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Abstract

Detoxification of dilute-acid lignocellulose hydrolysates by treatment with $Ca(OH)_2$ (overliming) efficiently improves the production of fuel ethanol, but is associated with drawbacks like sugar degradation and $CaSO_4$ precipitation. In factorial designed experiments, in which pH and temperature were varied, dilute-acid spruce hydrolysates were treated with $Ca(OH)_2$, NH_4OH or $NaOH$. The concentrations of sugars and inhibitory compounds were measured before and after the treatments. The fermentability was examined using the yeast *Saccharomyces cerevisiae* and compared with reference fermentations of synthetic medium without inhibitors. The treatment conditions were evaluated by comparing the balanced ethanol yield, which takes both the degradation of sugars and the ethanol production into account. Treatment conditions resulting in excellent fermentability and minimal sugar degradation were possible to find regardless of whether $Ca(OH)_2$, NH_4OH or $NaOH$ was used. Balanced ethanol yields higher than those of the reference fermentations were achieved for hydrolysates treated with all three types of alkali. As expected, treatment with $Ca(OH)_2$ gave rise to precipitated $CaSO_4$. The NH_4OH treatments gave rise to a brownish precipitate but the amounts of precipitate formed were relatively small. No precipitate was observed in treatments with $NaOH$. The possibility that the ammonium ions from the NH_4OH treatments gave a positive effect as an extra source of nitrogen during the fermentations was excluded after experiments in which NH_4Cl was added to the medium. The findings presented can be used to improve the effectiveness of alkali detoxification of lignocellulose hydrolysates and to minimize problems with sugar degradation and formation of precipitates.

List of papers

Paper 1
Sárvári Horváth, I., Sjöde, A., Alriksson, B., Jönsson, L.J., Nilvebrant, N.-O. (2005),
Critical conditions for improved fermentability during overliming of acid hydrolysates
from spruce. Appl. Biochem. Biotechnol., 124, 1031-1044.
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Paper 2
Alriksson, B., Sárvári Horváth, I., Sjöde, A., Nilvebrant, N.-O., Jönsson, L.J. (2005),
Ammonium hydroxide detoxification of spruce acid hydrolysates. Appl. Biochem.
Biotechnol., 124, 911-922.
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Paper 3
Alriksson, B., Sjöde, A., Nilvebrant, N.-O., Jönsson, L.J. (2006), Optimal conditions for
alkaline detoxification of dilute-acid lignocellulose hydrolysates. Appl. Biochem.
Biotechnol., 130, 599-611.
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Introduction

A transition from the utilization of petroleum-derived fuels in the transportation sector to renewable fuels, such as bioethanol, can be beneficial to society in many ways. Combustion of renewable materials will not contribute to a net increase of the greenhouse gas CO_2 in the atmosphere. CO_2 is believed to contribute to global warming (IPCC, 2001). Bioethanol can also contribute to the national energy security by reducing the dependency of petroleum imports. However, the strongest argument for switching to alternative fuels is that the global supply of petroleum is running low (Bentley, 2002). Ethanol can be produced from renewable raw materials and will function in the automobiles and buses of today without any major modifications of the engines.

The technique of making ethanol is ancient. People have been making alcoholic beverages, such as wine, through fermentation for thousands of years (Pretorius, 2000). The industrial ethanol production of today is mainly based on sugar and starch feedstocks, such as sugarcane and corn. However, the cost of these raw materials is calculated to be as high as 40-70% of the total production cost (Claassen *et al.*, 1999). Another disadvantage of using cultivated sugar and starch crops for ethanol production is the limited supply of agricultural land (Sun and Cheng, 2002). A way to reduce the cost of large-scale ethanol production would be to utilize a cheaper feedstock. Lignocellulose provides a cheap and abundant raw material, part of which can be converted to fermentable sugars through hydrolysis.

During hydrolysis of lignocellulose not only sugars are generated but also a wide range of by-products. Some of these by-products inhibit the fermenting microorganism and cause a decrease in the ethanol productivity. To obtain an effective fermentation process, the effect of the inhibitors has to be eliminated. There are several ways to deal with inhibitor problems. One of the most effective methods is alkali detoxification, which is the focus of this thesis.

