al., 1996; Watson et al., 1984]. The monomeric sugars and cell wall derived components of sugarcane bagasse, a model lignocellulosic substrate, has been summarized in Table 2. The formation of cell wall degrading components in the acid hydrolysate are depend upon multiple factors including nature of lignocellulosic material and its cell wall composition, thermochemical conditions, and reaction time of the hydrolysis [Palmqvist & Hahn-Hagerdal, 2000a, b]. These components are required to remove from the lignocellulose hydrolysates prior to the fermentation.

Lignocellulosic material	Inhibitors profile (g/l)	References
Sugarcane bagasse	Furans, 1.89; Phenolics, 2.75; Acetic acid, 5.45	Chandel et al., 2007 <sup>a</sup>
Wheat straw	Furfural, 0.15±0.02; acetic acid, 2.70±0.33	Nigam, 2001
Rice straw	Acetate, 1.43; HMF, 0.15; Furfural, 0.25	Baek & Kwon, 2007
Corn stover	Acetic acid, 1.48; Furans, 0.56; Phenolics, 0.08	Cao et al., 2009
Spruce	Phenolics, 0.44 ± 0.05; Furfural, 1.0 ± 0.1; HMF, 3.3 ± 0.2; Acetic acid, 5.0 ± 0.4; Levulinic acid, 0.2 ± 0.1; Formic acid, 0.7 ± 0.1	Alriksson et al., 2010
<i>Eucalyptus globulus</i>	Furfural, 0.26; 5-HMF, 0.07; Acetic acid, 3.41; Phenolics, 2.23	Villarreal et al., 2006
<i>Saccharum spontaneum</i>	Furfurals, 1.54 ± 0.04; Phenolics, 2.01 ± 0.08	Chandel et al., 2011a
Poplar	2-furoic acid, 0.3 microgram/g; 3,4-HBA, 2.5; Salicylic acid, 56; Syringaldehyde, 6.0; Ferulic acid, 4.7	Balan et al., 2009
Soft wood	Acetic acid, 5.3; Furfural, 2.2	Qian et al., 2006

Table 1. Plant cell wall derived inhibitors profile from different lignocellulosic substrates

HCl (%)	Xylose (g/l)	Arabinose (g/l)	Glucose (g/l)	Total sugars (g/l)	Total furans (g/l)	Total phenolics (g/l)	Acetic acid (g/l)
0.5	16.5	1.98	1.85	20.33	0.94	0.65	3.50
1.5	17.2	2.56	3.82	23.58	1.36	1.58	4.19
2.5	21.5	2.95	5.84	30.29	1.89	2.75	5.45
3.5	19.5	1.82	2.09	23.41	3.41	3.01	6.69

Table 2. Acid hydrolysis of sugarcane bagasse at 140 °C, 30 min and initial solid: liquid ratio (1:10) [Source: Chandel et al., 2007a] (The values are mean of three replicates. Standard deviation was within 10%)

### 3. Plant cell-wall derived inhibitors and microorganisms

The toxicity of inhibitors depends upon the concentration, type of fermentative organism, the mode of cultivation, and cultivation conditions (i.e. pH, inoculum, dissolve oxygen and temperature). The toxic component may lead to stop the growth of microorganism by affecting the rate of sugar uptake with simultaneous decay in product formation. These inhibitors affect the cellular physiology by disturbing the function of biological membranes, causes poor microbial growth lingering towards extended incubation time with poor metabolite production. However, the yield may remain unaltered. The mechanism of inhibition of some compounds such as phenolics and plant cell wall derived extractives are yet to be known.

Among sugar derived inhibitors, furfurals have been found to inactivate the cell replication that reduces the growth rate and the cell mass yield on ATP, volumetric growth rate and specific productivities [Taherzadeh et al., 1999; Palmqvist et al., 1999a]. Furfurals have been found toxic to *Pichia stipitis* under aerobic condition, whereas the growth of *Saccharomyces cerevisiae* was less affected under anaerobic condition by converting into furoic acid [Palmqvist et al., 1999a; Taherzadeh et al., 1999]. Adaptation of microorganisms on high furfural concentration has been found a successful option to decrease the furfural effect on growth. It may be due to the synthesis of new enzymes or co-enzymes for furfural reduction [Boyer et al., 1992; Villa et al., 1992]. Furans (furfurals and 5-HMF) in conjunction with acetic acid have been reported highly affective to the growth of *P. stipitis*, *Pachysolen tannophilus* and *Escherichia coli* [Martinez et al., 2000; Lohmeier-vogel et al., 1998].

