



## Review

# Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects



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## HIGHLIGHTS

- By-products of lignocellulose pretreatment inhibit microbial and enzymic biocatalysts.
- Groups of inhibitors from components of lignocellulose are reviewed.
- The review covers different strategies to alleviate inhibition problems.
- Industrial implementation increases the relevance of inhibitor management.

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## ABSTRACT

Biochemical conversion of lignocellulosic feedstocks to advanced biofuels and other commodities through a sugar-platform process involves a pretreatment step enhancing the susceptibility of the cellulose to enzymatic hydrolysis. A side effect of pretreatment is formation of lignocellulose-derived by-products that inhibit microbial and enzymatic biocatalysts. This review provides an overview of the formation of inhibitory by-products from lignocellulosic feedstocks as a consequence of using different pretreatment methods and feedstocks as well as an overview of different strategies used to alleviate problems with inhibitors. As technologies for biorefining of lignocellulose become mature and are transferred from laboratory environments to industrial contexts, the importance of management of inhibition problems is envisaged to increase as issues that become increasingly relevant will include the possibility to use recalcitrant feedstocks, obtaining high product yields and high productivity, minimizing the charges of enzymes and microorganisms, and using high solids loadings to obtain high product titers.

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## 1. Introduction

Dwindling fossil resources and environment pollution related to the exploitation of petroleum and coal make it necessary to consider a gradual transition towards a bio-based economy. While the future supply of energy is likely to be based on a wide range of alternative platforms, such as wind, water, solar fuels, and biomass, among others, the production of chemicals will increasingly depend on plant biomass (FitzPatrick et al., 2010). Lignocellulosic biomass from agriculture and forestry, which includes agro-industrial residues, forest-industrial residues, energy crops, municipal solid waste, and other materials, is the most abundant bioresource to consider as feedstock for biorefineries that complement oil refineries as sources of fuels and platform chemicals.

Utilization of lignocellulosic materials for biochemical conversion in biorefineries requires pretreatment for disrupting the close inter-component association between main constituents of the plant cell wall (Yang and Wyman, 2008). Pretreatment clears away the physical and chemical barriers that make native biomass recalcitrant and makes cellulose amenable to enzymatic hydrolysis, which is a key step in biochemical processing of lignocellulose based on the sugar platform concept. This effect is achieved by increasing the accessible cellulose surface area through solubilization of hemicelluloses and/or lignin, which are coating the cellulose of the native biomass.

While the aims of pretreatment are to uncover the cellulose for enzymatic saccharification and fractionating the main components of the feedstock, pretreatment often involves side reactions resulting in lignocellulose-derived by-products that are inhibitory to downstream biochemical processes. Inhibition problems become more significant as the by-products accumulate as a result of recirculation of process water, or as their concentration increases when

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high solids loadings are used to achieve concentrated sugar streams and high product titers.

There are several older reviews on inhibitors, for example Palmqvist and Hahn-Hägerdal (2000) and Klinke et al. (2002), as well as more recent ones, such as Pienkos and Zhang (2009), Jönsson et al. (2013), and Ko et al. (2015). The aims of this review are to provide, in brief, an updated overview of the origin and characteristics of different groups of inhibitory substances, and, with focus on the more recent literature, examine different remedies that can be used to alleviate inhibition problems. We provide a new scheme of groups of inhibitory substances, and pay attention to inhibition of both microbial cells and cellulolytic enzymes.

## 2. Pretreatment

Most lignocellulose-derived inhibitors form during pretreatment when hemicelluloses and/or lignin are solubilized and degraded (Fig. 1). Extractives and cellulose that is unintentionally affected by the pretreatment are other sources (Fig. 1). Since the formation of inhibitory substances is much dependent on the pretreatment process, this review includes a brief discussion on the most commonly used pretreatment techniques, as summarized in Table 1. Only pretreatment methods that are relevant with respect to formation of inhibitors and that are of interest for industrial implementation are covered.

### 2.1. Acid-based methods

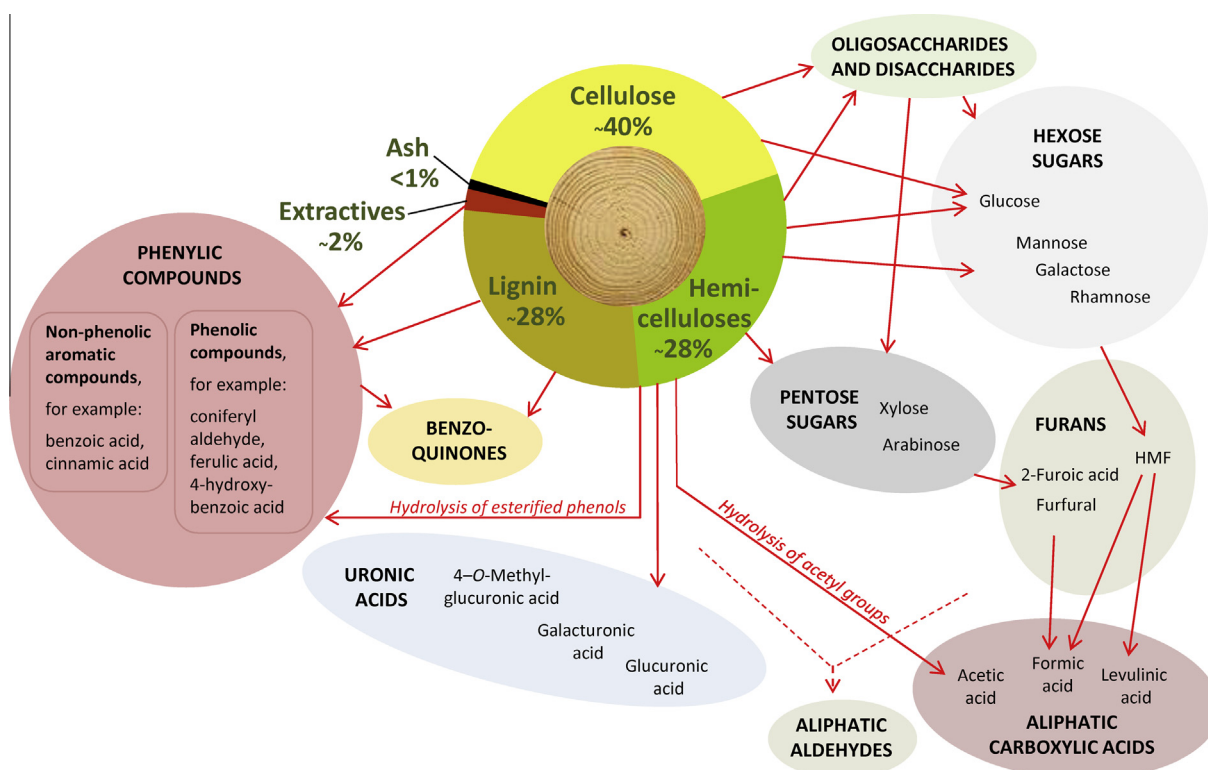
Acid hydrolysis is one of the most promising pretreatment methods with respect to industrial implementation. It is usually performed with mineral acids, but organic acids and sulfur dioxide are other options. Dilute sulfuric acid pretreatment has been studied for a wide range of lignocellulosic biomass (Yang and Wyman, 2008; Hu and Ragauskas, 2012). It results in high recovery of the hemicellulosic sugars in the pretreatment liquid, and in a solid