Dilute-acid spruce hydrolysates were detoxified using different kinds of alkali under various conditions (Papers 1-3). In all experiments, the effectiveness of the detoxification treatments was evaluated by using baker's yeast (*Saccharomyces cerevisiae*) as the fermenting microorganism. In Paper 1, an optimization of detoxification with $Ca(OH)_2$ is presented. The possibility of using NH_4OH for detoxification was explored and the results are reported in Paper 2. In Paper 3, optimized treatments with NH_4OH and $NaOH$ are compared with $Ca(OH)_2$ treatment.

Background

The history of fuel ethanol

The use of ethanol as an automobile fuel is not a new invention. Already in 1908, Ford's model T could be adjusted to run on either gasoline or alcohol (DiPardo, 2000). However, after World War II the interest in using ethanol as a fuel declined because cheap gasoline made from petroleum was available. In the 1970's, the interest in fuel ethanol was renewed due to the oil crisis (DiPardo, 2000). More recently, ethanol has become used as an additive in gasoline. MTBE (methyl tertiary butyl ether) is used as a gasoline additive to increase the oxygen content and the octane number. During the last few years, the use of MTBE has been banned in several states in the USA due to the risk of contamination of water. Many companies have replaced MTBE with ethanol to give the gasoline similar clean burning and octane boosting properties as MTBE-blended gasoline (F.O. Licht's World ethanol and biofuels report 2006; Sun and Cheng, 2002). Today there are several flexifuel automobile models (vehicles that can run on mixtures of ethanol and gasoline containing up to 85% ethanol) available from various manufacturers (BAFF, 2006).

About 99% of the fuel ethanol is produced from cultivated crops (BAFF, 2006). Brazil has for a long time been the leading ethanol producer of the world. However, during the last years USA has increased its production and today both countries have an annual production of about 16 000 000 m^3 (F.O. Licht's World ethanol and biofuels report, 2006). The Brazilian ethanol is mainly produced from sugarcane. Brazil is the world leader in the use of ethanol as an automobile fuel. In Brazil, the ordinary gasoline, which is used in about 7 000 000 cars, contains about 24% ethanol. In addition, 4 000 000 automobiles drive on a blend of 95% ethanol and 5% water (BAFF, 2006). In the USA, ethanol is mainly produced from corn.

In Sweden, about 55 000 m^3 of fuel ethanol is produced per year from wheat and about 18 000 m^3 from spent sulfite liquor (Agroetanol AB, 2006; Jordbruksverket, 2006). In Sweden, the ordinary gasoline typically contains 5% ethanol and the number of flexifuel automobiles is increasing (Jordbruksverket, 2006). The Swedish ethanol production does not cover the demand and therefore Sweden is a net importer of ethanol. However, initiatives have been taken to increase the future national ethanol production. In 2004 an ethanol-from-lignocellulose pilot plant was inaugurated in the city of Örnsköldsvik. Agroetanol AB plans to expand its production of ethanol from grain with 150 000 m^3 in 2008.

Environmental issues

Global warming is pointed out to be a serious environmental problem that is believed to be caused by human activities (IPCC, 2001). The mechanism behind global warming is the increased emission of greenhouse gases like CO_2. A considerable part of all greenhouse gases comes from the transport sector. The so called "greenhouse effect" is a natural phenomenon that is a prerequisite for life on earth. Shortwave radiation from the sun reaches the atmosphere of the earth and penetrates a layer of greenhouse gases, predominantly CO_2 but also CH_4, N_2O, and other gases. When the radiation reaches the earth, the major part is absorbed by its surface. The earth in turn emits thermal radiation to the atmosphere. The thermal radiation has a longer wavelength than the incoming radiation, which makes penetration of the greenhouse gas layer more difficult and thereby most of the thermal radiation is absorbed by the greenhouse gases. The thermal radiation is eventually emitted from the greenhouse gases and part of this radiation goes back to earth. This phenomenon keeps the earth warm. The more greenhouse gases there are in the atmosphere, the more thermal radiation will be emitted back to earth (Houghton, 2004; Maslin, 2002). Man's historically recent (1860-today) intensive combustion of fossil fuels like coal and petroleum has led to an accumulation of CO_2 in the atmosphere, which has led to a fast increase of the average temperature on earth. During the 20th century, the global average temperature has increased by about 0.6°C (IPCC, 2001). A rise in the average temperature of the earth is associated with problems like sea level rise, extreme weather, and disruption of ecosystems (IPCC, 2001). Carbon dioxide released from the combustion of fossil fuel products originates from plants and animals that lived on earth millions of years ago. These materials have been stored under the surface of the earth and there are no natural means for incorporating the large amounts of CO_2 that are quickly released in the atmosphere by combustion into new biomass. The combustion of renewable materials, like ethanol, does not contribute to a net increase of CO_2 in the atmosphere because they are a part of the natural closed carbon cycle (Fig. 1).