The presence of lignin derived inhibitors in the acid hydrolysate is highly effective for the growth of fermenting microorganisms. Lignin derived inhibitors include polyaromatic, phenolics and aldehydes. Their toxicity is thought to be proportional to the molecular weights. The lower molecular weight of phenolic compounds is generally lethal to the microorganisms than higher molecular weight compounds [Clarck & Mackie, 1984]. They are toxic to fermenting microorganism even more than furans and weak acids inhibiting the microbial growth. They cause a partition and disturb the membranes in turn affecting their ability to serve as selective barriers and enzyme matrices affecting the cell growth and sugar assimilation [Palmqvist & Hahn-Hagerdaal, 2000b]. Among lignin derived inhibitors vanillin, syringaldehyde, 4-hydroxybenzoic acid, catechol, acetosyringone, and 1-hydroxybenzotriazole (HBT) causes a partition and loss of integrity of biological membranes in microorganisms diminishing cell growth and sugar assimilation [Palmqvist et al., 1999]. Delgenes et al. [1996] reported that the inhibitory effect of lignin derived compounds on the sugar utilization efficiency of *C. shehatae*, *P. stipitis* and *S. cerevisiae* and *Z. mobilis*. Vanillin was found to be the strongest inhibitor of growth and ethanol production in both xylose and hexose fermenting yeasts. Hu et al., [2009] observed the p-hydroxy benzaldehyde and vanillin at 10 mM concentration inhibits the growth of *Rhodospiridium toruloides*.

Apart from the furans and phenolics, there are other compounds present in acid hydrolysates. These include acetic acid and raw material extractives (tannic and terpene acids). However their degree of severity on fermenting microorganisms is low compared to furans and phenolics [McMillan, 1994]. Among acidic inhibitors, the hemicellulose derived organic acid inhibitors, i.e. acetic acid, formic acid levulinic acid, acidic resins, tannic, and terpene acids mostly inhibit the growth and metabolism allowing the higher permeation of cell membrane in microorganisms [Zaldivar and Ingram, 1999; Takahashi et al., 1999; Imai and Ohno, 1995]. The toxicity of acetic acid also depends upon the culture conditions are being employed during the fermentative process. Felipe et al., [1995] reported limits of acetic acid concentrations (1g/l) in the fermentation medium that can improve the xylose-to-xylitol bioconversion, whereas the ethanol production was reported to be stimulated at higher concentration (10 g/l) when medium was free of other toxic compounds [Palmqvist et al., 1999]. Other weak acids at low concentration have been found to exert a stimulating effect on the ethanol production by *S. cerevisiae* [Pampulha & Loureiro-Dias, 1989].

#### 4. Strategies to overcome the fermentation inhibitors

There are several detoxification methods such as physical (evaporation, membrane mediated detoxification), chemical (neutralization, calcium hydroxide overliming, activated

charcoal treatment, ion exchange resins, and extraction with ethyl acetate) and biological detoxification (enzymatic mediated using laccase, lignin peroxidase), in-situ detoxification, in-situ microbial detoxification etc. Table 3 summarizes the hydrolysate detoxification using various non-biological methods employed to the variety of lignocellulosic hydrolysates. Each method represents its specificity to eliminate particular inhibitor from the hydrolysate.

<b>Lignocellulose Hydrolysae</b>	<b>Detoxification methods</b>	<b>Changes in hydrolysate composition</b>	<b>References</b>
Sugarcane bagasse	Neutralization	NA	Chandel et al., 2007a
<i>Saccharum spontaneum</i>	Over-liming	Removal of furfurals (41.75%), total phenolics (33.21%), no effect on acetic acid content. Reduction of reducing sugars (7.61%)	Chandel et al., 2011a
Oak wood	Activated charcoal	Removal of phenolics (95.40%)	Converti et al., 1999
Wheat straw	Ion exchange-D 311 + over-liming	Removal of furfurals (90.36%), phenolics (77.44%) and acetic acid (96.29%)	Zhuang et al., 2009
Wheat straw	Ethyl acetate + Over-liming	Removal of furfurals (59.76%), phenolics (48.23%) and acetic acid (92.19%)	Zhuang et al., 2009
Aspen	Roto-evaporation	Removal of acetic acid (54%), furfural (100%) and vanillin (29%)	Wilson et al., 1989
Spruce wood	Dithionite and sulfite	No major change in composition of hydrolysates	Alriksson et al., 2010
Corn stover	Membrane based organic phases alamine 336	60% acetic acid removal	Grzenia et al., 2008