**Table 1**

Overview of pretreatment methods for lignocellulosic feedstocks prior to enzymatic hydrolysis of cellulose.

Pretreatment methods	Main effect	Used chemicals	By-product formation
Acid-based methods	Hydrolysis of hemicelluloses to monosaccharides	Involve catalysts such as H <sub>2</sub> SO <sub>4</sub> , SO <sub>2</sub> , HCl, H <sub>3</sub> PO <sub>4</sub>	Aliphatic carboxylic acids, phenolic compounds, furans, etc. (see Fig. 1)
Hydrothermal processing	Solubilization of hemicelluloses without complete hydrolysis	No additives	Acetic acid, minor amounts of furan aldehydes
Mild alkaline methods	Removal of lignin and a minor part of hemicelluloses	Involve alkali such as NaOH, Ca(OH) <sub>2</sub> , NH <sub>3</sub>	Acetic acid, hydroxy acids, dicarboxylic acids, phenolic compounds
Oxidative methods	Removal of lignin and part of hemicelluloses	Involve oxidants such as H <sub>2</sub> O <sub>2</sub> and O <sub>2</sub> (alkaline conditions), and O <sub>3</sub>	Aldonic and aldric acids, furoic acid, phenolic acids, acetic acid
Chemical pulping processes	Methods that target lignin and to some extent hemicelluloses	Kraft pulping, sulfite pulping, soda pulping, organosolv pulping	Aliphatic acids
Alternative solvents	Dissolution of specific lignocellulosic components or the whole biomass	Ionic liquids	Dependent on solvent and conditions

cellulose fraction with enhanced enzymatic convertibility. Acid pretreatment has also some drawbacks, such as high cost of the materials used for construction of the reactors, gypsum formation



**Fig. 1.** Degradation products from lignocellulose as a result of pretreatment under acidic conditions. Numbers indicate fractions of constituents of wood of Norway spruce. Red arrows indicate tentative formation pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during neutralization after treatment with sulfuric acid, and formation of inhibitory by-products.

Steam explosion is a successful pretreatment option that involves heating lignocellulose with superheated steam followed by a sudden decompression. The high-pressure steam modifies the cell wall structure, yielding a slurry, which upon filtration renders a filtrate with hemicellulosic sugars and a cellulose-rich filter cake containing also lignin and residual hemicellulose. Steam explosion can be assisted by impregnation with an acid catalyst, for instance sulfuric acid or sulfur dioxide. If no impregnating agent is used, the process is catalyzed through autohydrolysis. Acetic acid and uronic acids released from hemicellulose, and formic and levulinic acids resulting from sugar degradation (Fig. 1) contribute to acidification, and can inhibit downstream biochemical processes.

## 2.2. Hydrothermal processing

Hydrothermal processing is an approach in which water in liquid phase or in vapor phase is used to pretreat lignocellulosic biomass (Hu and Ragauskas, 2012). It is a relatively mild pretreatment method that does not require any catalysts and does not cause significant corrosion problems. Under high pressure water penetrates into the biomass, hydrates cellulose, and removes most of the hemicelluloses and a minor part of lignin. The solubilization of hemicelluloses is catalyzed by hydronium ions resulting from water auto-ionization. Controlling the pH around neutral values minimizes the formation of fermentation inhibitors.

## 2.3. Mild alkaline methods

Alkaline treatment can be used for removing lignin and thereby increasing the digestibility of cellulose. Compared to acid and hydrothermal processes, mild alkaline pretreatments lead to less solubilization of hemicelluloses and less formation of inhibitory compounds, and they can be operated at lower temperatures. Sodium hydroxide and potassium hydroxide are the most commonly used forms of alkali, but their cost is a serious limitation. Other suitable forms of alkali are calcium hydroxide and ammonia, which can be used in processes such as lime pretreatment, ammonia recycled percolation (ARP) and ammonia fiber expansion (AFEX) (Yang and Wyman, 2008).

## 2.4. Oxidative methods

The use of oxidants for pretreating lignocellulosic biomass allows the reduction of cellulose crystallinity and disruption of association between carbohydrates and lignin. These methods include alkaline peroxide pretreatment, ozonolysis, and wet oxidation. Wet oxidation is achieved by treating biomass with water and air or oxygen at high temperatures for relatively short times. Hemicelluloses are extensively solubilized, and recovered mostly as oligosaccharides. Lignin is fragmentized and oxidized to aliphatic carboxylic acids and phenolic compounds (Klinke et al., 2002; Martín et al., 2007). The combination of wet oxidation with alkaline compounds minimizes the formation of furan and phenolic aldehydes.