Another environmental benefit of ethanol as a fuel is that the emission of carbon monoxide, nitrogen oxides and hydrocarbons in general is less compared to gasoline (Hsieh *et al.,* 2002). However, ethanol-containing fuels will contribute to an increased emission of formaldehyde and acetaldehyde (Leong *et al.,* 2002). Nevertheless, the environmental damage caused by reactive aldehydes is far less than that of poly-nuclear aromatic compounds, which are emitted when gasoline is combusted (Hsieh *et al.,* 2002).

The European Union has set up a biofuel directive, which states that 5.75% of all gasoline and diesel transport fuels should come from renewable resources in the year 2010. The

directive was established in 2003 as a response towards the issue of global warming (BAFF, 2006).
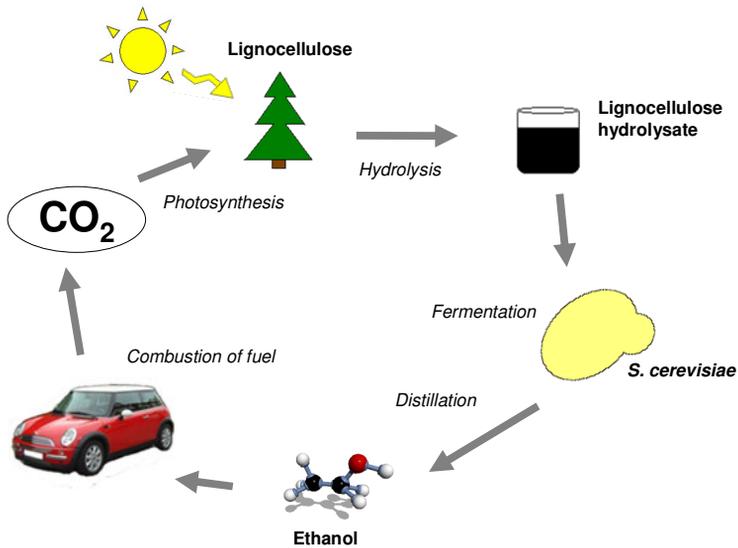
Figure 1. Production of ethanol from lignocellulose.

Energy security

Another driving force of ethanol research, besides the environmental benefits, is the possibility to create energy security and independence of petroleum imports. The president of USA, George W. Bush, announced in his State of the Union speak in January 2006 a national goal of decreasing the petroleum imports from the Middle East with 75% by 2025 (The White House, 2006). The energy from petroleum is to be replaced by energy from new technologies. In the transport sector, the new technologies that are of main interest are hydrogen-fueled automobiles, improvements in battery technology for hybrid automobiles, and development of ethanol production from cellulosic waste materials.

In Sweden, a commission appointed by the government for reducing the dependency of Sweden on the import of petroleum presented a new directive in June 2006. The directive included the goal of reducing the consumption of petroleum-derived fuels for road traffic with 40-50% by the year 2020. The tools that are expected to be useful for achieving the goal are new fuels and higher energy efficiency (BAFF, 2006). The move from petroleum to renewables could also lead to positive spin-off effects on the national economy, such as boosting the agricultural and forestry sector and creating new jobs (Sheehan and Himmel, 1999; Lin and Tanaka, 2006).

Net energy gain?

An important and debated issue is the net energy gain of ethanol production. A useful metric for measuring the energy balance is r_E. The definition of r_E is the total product energy divided by the nonrenewable energy input. An r_E value equal to one is a break-even situation, while values less than one mean a net loss of energy. Hammerschlag (2006) performed a comparison of six different studies on the production of ethanol from corn and four different studies on the production of ethanol from cellulose. The comparison showed that the r_E values from the corn ethanol studies were between 0.84 and 1.65. For the cellulosic ethanol, one of the four studies showed an r_E value of 0.69, while the other three studies showed r_E values between 4.40 and 6.61 (Hammerschlag, 2006). The study that resulted in an r_E value of 0.69 was assumed to be unreasonably calculated because the fact that the combustion of residual lignin can generate energy in the process was not taken into account. Considering that the production of corn ethanol is a mature technology it is not likely that there will be any major net energy improvements in the future. The production of cellulosic ethanol is an immature technology and it is not unlikely that the net energy gain will improve significantly as a result of future research and development efforts.