Table 3. Different detoxification strategies (Non-biological) applied to lignocellulose hydrolysates for the removal of fermentation inhibitors

It is difficult to compare detoxification methodologies based on the selection of lignocellulosic hydrolysates and types of the microorganisms been used in the fermentation media. Also, the lignocellulosic hydrolysates vary in their degree of inhibition, and microorganism reveals different inhibitor tolerances [Mussatto & Roberto, 2004; Palmqvist & Hahn-Hagerdal, 2000a, b].

#### **4.1. Physical methods**

##### **4.1.1 Evaporation**

The evaporation under vacuum can eliminate volatile compounds such as acetic acid, furfural and vanillin from lignocellulosic hydrolysate. However, this method retains the concentration of non-volatile toxic compounds (extractives and lignin derivatives) in the hydrolysates. Palmqvist et al., [1996] observed the removal of most volatile fraction (10% v/v) from willow hemicellulose hydrolysate by roto-evaporation. Wilson et al., [1989] found a decrease in the concentration of acetic acid, furfural and vanillin by 54%, 100% and 29%, respectively, compared with the concentrations in the hydrolysate. Larsson et al., [1999] observed the removal of furfural (90%) and HMF (4%) using vacuum evaporation from wood hemicellulosic hydrolysate. The improved production of xylitol was reported from hemicelluloses hydrolysate after removal of acetic acid, furfural and other volatile compounds [Converti et al., 2000].

Another potential substrate sugarcane bagasse was hydrolyzed and vacuum evaporated followed by activated charcoal treatment, revealed 89% removal of furfural [Rodrigues et al., 2001] with partial elimination of acetic acid. Zhu et al., (2011) applied the complex extraction to detoxify the prehydrolysate corn stover using mixed extractant (30% trialkylamine-50% n-octanol-20% kerosene). The detoxification resulted into removal of 73.3% acetic acid, 45.7% 5-HMF and 100% furfural. The effect of evaporation on the removal of fermentation inhibitors has been summarized in Table 3.

##### **4.1.2 Membrane separations**

Adsorptive micro porous membranes have surface functional groups attached to their internal pores, which may eliminate the cell wall derived inhibitors from the lignocellulose acid hydrolysates. During clarification of inhibitors, the feed is being pumped through the membrane pores that bind to the solute predominantly by convection. This phenomenon can greatly reduce the required processing time. Also, the drop in the pressure for flow through adsorptive membranes changes significantly compared to the typical packed beds. Wickramasinghe & Grzenia [2008] observed better performance of membrane assisted system for acetic acid removal from the biomass hydrolysates than ion-exchange resins. The effect of various diluted organic phases (alamine 336, aliquat 336) for the removal of acetic acid (60%) from corn stover hydrolysates from alamine 336 was further investigated [Grzenia et al., 2008]. Later, Grzenia et al. [2010] used the membrane extraction for removal of inhibitors from sulfuric acid derived hemicellulosic hydrolysate of corn stover. Extraction of sulphuric, acetic, formic and levulinic acid as well as 5-hydroxymethylfurfural and furfural was removed when alamine 336, octanol and oetyl alcohol used in the organic phase. Thus, the adsorptive membranes may offer significant improvements over traditional ion-exchange resins. Effect of membrane separation on the removal of fermentation inhibitors has been summarized in Table 3.

## 4.2 Chemical methods

### 4.2.1 Neutralization

Due to highly acidic nature of hemicellulosic hydrolysates, the neutralisation of is unavoidable step before using the hydrolysate for fermentation. Alkali most preferably calcium hydroxide or sodium hydroxide are used for neutralization of hydrolysates (pH-6.0-7.0). During the process, furfurals and phenolics may be removed by precipitation to the some extent. Table 3 summarizes the neutralization effect on the removal of fermentation inhibitors from lignocellulose hydrolysates.