## 2.5. Chemical pulping processes

Although pulping processes are primarily used for manufacturing of paper and cellulose derivatives (Sjöström, 1993), the integration of ethanol production to pulp mills has been demonstrated at commercial scale (Rødsrud et al., 2012). Chemical pulping can be applied to both softwoods and hardwoods, and the major technologies used are Kraft and sulfite pulping. In the Kraft

process, which is based on NaOH and Na<sub>2</sub>S, lignin and parts of the hemicelluloses are degraded into black liquor, which is typically used for energy purposes. In sulfite pulping, which is based on an aqueous mixture of bisulfite (HSO<sub>3</sub><sup>-</sup>) and sulfite (SO<sub>3</sub><sup>2-</sup>), the hemicelluloses are hydrolyzed and removed to the spent sulfite liquor (SSL), while the cellulose is maintained almost intact. SSLs from softwood are rich in hexoses, and can be fermented to ethanol by *Saccharomyces cerevisiae*, whereas those from hardwoods are more difficult to ferment because of their high pentose content (Pereira et al., 2013). A recently developed modification of sulfite pulping, known as the BALI™-process, can produce easily-convertible cellulose from softwood, hardwood and agricultural residues, and it is characterized by low generation of fermentation inhibitors (Rødsrud et al., 2012). Another sulfite-based process is SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose), which includes sulfite treatment followed by a mechanical size reduction. In the SPORL pretreatment the hemicelluloses are hydrolyzed to sugars with limited formation of fermentation inhibitors (Zhu et al., 2009).

Soda pulping is typically used for pulping of non-wood plants with higher contents of inorganic material than wood. A difference compared to Kraft and sulfite pulping is that it does not require sulfur-containing chemicals.

In organosolv pretreatment, which was initially investigated as an alternative to conventional chemical pulping processes, organic solvents are used for solubilizing lignin (Pan et al., 2008). The pretreatment is typically performed at around 200 °C, but if acid catalysts are used the process can be run at lower temperatures. The solvents must be removed from the system to avoid inhibition of enzymatic hydrolysis and fermentation, and should be recycled to reduce operational costs. The most economic option is to use low-molecular weight alcohols, but the risk of operations with volatile and flammable solvents has motivated the interest for nonvolatile organic compounds (Martín et al., 2013).

## 2.6. Ionic liquid/alternative solvent pretreatment

The use of ionic liquids (ILs), is another alternative for pretreatment of lignocellulosic materials (Karatzos et al., 2012). ILs disrupt the non-covalent interactions between lignocellulose components without leading to significant degradation. Cellulose regenerated from IL solutions has increased enzymatic convertibility. The development of energy-efficient recycling methods, and the implementation of effective strategies for recovery of hemicelluloses and lignin from pretreatment liquids is required for the industrial application of ILs. Even though the formation of inhibitors is limited, the minor amounts of ILs remaining in the pretreated materials are potentially toxic to enzymes and fermentative microorganisms (Yang and Wyman, 2008).

## 3. Feedstocks composition and by-product formation

In general it can be assumed that lignocellulosic feedstocks contain about 40% of the carbon bound as cellulose, 30% as lignin and 26% as hemicelluloses and other polysaccharides. While cellulose is a uniform component of most types of cellulosic biomass, the proportions and composition of hemicelluloses and lignin differ between species (Fengel and Wegener, 1989; Sjöström, 1993). The chemical differences between feedstocks have a major impact on the formation of inhibitors during pretreatment.

### 3.1. Glucans

Cellulose, the structural base of the plant cells, is a linear homopolysaccharide composed of anhydroglucose units linked by

$\beta$ -1,4-glycosidic bonds. In native cellulose, the degree of polymerization can be as high as 15,000, and the individual molecules form microfibrils stabilized by hydrogen bonds, thus making the macromolecule highly crystalline and difficult to hydrolyze. Amorphous regions comprise a minor part of native cellulose and alternate with crystalline regions (Fengel and Wegener, 1989).

Another glucan of high interest if a whole-crop approach will be used is starch, which is the main reserve polysaccharide in plants. In starch the anhydroglucose units are linked by  $\alpha$ -1,4-glycosidic bond, which are easy to split and therefore the hydrolysis can be performed under relatively mild conditions.

### 3.2. Hemicelluloses

Differently to cellulose, hemicelluloses are heteropolysaccharides, they are often branched, have low degree of polymerization, and are easy to hydrolyze. The main softwood hemicelluloses are *O*-acetyl-galactoglucomannans and arabino-4-*O*-methylglucurono-*D*-xylans, while in hardwoods *O*-acetyl-4-*O*-methylglucurono-*D*-xylans are the most relevant ones (Fengel and Wegener, 1989; Sjöström, 1993). In annual plants the most important kind of hemicelluloses are arabino-(*O*-acetyl-4-*O*-methylglucurono)-*D*-xylans, which also have *p*-coumaric and ferulic acids attached to the arabinose moieties (Xiao et al., 2001) (Fig. 1). Hydrolysis of the backbone of hemicelluloses leads to the formation of pentoses (predominant in hardwoods and annual plants), hexoses (mainly in softwoods), and uronic acids (Fig. 1). Acetic acid, resulting from the hydrolysis of acetyl groups, is another important component of the hydrolysates of hardwoods and annual plants (Fig. 1). Additionally, the hemicellulosic hydrolysates formed during pretreatment of annual plants generally contain phenolic acids.

### 3.3. Lignins and esterified phenols

Lignin is a complex aromatic polymer composed of phenylpropanoid units, and representing 25–39% of the dry weight of softwoods, and 17–32% of hardwoods (Fengel and Wegener, 1989). Softwood lignin is comprised predominantly by guaiacyl units. Hardwood lignin contains mainly syringyl units, but also important amounts of guaiacyl units. Apart from guaiacyl and syringyl units, lignins of annual plants contain also *p*-hydroxyphenyl units. Phenylpropanoid units are linked through a complex network of ether and carbon–carbon bonds. Lignin binds the cell wall components together, giving lignocellulosic biomass its structural integrity.

Other phenolic compounds that are relevant for lignocellulose processing are *p*-coumaric, ferulic and diferulic acids, which are typical in grasses. They are not lignin components, but contribute to crosslinking with hemicelluloses. They are esterified to arabinoxylans and ether- or ester-linked to lignin (Bidlack et al., 1992).