A comparison of greenhouse gas reduction can also be evaluated on basis of r_E values because the energy content of any fossil fuel is approximately proportional to the CO_2 emission when combusted. The r_E value of gasoline is 0.76, which means that ethanol produced for replacing gasoline must exceed this r_E value for contributing to a net reduction of the emission of CO_2 (Hammerschlag, 2006).

The end of cheap petroleum

The end of cheap petroleum will become a reality the day when the peak in the production is reached, unless the demand declines as much as the production. Petroleum stands for about 40% of the world's primary energy demand and about 50% of all the petroleum is consumed by the transportation sector (IEA, 2002). During the period of

1971 to 2000 the consumption of petroleum increased by 47%. The demand is calculated to increase by 60% from the year 2000 to 2030 (IEA, 2002). However, the petroleum of today was formed in two brief periods for about 90 and 150 million years ago, which means that the petroleum is a finite resource that can be depleted (Campbell, 2006). The rate of discovery of new deposits of petroleum has declined since the 1960's and in the early 1980's the consumption started to exceed the discoveries of new petroleum (Campbell, 2006). The global production of conventional petroleum is predicted, by the pessimists, to reach its peak around the year 2010 (Campbell and Laherrere, 1998; Bentley, 2002). A more optimistic prediction was made by Odell (1999) who predicted that the peak of conventional petroleum production will be reached in the year 2030. The definition of conventional petroleum is ambiguous but in general conventional petroleum is considered to be low density liquid petroleum. Examples of unconventional petroleum are petroleum with higher density than water, oil sands, and oil shales (Greene *et al.*, 2006). Unconventional petroleum as well as coal can be used to produce liquid fuels but they have so far been of little economic interest. Nevertheless, they are potential resources that can be used to replace conventional petroleum (Greene *et al.*, 2006). However, if these raw materials will compete with biomass-derived fuels to replace conventional petroleum, the biomass fuels would have the benefits of being renewable and more environmentally friendly.

Traditional ethanol production
Ethanol can be produced synthetically from petroleum or by microbial fermentation of sugars. The three main groups of raw materials for production of ethanol by fermentation are sugar, starch, and lignocellulose (Lin and Tanaka, 2006). Sugar-containing raw materials include sugarcane, sugar beets, fruits, sweet sorghum, and molasses. The advantage with the sugar-based raw materials is that they can be converted into ethanol directly without the use of hydrolysis. A disadvantage is that many of these raw materials are considered to be a human food resource and will therefore be too expensive to use for fuel ethanol production (Badger, 2002). Starch-based materials that commonly are used for ethanol production include corn, potatoes, cassava, and various cereal grains. Prior to fermentation, the starch must be converted to sugars. Starch hydrolysis is typically performed by cooking the starch at high and low temperatures. Dextrin oligosaccharides are generated by adding α-amylase and glucoamylase is added to obtain glucose (Wheals *et al.*, 1999). A disadvantage of using starch-based materials for ethanol production is that the hydrolysis cost is high due to high energy costs of the cooking step and high costs for the amylolytic enzymes (Lin and Tanaka, 2006). Lignocellulose-based feedstocks that can be considered for ethanol production are wood residues, agricultural residues, and spent sulfite liquor from pulp and paper mills. The advantages of using lignocellulose as the raw

material for ethanol production are that it is abundant and relatively cheap (Wheals *et al.*, 1999). A disadvantage is that lignocellulose is relatively recalcitrant to hydrolysis (Gray *et al.*, 2006). Production of ethanol from lignocellulosic waste materials, such as sawdust from the forestry industry, benefits from the fact that the energy input for the overall process can be kept low. Energy-related expenses for planting, fertilization and harvesting can be avoided if waste materials are used. If lignocellulosic waste materials are used, there will not be any competition for the limited agricultural land available, which instead might be needed for food production (Sun and Cheng, 2002). The production of ethanol from lignocellulose is described in detail in the next section.