### 4.2.2 Calcium hydroxide over-liming

Over-liming with a combination of high pH and temperature has for a long time been considered as a promising detoxification method for dilute sulfuric acid-pretreated hydrolysate of lignocellulosic biomass [Chandel et al., 2007a; Martinez et al., 2001]. This process has been demonstrated to help with the removal of volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) from the hydrolysate additionally causing a sugar loss (~10%) by adsorption [Chandel et al., 2011a, b; 2009; Martinez et al., 2000; Ranatunga et al., 2000]. The dried calcium hydroxide is added in acidic hydrolysates converting into gypsum which can be used as plaster of paris having many commercial values. The effect of overliming on the removal of fermentation inhibitors from the variety of lignocellulose hydrolysates has been summarized in Table 3.

### 4.2.3 Activated charcoal treatment

The detoxification of hemicellulose hydrolysates, by activated charcoal is known to be a cost effective with high capacity to absorb compounds without affecting levels of sugar in hydrolysate [Canilha et al., 2008; Chandel et al., 2007a; Mussatto & Roberto, 2001]. The effectiveness of activated charcoal treatment depends on different process variables such as pH, contact time, temperature and the ratio of activated charcoal taken versus the liquid hydrolysate volume [Prakasham et al., 2009]. A summarized description of charcoal treatment on detoxification of lignocellulose hydrolysate has been summarized in Table 3.

### 4.2.4 Ion exchange resins

Treatment with ion exchange resins has been known to remove lignin-derived inhibitors, acetic acid and furfurals respectively, leading to hydrolysate that show a fermentation similar to that of an inhibitor-free model substrate. The ion-exchange resins based separation of fermentative inhibitors may not be cost effective [Lee et al., 1999], however, it provides most effective means of inhibitor separation when the hydrolysate being adjusted to a pH of 10 which requires significant quantities of base chemicals [Wilson and Tekere, 2009; Ranjan et al., 2009]. Further, the anion treatment also helps to remove most inhibitors (i.e. levulinic, acetic, formic acids, and furfural and 5-HMF). Villarreal et al. (2006) investigated the effect of four different ion exchange resins (cation and anion) for the detoxification of *Eucalyptus* hemicellulosic hydrolysates for the improved xylitol production by *Candida guilliermondii*. The ion exchange detoxification drastically enhanced the fermentability of the hydrolysate. Total 32.7 g/l of xylitol was achieved after 48 h fermentation, which correspond to 0.68 g/l/ h volumetric productivity and 0.57 g/g xylitol yield factor [Villarreal et al. 2006]. The ion exchange resins also led to a considerable loss of fermentable sugars from the hydrolysate. Chandel et al., [2007a] observed that ion exchange

resins diminish furans (63.4%) and total phenolics (75.8%) from sugarcane bagasse acid hydrolysates. Although the ion exchange resins is effective, however is not cost effective that reflects its limited feasibility in commercial industrial purpose in lignocellulosics derived products synthesis. Table 3 summarizes the effect of different ion exchange resins treatment on detoxification of lignocellulose hydrolysate.

#### 4.2.5 Extraction with ethyl acetate

The extraction of fermentation inhibitors using ethyl acetate has been found to increase the ethanol yield in fermentation by *P. stipitis* from 0 to 93% of that obtained in the reference fermentation [Wilson et al., 1989]. The extraction procedure could eliminate acetic acid (56%), and total furfural, vanillin, and 4-hydroxybenzoic acid. 84% phenolics was removed from the *Eucalyptus* wood hemicelluloses hydrolysate with the extraction using ethyl acetate or diethyl ether [Cruz et al. 1999]. Ethyl acetate extraction has been shown to increase the rate of glucose consumption in hydrolysate of pine wood [Clark & Mackie, 1984]. The low molecular weight phenolic compounds were suggested to be the most inhibiting compounds in the ethyl acetate extract [Zhuang et al., 2009]. Pasha et al., [2007] detoxified the *Prosopis juliflora* hydrolysate with calcium hydroxide overliming in conjunction with ethyl acetate. In these studies, the ethanol yield of  $0.459 \pm 0.012$  g /g, productivity of  $0.67 \pm 0.015$  g/l/h and fermentation efficiency of 90% after fermentation of this detoxified sugar syrup with fusant *S. cerevisiae* VS<sub>3</sub>. Table 3 shows a summarized effect of extraction using ethyl acetate on detoxification of lignocellulose hydrolysate.