### 3.4. Extractives

Wood extractives are a heterogeneous group of compounds that can be extracted with polar or non-polar solvents. They consist mainly of terpenes, fats, waxes, and phenolics, and their content and composition vary among species, location and season. They are present in small quantities, but are crucial for some biomass properties, such as color, odor and protection from parasites (Fengel and Wegener, 1989).

### 3.5. Inorganic components

The content of inorganic matter of wood is determined as the ash remaining after incineration of a sample. Ash comprises around 0.1–1% of wood of temperate zones and up to 5% of tropical

species (Fengel and Wegener, 1989). Some agricultural residues can have an ash content above 15% (López et al., 2010). The ash content of agricultural residues can partially be affected by contamination from soil.

### 3.6. By-product formation

During biomass pretreatment, in order to achieve a good enzymatic digestibility of cellulose, the operational conditions are tuned to remove hemicelluloses and/or lignin from the lignocellulosic matrix. However, while aiming at optimization of a desirable goal other factors are also affected. For example, achieving high degrees of solubilization of hemicelluloses and/or lignin unavoidably leads to degradation of the solubilized fragments as a result of the severe conditions they are exposed to. The amount and nature of the formed degradation products, many of which are inhibitory to downstream biocatalytic processes, is directly related to the pretreatment method and conditions.

#### 3.6.1. Acidic conditions

Under the acidic conditions typical for processes, such as acid hydrolysis, acid pretreatment and sulfite pulping, the pentoses and uronic acids resulting from hydrolysis of the hemicelluloses undergo dehydration with formation of 2-furaldehyde, hereafter referred to as furfural, while the hexoses are dehydrated to 5-hydroxymethyl-2-furaldehyde, hereafter referred to as HMF (Fig. 1). Under severe pretreatment conditions, such as long reaction time and high temperature and acid concentration, HMF is further degraded to levulinic and formic acids (Fengel and Wegener, 1989). As HMF, furfural is also unstable in the dehydrative medium, and can be subjected to further degradation to formic acid and to condensation reactions with formation of resins (Danon et al., 2013). Acetic acid, which is not a sugar degradation product but a result of the hydrolysis of the acetyl groups of hemicelluloses, is another acid found in the liquors from acidic treatments of biomass (Fig. 1).

The splitting of  $\beta$ -O-4 ether and other acid-labile linkages in lignin macromolecules during acidic treatments results in the formation of a high number of phenolic compounds, which differ depending on the sort of biomass and treatment conditions (Jönsson et al., 1998; Larsson et al., 1999a; Martín et al., 2002, 2007; Du et al., 2010; Mitchell et al., 2014). Some of the most common phenols formed during acid pretreatment of wood are 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, syringic acid, and Hibbert's ketones (Larsson et al., 1999a; Mitchell et al., 2014). Phenolic acids, such as *p*-coumaric acid and ferulic acid, are common products in pretreatment of annual plants (Martín et al., 2007).

As a fraction of the extractives are phenolic compounds, some phenols in lignocellulosic hydrolysates are likely to originate from extractives. Although many fatty extractives would precipitate and be removed with the filter cake, some soluble phenols would remain in the hydrolysates. Pyrogallol and gallic acid resulting from hydrolysable tannins are present in hardwood SSLs (Pereira et al., 2013), and gallic acid has also been found in pretreated lignocellulose (Du et al., 2010; Kim et al., 2013; Mitchell et al., 2014).

Apart from phenolic compounds, non-phenolic aromatic compounds such as benzoic acid (Martín et al., 2002; Du et al., 2010; Mitchell et al., 2014), benzyl alcohol (Mitchell et al., 2014), cinnamic acid (Persson et al., 2002; Kim et al., 2013), cinnamaldehyde (Persson et al., 2002), 3,4-dimethoxy-cinnamic acid (Mitchell et al., 2014), and *para*- and *ortho*-toluic acid (Du et al., 2010) have been found in lignocellulosic hydrolysates. Together with the phenolic compounds, these non-phenolic aromatics are the phenolic constituents of the lignocellulosic hydrolysates (Fig. 1).



As both hydroquinone and catechol are found in hydrolysates (Larsson et al., 1999a; Mitchell et al., 2014), it is a likely assumption that hydrolysates also contain *para*- and *ortho*-benzoquinones (Fig. 1). Formation of benzoquinones from phenolic compounds is likely to occur during pretreatment (Fig. 1). Toxic concentrations of *p*-benzoquinones in pretreated biomass were recently discovered (Stagge et al., 2015).

Apart from furan aldehydes and phenylic aldehydes, it is likely that small aliphatic aldehydes form during pretreatment (Fig. 1). As these are volatile and could potentially evaporate, more research is needed to understand the significance of aliphatic aldehydes. Recent findings indicate that small aliphatic aldehydes are ubiquitous in biomass after pretreatment under acidic conditions (Cavka et al., submitted for publication).

Metal ions can also be formed during acidic processing of biomass. Acidic conditions can cause corrosion of pretreatment equipment, resulting in the liberation of heavy metal ions, such as copper, nickel, chromium and iron, which can be inhibitory to fermenting microorganisms (Watson et al., 1984). Other cations, such as sodium, calcium and magnesium, can come from pretreatment chemicals or from adjustment of the pH.

Although in lower amounts than in acid pretreatments most of the above-mentioned products can also be formed during hydrothermal pretreatments, which generally start at a pH that is close to neutrality but get acidified as the reaction proceeds and acetic acid and uronic acids are released (Garrote et al., 2008).

### 3.6.2. Alkaline conditions

Under alkaline conditions the carbohydrates are better preserved than at low pH values, but some degradation also occurs leading to the formation of carboxylic acids. The peeling reactions occurring during alkaline treatments lead to endwise degradation of polysaccharides with formation of saccharinic acids, and also some amounts of lactic acid, formic acid and different dihydroxy and dicarboxylic acids (Fengel and Wegener, 1989). Acetic acid, formed by saponification of the acetyl groups, is another typical product of alkaline treatments. Phenolic compounds are also formed (Lawther and Sun, 1996), and in processes, such as alkaline wet oxidation they are further oxidized to carboxylic acids (Klinke et al., 2002).