Ethanol from lignocellulose

Lignocellulose

Lignocellulose consists mainly of cellulose, hemicellulose, and lignin (Sjöström, 1993). Cellulose and hemicellulose are supporting materials of the cell wall. The content of lignin is high in the middle lamella of the cell but due to volume differences between the cell wall and the middle lamella most of the lignin is located in the cell wall. Lignin and hemicellulose can form lignin-carbohydrate bonds, which result in complexes that provide a hydrolysis-resistant protecting sheet around the cellulose. The ratio of the three main components varies among different plant species. Dry wood typically consists of 40-45% cellulose, 20-30% hemicellulose, and 20-32% lignin. Other constituents of lignocellulose are extractives, which usually represent a fraction of less than 10%, and ash, the content of which is usually less than 1%. In general, softwood has a higher lignin content than hardwood. The composition and structure of hemicellulose differ vastly between softwood and hardwood. As is also the case for lignocellulose from agricultural residues, hardwood generally has a high content of xylan. Softwood, such as spruce has a high content of mannan. In the current study, a hydrolysate of Norway spruce (*Picea abies*) was utilized. Norway spruce consists of about 42% glucan, 27% lignin, 16% mannan and galactan, 9% xylan and arabinan, 3% other polysaccharides, 2% extractives, and 1% residual constituents (Sjöström, 1993).

Cellulose

Cellulose consists of repeating units of cellobiose. The D-glucopyranose units are linearly linked together by β-1,4-glucosidic bonds. The number of glucose units in one cellulose molecule (*i.e.* the degree of polymerization) is on average about 10 000 (Rowell *et al.*, 2005). Cellulose molecules can aggregate with each other due to hydrogen bonding and form microfibrils, which are the building blocks of fibrils, which in turn build the cellulose fiber (Sjöström, 1993). Cellulose consists of crystalline and amorphous regions. The

crystalline regions are formed when the packing density of the cellulose increases. The cellulose of wood is highly crystalline and can consist of up to 65% of crystalline regions (Rowell *et al.*, 2005).

Hemicellulose

Hemicellulose is a polymer that is composed of several different sugars like D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, and L-rhamnose, and also of different uronic acids (Sjöström, 1993). The degree of polymerization of hemicellulose is on average about 100-200 and the molecules can be highly branched (Rowell *et al.*, 2005).

Lignin

Lignins are complex polymers consisting of phenylpropane units linked together by ether or carbon-carbon bonds (Brunow *et al.*, 1999) There are three main precursors of lignin biosynthesis; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignins can be classified according to their structural elements. Guaiacyl lignin is to a high extent a polymer of coniferyl alcohol and is common in softwood. Guaiacyl-syringyl lignin is common in hardwood and is a polymer of coniferyl alcohol, and sinapyl alcohol (Sjöström, 1993).

Extractives

Extractives are a group of chemicals that can be extracted from wood by the use of various solvents. The extractives can be categorized as terpenoids, steroids, fats, waxes, and phenolic constituents (Sjöström, 1993). The roles of the extractives are diverse. Some extractives are involved in the defense system of the plant, others are precursors of certain chemicals, and for many others the role has not yet been established (Rowell *et al.*, 2005).

Hydrolysis

There are three general methods for hydrolysis of lignocellulose polysaccharides to sugars. These methods are (*i*) concentrated-acid hydrolysis, (*ii*) dilute-acid hydrolysis, and (*iii*) enzymatic hydrolysis.

(*i*) Hydrolysis with concentrated acid is an old technique that was available at the end of the 19th century (Sheehan and Himmel, 1999). A concentrated acid, such as H_2SO_4, is applied at moderate temperatures. The acid breaks the hydrogen bonding between the cellulose chains and the cellulose becomes extremely susceptible to hydrolysis. The addition of water leads to rapid hydrolysis to glucose (Sheehan and Himmel, 1999). The main advantages of concentrated-acid hydrolysis are that the process can be performed at low temperatures and results in very high yields. The disadvantage is that the large

amounts of acid that are used must be recovered and reused for making the process economically viable. Another problem is equipment corrosion (Galbe and Zacchi, 2002).

(*ii*) Dilute-acid hydrolysis is the oldest technique for converting lignocellulose to ethanol. (Sheehan and Himmel, 1999). An acid, such as H_2SO_4, is added to a concentration of approximately 0.5% and high temperatures are applied. The advantages of the method are the fast reaction rate and the low acid consumption and the disadvantages are the low sugar yield, the requirement of high temperatures, hemicellulose sugar degradation, and generation of fermentation inhibitors. A way to minimize the degradation of the hemicellulose sugars and increasing the conversion yield of cellulose to glucose is to apply a two-step hydrolysis. In the first step, the hemicellulose fraction is hydrolyzed under relatively mild conditions (approx. 170-190°C) to generate sugars, like arabinose, galactose, glucose, mannose, and xylose. In the second step, harsher conditions (approx. 200-230°C) are applied to hydrolyze the cellulose fraction into glucose. The two fractions can then be pooled together prior to fermentation (Galbe and Zacchi, 2002).