### 4.3 Biotechnological routes for detoxification

Due to concerns of feasibility and affordability of physico-chemical treatments, the biotechnological methods encompass the application of living microorganisms and/or the microbial enzymatic applicability for the detoxification of lignocellulose hydrolysates. These microorganisms and/or the enzymes have potential to alter the chemical nature of inhibitors in hydrolysates. The biological methods of detoxification are more feasible, environmental friendly, with fewer side-reactions and less energy requirements [Parawira and Tekere, 2011.]. The slow reaction time of microbial/ enzymatic detoxification and the loss of fermentable sugars make them unattractive [Yang & Wyman, 2008]. However, it is unavoidable to explore the biotechnological routes of detoxification towards process economization.

#### 4.3.1 Microbial pretreatment of lignocellulosics

The microorganisms are able to degrade lignin during the incubation by leaving cellulose and hemicelluloses behind with loose bonding of both in the raw material. The lignocellulosic substrate after pretreatment with microorganism could easily be hydrolyse into fermentable sugars requiring less acid load, lower temperature, and less hydrolysis time [Kuhar et al., 2008]. The microbial mediated pretreatment resulted into the maximum de-polymerisation of carbohydrate polymers into mixture of simple sugars with less fermentation inhibitors. The pretreated lignocellulose substrate when hydrolysed leads to maximum sugar recovery with minimum inhibitors in short period of time by eliminating the requirement of detoxification step [Liang et al. 2010; Kuhar et al., 2008; Keller et al. 2003].

#### 4.3.2 Microbial acclimatization

Often, microorganisms can adapt to a variety of fermentation media. However, multiple essential nutrients or compounds along with suitable carbon and nitrogen sources are required for any fermentation reaction. The adaptation of microorganisms to lignocellulosic hydrolysates is another parameter to get the improved product yields [Silva and Roberto, 2001; Sene et al., 2001]. Acclimatization of fermentative microorganisms to the hydrolysates containing inhibitors prior to fermentation of lignocellulose hydrolysates provide improved productivities [Parawira & Tekere, 2011]. Microorganisms, *P. stipitis*, *C. shehatae* and *P. tanophilus* are being explored to overcome inhibition and improve fermentation ability of xylose-containing hydrolysates by adaptation [Tomas-Pejo et al., 2010; Zhu et al., 2009; Martin et al., 2007]. Parekh et al. [1987] employed *P. stipitis* on a steam-stripped hardwood hydrolysate that improved ethanol production (46%), with >90% xylose utilization compared with steam-unstripped hardwood hydrolysate. Microorganism *P. stipitis* NRRL Y-7124 adapted on wheat straw hemicellulosic hydrolysates, showed improved ethanol production (yield,  $0.41 \pm 0.01$  g/g) equivalent to  $80.4 \pm 0.55\%$  theoretical conversion efficiency [Nigam, 2001]. The adaptation of *C. guilliermondii* to rice straw hemicellulose hydrolysate for xylitol production was found to be an effective and inexpensive method to alleviate the inhibitory effect of toxic compounds on the xylose- to-xylitol bioconversion [Silva & Roberto, 2001].

#### 4.3.3 In-situ microbial detoxification

Under in-situ microbial detoxification, the microorganisms are being grown in lignocellulose hydrolysate to detoxify the inhibitory substances by transforming their chemical nature [López et al., 2004]. The in-situ detoxification of impurities could be achieved at higher rate that may economize the overall ethanol production in the same vessel. Attempts were made to detoxify the lignocellulose hydrolysates directly with the employing wild (yeasts, fungi, bacteria) and/or recombinant microorganisms expressing the laccase or peroxidases. Palmqvist et al. [1997] used *Trichoderma reesei* to degrade the inhibitors from willow hemicellulosic hydrolysate that directly assisted improvements in ethanol productivity by three fold and yield four fold. Later, Larsson et al. [1999] detoxified the dilute-acid hydrolysate of spruce by *T. reesei* and found to be most efficient compared to anion exchange, over-liming, and treatment with laccase enzyme, however, with higher consumption of fermentable sugars (35%). A fungal isolate, *Coniochaeta ligniaria*, (NRRL30616), was also reported to metabolize furfural, HMF, aromatic and aliphatic acids, and aldehydes present in corn stover hydrolysate [Nichols et al., 2008].