### 3.6.3. Oxidative conditions

The occurrence of oxidation reactions during pulping results in the formation of gluconic and glucaric acids (Fengel and Wegener, 1989). In sulfite pulping the demethylation of 4-*O*-methylglucuronic acid results in glucuronic acid formation, which can then further result in decarboxylation and oxidation leading to formation of xylonic acid.

Under alkaline wet oxidation the phenolic compounds and furan aldehydes resulting from lignin and carbohydrate degradation can be subjected to further reactions. Phenolics are oxidized to different carboxylic acids, and furfural to furoic acid. Furthermore, the oxidative cleavage of the side chain of phenylpropane derivatives leads to phenolic acids, such as 4-hydroxyphenolic, vanillic and syringic acids (Klinke et al., 2002; Martín et al., 2007).

## 4. Inhibitory effects

### 4.1. Inhibition of microorganisms

By-products of pretreatment of lignocellulose under acidic conditions can be divided into groups on basis of chemical functionality, origin, and effects on the fermenting microorganism (Fig. 1). Carbohydrate degradation products such as the common aliphatic carboxylic acids acetic acid, formic acid, and levulinic acid, and the

furan aldehydes furfural and HMF exhibit relatively low toxicity, but can be present in high concentrations depending on the pretreatment conditions and the feedstock.

Larsson et al. (1999b) studied the concentrations and effects of acetic acid, formic acid, and levulinic acid in acid hydrolysates of Norway spruce and in fermentations with *S. cerevisiae*. They found that concentrations of around 100 mM were required to observe inhibitory effects. Due to the low acetyl content, softwood hydrolysates have relatively low concentrations of acetic acid. Formation of formic acid and levulinic acid occurs at the expense of sugars (Fig. 1) and it is therefore desirable to use pretreatment conditions in which the formation of these acids is minimized. For these reasons the concentrations of aliphatic carboxylic acids in pretreated softwood may be low enough to stimulate rather than inhibit ethanol formation. This is the effect of increased demand for ATP and/or less efficient production of ATP due to uncoupling of the respiratory chain and the oxidative phosphorylation of ADP, which leads to increased ATP-generating glycolytic activity at the expense of biomass formation. The use of hardwood and agricultural residues with high acetyl content as feedstocks as well as the development of high-solid processes (Kristensen et al., 2009) contribute to making inhibition by aliphatic carboxylic acids more important.

Aromatic carboxylic acids are found within the group of phenylic compounds (Fig. 1), which include both phenolic aromatic carboxylic acids, such as for example ferulic acid and 4-hydroxybenzoic acid, and non-phenolic aromatic carboxylic acids, such as cinnamic acid (Fig. 1). There are good reasons to group aromatic carboxylic acids with other phenylic compounds rather than with the aliphatic carboxylic acids. As suggested by the phenylpropanoid structure of some of the aromatic acids, as well as of the presence of S (syringyl), G (guaiacyl) and H (4-hydroxyphenyl) moieties, these compounds originate from lignin or from hydrolysis of esterified phenols (Fig. 1). Furthermore, in contrast with the carbohydrate-derived aliphatic carboxylic acids mentioned in Fig. 1, each of the aromatic carboxylic acids are present in relatively low concentrations in lignocellulosic hydrolysates, and their inhibitory effect is typically stronger than that of the aliphatic carboxylic acids. For example, Larsson et al. (2000) found that ferulic acid was inhibitory to *S. cerevisiae* at 0.20 g/L (1.0 mM). As judged by these experiments, the inhibitory effect of ferulic acid would tend to occur at concentrations that are two order of magnitudes lower than those of the common aliphatic carboxylic acids acetic acid, formic acid, and levulinic acid. While pretreated corn stover contained up to 6.6 mg/L (0.033 mM) ferulic acid (Du et al., 2010), up to 210 mg/L (1.1 mM) was found in sugarcane bagasse hydrolysates (Martín et al., 2002). Thus, although the concentrations are much lower than those of the common aliphatic carboxylic acids, the much stronger inhibitory effects make it possible that aromatic carboxylic acids contribute to inhibitory effects.

As with regard to formic acid and levulinic acid, formation of furan aldehydes means decreased sugar yields (Fig. 1). It is therefore desirable to minimize their formation during pretreatment. Analyses of pretreated corn stover, poplar, and pine showed HMF concentrations up to 0.17 g/L (1.3 mM) and furfural concentrations up to 0.22 g/L (2.3 mM) (Du et al., 2010). Larsson et al. (1999b) found up to around 50 mM HMF and up to around 40 mM furfural in acid hydrolysates of Norway spruce, and used these concentrations in studies of inhibition. Banerjee et al. (1981) studied inhibition of *S. cerevisiae* by furfural in the range 0.5–4 g/L (5–40 mM). While furan aldehydes may in some cases be present in relatively high concentrations (such as tens of mM, several grams per liter), the inhibitory effects are lower than for aromatic aldehydes such as for instance coniferyl aldehyde (Fig. 1). Larsson et al. (1999a) found 35 mg/L (0.2 mM) coniferyl aldehyde in a hydrolysate of Norway spruce. Inhibitory effects on *S. cerevisiae* were found

already at a concentration of 0.02 g/L (0.1 mM) (Larsson et al., 2000). Apart from coniferyl aldehyde, lignocellulosic hydrolysates typically contain many aromatic aldehydes that could contribute to microbial inhibition (Larsson et al., 1999a; Martín et al., 2002; Du et al., 2010; Mitchell et al., 2014). The inhibitory effects of aromatic aldehydes and other aromatic substances vary and can be predicted on basis of their functional groups (Larsson et al., 2000). Inhibition by aldehydes appears to be similar to inhibition by carboxylic acids in the sense that carbohydrate-derived furan aldehydes can be present in relatively high concentrations, but the toxicity is low, while lignin-derived aromatic aldehydes have relatively high toxicity, although the concentrations found in hydrolysates are generally low.

Other inhibitory compounds that can tentatively form through pretreatment under acidic conditions include quinones and small aliphatic aldehydes (Fig. 1). Although the presence of these groups of compounds in lignocellulosic hydrolysates warrants further attention, it is clear that compounds such as benzoquinone are strongly inhibitory to yeast. Inclusion of 20 mg/L of benzoquinone in fermentation experiments with *S. cerevisiae* was sufficient to completely inhibit growth and ethanol formation (Larsson et al., 2000).

#### 4.2. Inhibition of cellulolytic enzymes

The catalytic action of cellulolytic enzymes can be inhibited by non-productive binding to constituents of the solid fraction, such as lignins (Nakagame et al., 2011; Rahikainen et al., 2013; Pareek et al., 2013) and residual hemicelluloses (Pareek et al., 2013; Kumar and Wyman, 2014). The positive effect on enzymatic hydrolysis of cellulose achieved by adding bovine serum albumin could be attributed to prevention of unproductive binding of cellulase onto lignin (Brethauer et al., 2011).

Inhibition of cellulases is also caused by soluble carbohydrates and aromatic substances in the pretreatment liquid. Product inhibition of cellulolytic enzymes by monosaccharides, such as glucose, and disaccharides, such as cellobiose, is a well-known problem (Berlin et al., 2007; Teugjas and Våljamäe, 2013). More recently, the inhibitory effects of oligosaccharides derived from xylan and mannan have been investigated (Qing et al., 2010; Kumar and Wyman, 2014). The presence of such oligosaccharides is dependent on the pretreatment method, and also on the potential inclusion of enzymes that degrade hemicellulose-derived oligosaccharides in the enzyme preparation.

Solubilized aromatics, such as phenolics, may also affect enzymatic saccharification negatively (Ximenes et al., 2011). Another finding that supports the significance of aromatic substances as enzyme inhibitors is that inhibition of cellulolytic enzymes can be alleviated through addition of sulfur oxyanions, such as sulfite and dithionite, which react with many aromatic compounds but not with sugars (Alriksson et al., 2011; Cavka et al., 2011; Cavka and Jönsson, 2013). Furthermore, when sodium borohydride was used for detoxification rather than sulfite or dithionite, inhibition of the fermenting microorganism was alleviated, but not inhibition of the cellulolytic enzymes (Cavka and Jönsson, 2013). Treatment with sulfur oxyanions results in sulfonation of aromatic compounds making them less reactive, negatively charged and strongly hydrophilic (Fig. 2), while treatment with sodium borohydride makes them less reactive without changing the hydrophilicity very much (Cavka and Jönsson, 2013). This indicates that aromatic compounds play an important role in inhibition of cellulolytic enzymes and also that hydrophobic interactions between aromatic substances and cellulolytic enzymes are the cause of the problem. The role played by soluble aromatic compounds in inhibition of enzymes is further supported by fractionation of cellulase and fermentation inhibitors from steam-pretreated mixed hardwood (Kim et al., 2013).

Fractionation by washing with hot and cold water furthermore indicated that phenolic compounds that were more hydrophobic were more inhibitory than those that were less hydrophobic (Kim et al., 2013). Thus, although identification of soluble substances that inhibit cellulolytic enzymes warrants further attention, results obtained so far point towards contributions by hemicellulose- and cellulose-derived carbohydrates (such as mono-, di-, and oligosaccharides, Fig. 1) as well as aromatic substances (such as phenylic compounds, Fig. 1).

### 5. Strategies to counteract inhibition problems

Table 2 summarizes different strategies employed to avoid or tackle problems with inhibition of biocatalysts after pretreatment of lignocellulose under acidic conditions. As the scientific and technical literature in the area is prolific while space is limited, examples of different strategies are provided (Table 2).

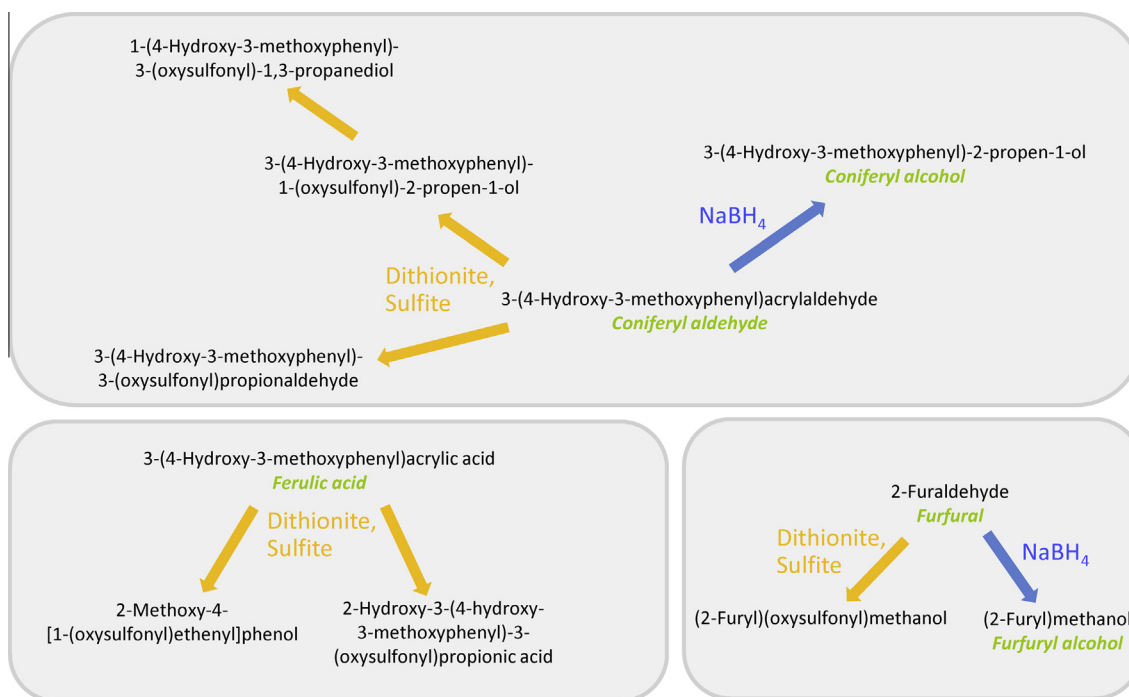
#### 5.1. Feedstock selection and engineering