The microbial mediated detoxifications are been considered effective than the soft rot fungi resulted into less sugar consumption with shorter incubation time. In a related study, López et al. [2004] isolated five bacteria related to *Methylobacterium extorquens*, *Pseudomonas sp.*, *Flavobacterium indologenes*, *Acinetobacter sp.*, *Arthrobacter aurescens*, and fungus *C. ligniaria* C8 (NRRL30616), capable of depleting toxic compounds from defined mineral medium containing a mixture of ferulic acid, HMF, and furfural as carbon and energy sources. Organism *C. ligniaria* C8 (NRRL30616) was effective in removing furfural and HMF from corn stover hydrolysate.

Okuda et al. [2008] investigated the biological detoxification of a waste house wood (WHW) hydrolysate by thermophilic bacterium *Ureibacillus thermosphaericus*. Chromatographic analysis confirmed that *U. thermosphaericus* 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. In another example of in-situ detoxification, Tian et al. [2009] isolated yeast strains namely Y1, Y4 and Y7 and evaluated their efficiency for ethanol production after in-situ detoxification of hydrolysates. Strains Y1 and Y4 yielded 0.49 g and 0.45 g ethanol/g glucose, equivalent to maximum theoretical values of 96% and 88.2%, respectively. Further, attempts are underway to detoxify the lignocellulose hydrolysates through direct application of microorganisms in the hydrolysate. Table 4 summarizes the microorganism used for in-situ detoxification of lignocellulose hydrolysates.

Lignocellulose hydrolysate	Enzyme/ Microorganisms	Effect of the method	References
Sugarcane bagasse	Laccase	80% removal of phenolics	Martin et al., 2002
Lignocellulose hydrolysate	Peroxidase from <i>C. cinereus</i> IFO 8371	100% removal of p-coumaric acid, ferulic acid, vanillic acid and vanillin	Cho et al., 2009
Spruce	Residue lignin	53% removal of phenolics and 68% removal of furans	Bjorklund et al., 2002
Willow	<i>T. reesei</i>	Considerable removal of phenolics, furans and weak acids	Palmqvist et al., 1997
Corn stover	<i>Coniochaeta ligniaria</i>	80% Removal of furfural and 5-HMF	López et al., 2004
Sugarcane bagasse	<i>Issatchenkia occidentalis</i> CCTCC M 206097	Reduction of syringaldehyde (66.67%), ferulic acid (73.33%), furfural (62%), and 5-HMF (85%)	Fonesca et al., 2011
Spruce	Continuous fermentation	Elimination of detoxification step; improved ethanol yield, 0.42-0.46 g/g	Purwadi et al., 2007
Willow	High cell density fermentation (10 g/l dry weight)	High ethanol productivity even in un-detoxified hydrolysate	Palmqvist et al., 1996

Table 4. Different Biotechnological strategies applied to lignocellulose hydrolysates for the removal of fermentation inhibitors

#### 4.3.4 Alterations in fermentation conditions

The presence of lignocellulose inhibitors in fermenting medium affects the ethanol and biomass productivities as microorganism take more incubation times to convert into products [Chandel et al., 2007a; Nilvebrant et al., 2001; Zaldivar et al., 2001]. Usually the ethanol productivity is determined by cell-specific productivity and cell mass concentration,

cumbersome by lignocellulose-derived inhibitors. To overcome by inhibitors, high cell-mass inocula are effective to tolerate the stress of inhibitory substances [Purwadi et al., 2007]. The ethanol productivity has been increased by maintaining the initial cell-mass at higher density [Brandberg et al., 2007]. By altering the initial cell density, the increased production of ethanol (0.44 g/g) was reported at initial cell density (10 g/l dry weight) [Palmqvist et al., 1996]. The ethanol productivity in fed-batch fermentation was limited by the feed rate that in turn, was limited by the cell-mass concentration (Taherzadeh et al. 1999).

In continuous fermentation, the ethanol productivity also depends upon the rate of dilution. Since the microbial growth rate is known to decrease by the inhibitors, the productivity in continuous fermentation of lignocellulosic hydrolysates remains low [Lee et al., 1996; Palmqvist et al., 1998]. Purwadi et al. [2007] has achieved the ethanol yield of 0.42-0.46 g/g sugar utilized from the crude hydrolysates of spruce wood as carbon source under continuous fermentations using the flocculating *S. cerevisiae* CCUG 53310. Cellular recirculation strategy was employed in the fermentation of an enzymatic hydrolysate of spruce [Palmqvist et al., 1998], and in fermentation of bagasse hydrolysate [Ghose and Tyagi, 1979].