Efforts to commercialize bioconversion of lignocellulosic feedstocks could potentially focus on feedstocks with relatively low recalcitrance, which makes it possible to perform pretreatment under mild conditions. Examples include hydrothermal pretreatment without any addition of acid catalysts for bioconversion of *Miscanthus* grass (Chiaromonti et al., 2012) and wheat straw (Larsen et al., 2012). These conditions led to low concentrations of furan aldehydes and phenols, but the concentrations of acetic acid were reported to reach 17 g kg<sup>-1</sup> for the *Miscanthus* and 5.1 g kg<sup>-1</sup> for the wheat straw. Collections of natural varieties of feedstocks that are of interest for bioconversion using a sugar platform concept, such as *Populus trichocarpa* trees (Studer et al., 2011), can be screened to identify varieties that exhibit lower recalcitrance. Feedstock engineering targeting components such as lignin, hemicellulose, and pectin is another approach to decrease recalcitrance and thereby reduce inhibitor release. By selecting or engineering plants with low acetyl content, the risk for formation of inhibitory concentrations of acetic acid can potentially be minimized. These strategies are of interest mainly with regard to short-rotation crops dedicated to biorefining through a sugar platform process.

#### 5.2. Detoxification/conditioning

Detoxification or conditioning of lignocellulosic hydrolysates and slurries is one of the most powerful ways to counteract inhibition problems (reviewed by Pienkos and Zhang, 2009; Jönsson et al., 2013). This strategy includes techniques such as using chemical additives, e.g. alkali (Alriksson et al., 2006), reducing agents (Alriksson et al., 2011; Cavka and Jönsson, 2013) (Fig. 2), and polymers (Cannella et al., 2014). Other possibilities are to use enzymatic treatment, heating and vaporization, liquid-liquid extraction, and liquid-solid extraction (reviewed by Jönsson et al., 2013). Liquid-solid extraction covers techniques such as ion exchange and treatment with activated carbon (e.g. Duque et al., 2015).

A drawback with many detoxification methods is that a separate process step is required. Addition of reducing agents (Alriksson et al., 2011; Cavka and Jönsson, 2013) and adsorption to PEI polymer (Cannella et al., 2014) should, however, be compatible with biocatalytic conversion steps. An additional advantage with some reducing agents, such as the sulfur oxyanions sulfite and dithionite (Fig. 2), is that they improve both fermentability and enzymatic saccharification of cellulose.



**Fig. 2.** Comparison of reactions effected by treatment of inhibitors with sulfur oxyanions (sulfite, dithionite) and sodium borohydride. While treatments of lignocellulosic hydrolysate with sulfur oxyanions or sodium borohydride were beneficial with regard to alleviating the inhibition of yeast, only the treatment with sulfur oxyanions was beneficial for alleviating inhibition of cellulolytic enzymes. This indicates that the hydrophilization of aromatic substances caused by sulfonation was essential for the positive effect on enzymatic hydrolysis.

**Table 2**

Overview of strategies to circumvent problems with soluble lignocellulose-derived inhibitors formed during pretreatment under acidic conditions.

Strategy	Approach (examples)	Considerations/potential drawbacks	References (examples)
Feedstock selection and engineering	Using less recalcitrant feedstocks and feedstocks that generate less inhibitors during pretreatment	Desirable to use broad range of feedstocks; Option for short-rotation crops dedicated to biorefining through sugar platform process	Studer et al. (2011), Chiaramonti et al. (2012), Larsen et al. (2012)
Detoxification/conditioning	Chemical additives, e.g., alkaline treatment, reducing agents, polymers	More chemicals needed; some methods require additional process step	Alriksson et al. (2006, 2011), Cannella et al. (2014)
Bioabatement	Microbial treatment	Could be time-consuming and affect sugar content	Cao et al. (2013, 2015)
Culturing schemes	SSF/CBP decrease feed-back inhibition by sugars; use large inoculum size	Effects on productivity and product yield; inoculum adds to cost of an industrial process	Pienkos and Zhang (2009), Hoyer et al. (2010), Olofsson et al. (2010), Olson et al. (2012), den Haan et al. (2013)
Selection of microorganism	Screening of microbial collections from natural or industrial environments	Selection ought to be made primarily on basis of specific productivity and product yields	Favaro et al. (2013), Wimalasena et al. (2014)
Evolutionary engineering	Adaptive evolution using specific inhibitors and lignocellulosic hydrolysates	Cause of inhibition problems varies depending on feedstock, pretreatment conditions	Koppram et al. (2012), Almario et al. (2013), Smith et al. (2014)
Genetic/metabolic engineering	Engineering of resistance to phenolics, furfural, and carboxylic acids	GMM-based process	Larsson et al. (2001), Wang et al. (2013), Sanda et al. (2011)

While there are numerous ways to perform detoxification (Jönsson et al., 2013), only few have been the topic of techno-economic evaluations considering for example process inputs, energy requirements, capital equipment, performance, and contribution to the ethanol cost. Techno-economical aspects of inclusion of a sodium sulfite conditioning step in bioconversion of steam-pretreated Norway spruce through simultaneous saccharification and fermentation (SSF) with *S. cerevisiae* have recently been investigated (Cavka et al., 2015). The study indicated that sodium sulfite conditioning would be economically justified if the yeast inoculum or the enzyme dosage could be lowered by about 0.7 g/L (dry weight) and 1 FPU/g water-insoluble solids. These thresholds were far below the gains of sodium sulfite conditioning found in the study. The approach was validated in a biorefinery demonstration

plant with 10 m<sup>3</sup> bioreactors. The evaluation also indicated that conditioning with sodium sulfite could shorten the SSF time from 72 h to 24 h using the same yeast and enzyme loads and without affecting the overall ethanol yield. Shortening of residence time in a full-scale plant producing 60,000 m<sup>3</sup> of ethanol per year from 72 h to 24 h would lead to a reduction of the number of the SSF bioreactors from 7 to 3, which would result in a decrease of the capital investment cost equivalent to 10–11.4 MUSD, which considerably surpassed the cost of the addition of sodium sulfite (Cavka et al., 2015).