#### 4.3.5 Enzymatic clarification

Enzymatic detoxification is the most preferred biotechnological route of inhibitors' clarification. Laccase and peroxidases enzymes derived from white rot fungi (*Trametes versicolor*, *Phenorchete chrysosporium*, *Cythus bulleri*, *C. stercoreous*, and *Pycnoporous cinnabarinus*) have been found effective for the removal of phenolics from the lignocellulose hydrolysates. Jonsson et al. [1998] explored laccase and peroxidase enzymes of the white-rot fungus *T. versicolor* to detoxify the willow hemicellulosic hydrolysate. The detoxification mechanism of these enzymes probably involves oxidative polymerization of low-molecular-weight phenolic compounds in turn increasing the ethanol productivity. 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. About 80% of the phenolic compounds were specifically removed by the laccase treatment.

The effect of laccase enzyme derived from *C. bulleri* for the detoxification of sugarcane bagasse hemicellulosic hydrolysate showed 77% phenolics were eliminated from the acid hydrolysate [Chandel et al. 2007a]. The ethanol production efficiency was superior in the laccase treated hydrolysate (6.50 g/l, 0.37 g/g) compared to the untreated (3.46 g/l, 0.22 g/g) [Chandel et al. 2007a]. Cho et al., [2009] studied the effect of peroxidase enzyme derived from *Coprinus cinereus* IFO 8371 on the detoxification of phenolics enriched fermentation medium for the butanol production from *Clostridium beijerinckii* NCIMB 8052 with 100% removal of phenolic compounds (e.g. p-coumaric acid, ferulic acid, vanillic acid, and vanillin) using 0.01  $\mu$ M of peroxidase enzyme. This peroxidase treated fermentation solution along with acidification and precipitation (A/P) revealed amelioration in butanol production from 0.6 $\pm$ 0.15 to 8.9 $\pm$ 0.43 g/l. Enzymatic detoxification could be cost effective, if immobilized laccases and peroxidases on robust matrices (Diaion-HP-87H, Sepa beads, Eupergit-C etc) are employed for detoxification of lignocellulose hydrolysates. The immobilized enzymes could be promising solution towards the development of a reliable process for detoxification at industrial scale in the biorefineries.

## 5. Technological integration of detoxification process

A single method may not be sufficient to remove variety of inhibitors from lignocellulosic hydrolysate. Based on the multitask process, a common detoxification strategy could provide a better performance and effective ethanol yield at industrial scale. The integration of multi-step processes of detoxification in one experiment will provide a better solution to overcome the inhibitors. However, increased cost is the inevitable if integrated steps are followed. A comparative account of the different detoxification steps (i.e. over liming, activated charcoal, ion-exchange and laccase treatment) in conjunction with neutralization for the detoxification of sugarcane bagasse hemicellulosic hydrolysate was explored and summarized in Table 5 that shows the effect of detoxification treatments on the ethanol production by *Candida shehatae* NCIM3501 [Chandel et al. 2007a]. Fermentation of these hydrolysates with *Candida shehatae* NCIM 3501 showed maximum ethanol yield (0.48 g/g) from ion exchange treated hydrolysate, followed by activated charcoal (0.42 g/g), laccase (0.37 g/g), over-liming (0.30 g/g), and neutralized hydrolysate (0.22 g/g).

Treatment	Sugar fermented (%)	Ethanol (gp/l)	Biomass (gx/l)	Ethanol yield (gp/g)	Volumetric ethanol productivity (gp/l)/h	Specific ethanol production (gp/gx)
Neutralization	78.8	3.46	9.8	0.22	0.144	0.353
Overliming	85.9	5.19	12.3	0.302	0.216	0.421
Laccase	86.89	6.50	14.8	0.374	0.270	0.439
Activated charcoal	87.41	7.43	15.5	0.425	0.309	0.476
Ion exchange	89.93	8.67	16.0	0.482	0.361	0.535

Table 5. Fermentation profile of sugarcane bagasse hydrolysate detoxified with different methods [Source: Chandel et al., 2007a] (The values are mean of three replicates. Standard deviation was within 10%. Initial total sugar concentration-20.0 g/l. Ethanol productivities were calculated after 24 h of fermentation. Biomass production rate was continuously increased till the completion of the fermentation batch.)