Humbird et al. (2011) presented a thorough techno-economic model with detailed material and energy balances and capital and operating costs for the entire process of ethanol production from corn stover by dilute-acid pretreatment, enzymatic

saccharification, and co-fermentation with a recombinant *Zymomonas mobilis* strain. The model, for an assumed plant size of 2000 tons of ethanol per day, allows quantifying the economic impact of individual conversion performance targets, and addresses the replacement of overliming by ammonium hydroxide conditioning, which eliminates significant sugar losses and gypsum disposal, and allows treating the whole slurry without a solid–liquid separation step. This makes ammonia conditioning a more economical alternative than overliming in spite of the higher cost of ammonia compared to that of lime, and despite of the need of redesigning the wastewater treatment section because of the high levels of ammonium salts remaining in the stillage.

Duque et al. (2015) recently reported an economic analysis of the production of anhydrous ethanol from different agricultural residues by acid pretreatment, enzymatic hydrolysis, fermentation with *Pichia stipitis* and *S. cerevisiae*, distillation, and dehydration with molecular sieves. The study, which is based on material and energy balances generated by simulation for a feed rate of 1000 kg/h of raw material, addresses the energy consumption, equipment characteristics and the utilities requirements of each stage, including detoxification with activated charcoal, and resulted in an estimated ethanol production cost of around 0.65 USD/L.

### 5.3. Bioabatement

Microbial treatment, also referred to as bioabatement, can be used to improve both fermentability and enzymatic hydrolysis of cellulose (Cao et al., 2013, 2015). Challenges connected with bioabatement include the time required to perform the microbial process step, and the tendency of the microbes to consume sugar and thereby decrease the overall process yield.

### 5.4. Culturing schemes

The bioconversion process can be designed in a way that makes it less prone to inhibition problems. Product inhibition of cellulolytic enzymes can be avoided by performing enzymatic hydrolysis of cellulose and microbial fermentation simultaneously. Process designs that have recently been studied include SSF (simultaneous saccharification and fermentation) in fed-batch mode (Hoyer et al., 2010; Olofsson et al., 2010) and CBP (consolidated bioprocessing), in which the fermenting microorganism also contributes to the supply of enzyme (Olson et al., 2012; den Haan et al., 2013). Issues with modifications of the basic process design include the effects on productivity, yield, and final product titer.

A large inoculum size can compensate for inhibitory environment (reviewed by Pienkos and Zhang, 2009). The cost of the fermenting microorganism is, however, a significant part of the total process cost (Wingren et al., 2003).

### 5.5. Selection of microorganism

Screening of collections of microorganisms gathered from natural or industrial environments can be used to identify strains with high resistance to inhibitors. A study of strains of *S. cerevisiae* gathered from grape marc in a winery revealed strains with relatively high resistance to aliphatic carboxylic acids, furan aldehydes, and a sugarcane bagasse hydrolysate (Favaro et al., 2013). A screening of 90 strains of *Saccharomyces* spp. included an assessment of tolerance to acetic acid, formic acid, furfural, HMF, and vanillin (Wimalasena et al., 2014). An important issue is the specific productivity of the microorganism, as resistance to inhibitors is not sufficient to make a microorganism suitable for an industrial production process.

### 5.6. Evolutionary engineering

The inhibitor resistance of a selected fermenting microorganism can be improved through adaptive evolution. Recent examples of evolutionary engineering includes yeast strains exhibiting improved resistance to spruce wood hydrolysate (Koppram et al., 2012), corn stover hydrolysate (Almario et al., 2013), and triticale straw (Smith et al., 2014).

### 5.7. Genetic and metabolic engineering

Using genetic engineering, recombinant microorganisms exhibiting improved resistance to lignocellulosic hydrolysates have been developed. *S. cerevisiae* yeast expressing laccase from the white-rot fungus *Trametes versicolor* exhibited enhanced resistance to spruce wood hydrolysate (Larsson et al., 2001). Engineering of furfural resistance of *Escherichia coli* resulted in improved resistance to a sugarcane bagasse hydrolysate (Wang et al., 2013). Overexpression of transaldolase and alcohol dehydrogenase in *S. cerevisiae* resulted in a small increase of the ethanol yield when the yeast was cultured in a lignocellulosic hydrolysate supplemented with large amounts of yeast extract and peptone (Hasunuma et al., 2014). The increase in yield was attributed to improved performance in the presence of furfural (Hasunuma et al., 2014), although the furfural content of the hydrolysate was merely 7.8 mM. Sanda et al. (2011) engineered *S. cerevisiae* for increased resistance to acetic acid and formic acid by enhancing the activity of transaldolase and formate dehydrogenase and studied the performance of the yeast using a rice straw hydrolysate. Guadalupe Medina et al. (2010) proposed deletion of *S. cerevisiae* genes encoding glycerol-3-phosphate dehydrogenase and expression of an acetylating acetaldehyde dehydrogenase from *E. coli* as a way to achieve conversion of inhibitory acetic acid to ethanol and eliminate glycerol formation in anaerobic cultures of yeast. Further strain development using evolutionary engineering resulted in production of small amounts of glycerol and improved performance in medium with high sugar concentrations (Guadalupe-Medina et al., 2014). Wei et al. (2013) combined the pathway for conversion of acetic acid to ethanol by expression of the acetylating acetaldehyde dehydrogenase with a pathway for co-utilization of xylose through expression of xylose reductase and xylitol dehydrogenase.

Although there are many studies on strain selection, evolutionary engineering and metabolic engineering to enhance microbial resistance, the performance of the resulting microbial strains are seldom benchmarked against other microorganisms or against well-established detoxification methods such as treatment with alkali. A comparison of an engineered microorganism and alkali detoxification with regard to fermentation of a spruce wood hydrolysate (Jönsson et al., 2013) indicated that even though engineering of the microorganism greatly enhanced its resistance to inhibitors, chemical detoxification was a considerably more powerful approach to reach a fermentability comparable with that of a medium without inhibitors.

## 6. Conclusions

Rapid progress is being made with regard to understanding feedstock recalcitrance, and feedstock engineering has emerged as a rapidly developing field. Identification and characterization of previously unknown inhibitory substances in pretreated biomass is still a field under development. Novel methods for chemical detoxification without the need for separate process steps have recently been developed and are ready for industrial implementation. Selection and engineering of biocatalysts with improved



resistance has progressed with regard to some well-known inhibitors, but more efforts are needed to cover all groups of inhibitory compounds and to benchmark the engineered strains.

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