Over-liming in combination with sulphite addition was also tried by Olsson & colleagues [1995] that showed four times higher fermentation rate with recombinant *E. coli* than detoxification by overliming only, whereas the ethanol production from untreated willow hemicellulosic hydrolysate was uncomparable. The simultaneous detoxification and enzyme production has been reported to occur when the inhibitor-containing hemicellulose hydrolysate from the pretreatment stage was used as substrate for *T. reesei* [Palmqvist et al., 1997].

A similar concept, simultaneous detoxification and fermentation (SDF) was designed to improve production of ethanol from lignocellulose hydrolysates by appropriate ethanologen in conjunction with detoxification of sugar stream using detoxifying biocatalyst simultaneously together. This approach could have potential impact in bio-refineries, merging both individual steps detoxification and fermentation in one vessel. The enzyme-

containing inhibitor-free liquid can then be used to hydrolyse the cellulose fraction. This detoxification method could further improve the process economy since all the wood-derived sugars were utilized. Gyalai-Korpos et al. [2010] detoxified the steam pretreated rice straw hydrolysate through dual detoxification strategy (vacuum evaporation and calcium hydroxide overliming) for the cellulase production by *Trichoderma reesei* RUT C30. These studies revealed maximum activity of filter paperase ( $1.87 \pm 0.05$  FPU/ml) and Beta-glucosidase ( $1.74 \pm 0.03$  IU/ml) after 11 days of incubation.

## 6. Detoxification and future perspectives

Beyond lignocellulosic hydrolysis, constituents releases different monomeric sugars and widen the ethanol fermentability in bio-refinery. However, a wide range of compounds that are inhibitory to microorganisms are formed or released during the fermentation reaction. Based on the origin, inhibitors are usually divided in to three major groups' i.e. weak acids, furan derivatives, and phenolic compounds. It is unavoidable to economize overall process, as the inhibitors directly affect cellular growth and kinetics of biocatalysts used in the fermentation reaction. These compounds have a significant role individually and more toxic if they are used synergistically.

A number of methods could effectively be optimized at industrial scale including physical (evaporation, membrane based filtrations), physico-chemical (Rota evaporation with organic solvents), chemical (Calcium hydroxide over-liming, application of other alkalis such as sodium hydroxide, sodium di thionite, sodium di sulphite, adsorption on activated charcoal, ion-exchange) and biological (changes in fermentation strategies, laccase, peroxidases, using of microorganisms such as *T. reesei*, *C. ligniaria*, *I. occidentalis* in the hydrolysates) for detoxification of lignocellulose hydrolysates. Apart from conventional methods, recent genetic engineering approaches and directed evolution methods to make the competitive strains combating the inhibitors are also in vague. Larsson et al. [2001] have successfully attempted for the heterologous expression of laccase in *S. cerevisiae*. All these methods have been quite successful in terms of removal of inhibitors and simultaneously improving the product titers from the fermentation reaction.

## 7. Conclusion

Presence of inhibitors in lignocellulosic hydrolysate is an industrial malaise. Efficient detoxification can be prescribed as medicine to eliminate fermentation inhibitors present in lignocellulosic hydrolysate. Among all the detoxification methods, the biological strategies to eliminate inhibitors are most promising. A search for novel microbial strains for detoxification of lignocellulose hydrolysates without affecting the sugar and other nutrient fraction from the fermented media is yet to come. *T. reesei* has been found affective; however, has limitations with inefficient sugar utilization proven to be an expensive affair for industrial bioprocess. An optimal design of the fermentation process, rate of bioconversion, and the adaptive response of the microorganism to the toxic compounds in the hydrolysate could be established. Technologies are required to utilize cheaper and highly affective chemicals, which have more affinity towards inhibitors without affecting the original sugar content in lignocellulosic hydrolysates.

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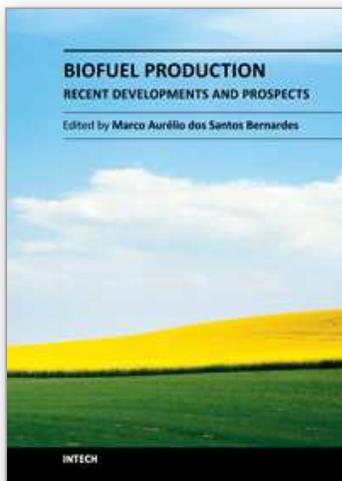
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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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