(*iii*) Enzymatic hydrolysis is a method in which cellulases are utilized for the hydrolysis. This is a quite new approach compared to concentrated-acid and dilute-acid hydrolysis. Cellulolytic enzymes were discovered during World War II when American scientists found the agent that was responsible for army clothing deterioration in the jungles of the South Pacific. The organism responsible for producing the cellulolytic enzymes was *Trichoderma reesei*, which today is used in the enzyme industry for producing a wide range of commercial enzymes (Sheehan and Himmel, 1999). The cellulases involved in the hydrolysis of lignocellulose include endoglucanases, which attack low-crystallinity regions of the cellulose fiber and generate free chain-ends, and exoglucanases, which remove cellobiose from the free chain ends. Then, β-glucosidase hydrolyses cellobiose to glucose (Sun and Cheng, 2002). However, a pretreatment of the lignocellulose prior to the enzymatic hydrolysis is necessary to achieve feasible reaction rates. The aim of the pretreatment is to make the cellulose more accessible to enzymatic attack due to weakening of the protecting lignin and hemicellulose matrix or due to alteration of the pores in the material. There are a wide range of different pretreatment methods. Steam explosion and dilute-acid pretreatment are among the most common (Sun and Cheng, 2002). The advantages of enzymatic hydrolysis are high yields, due to the highly specific cellulose conversion, and that the reaction is performed at moderate temperatures. Furthermore, the by-product formation is low. The disadvantages are the slow reaction rate of the enzymes and the high enzyme cost.

Ethanol production by enzymatic hydrolysis can be performed in a Separate Hydrolysis and Fermentation (SHF) mode or in a Simultaneous Saccharification and Fermentaion (SSF) mode. In the SHF process, hydrolysis is performed separately from fermentation, which means that the optimal temperatures for both the enzymatic hydrolysis and fermentation can be applied. A drawback with SHF is that the generated cellobiose functions as cellulase inhibitor (Mandels and Reese, 1963). It has also been proved that β-glucosidase can be inhibited by glucose (Holtzapple *et al.*, 1990). Another drawback is that SHF is a two-step process. To reduce the risk for enzyme inhibition and reduce the number of process steps, SSF can be used. In SSF, hydrolysis and fermentation occur at the same time, which means that the glucose that is generated is immediately consumed by the fermenting microorganism and inhibition of β-glucosidase is therefore prevented. The disadvantage of the SSF process is that the optimal temperatures for the cellulases and the fermenting microorganism are not the same so the selected temperature is a compromise, which means that neither hydrolysis nor fermentation will be performed under optimal conditions.

Recently, efforts have been made to combine cellulase production, hydrolysis and fermentation in one single step. This concept is called Consolidated BioProcessing (CBP) and the aim is to create a microorganism that is able to perform these three steps simultaneously (Lynd *et al.*, 2005). There are two different strategies to create a CBP microorganism: A naturally occurring cellulolytic microorganism can be modified by genetic engineering to gain important properties, such as the ability to give high ethanol yields, or alternatively a non-cellulolytic microorganism that gives high ethanol yields can be altered by genetic engineering to express heterologous cellulases (Lynd *et al.*, 2005).

 Today, most research efforts are focused on the enzymatic hydrolysis because of the high development potential. The dilute-acid and concentrated-acid hydrolysis are relatively mature techniques for which no major improvements are likely to happen. Nevertheless, the enzyme cost is still high for the enzymatic process. The cost of enzyme in the SSF and SHF processes has been calculated to be 10-20% of the total ethanol production cost (Wingren *et al.*, 2005).

Inhibitors
During dilute-acid hydrolysis of lignocellulose not only sugars are formed but also a wide range of by-products (Fig. 2). Some of these by-products can inhibit the fermenting microorganism. The generation of by-products is strongly dependent on the raw material, the hydrolysis method, and the hydrolysis conditions. Four groups of compounds that

may act as inhibitors are phenolic compounds, aliphatic acids, furan aldehydes, and inorganic ions.
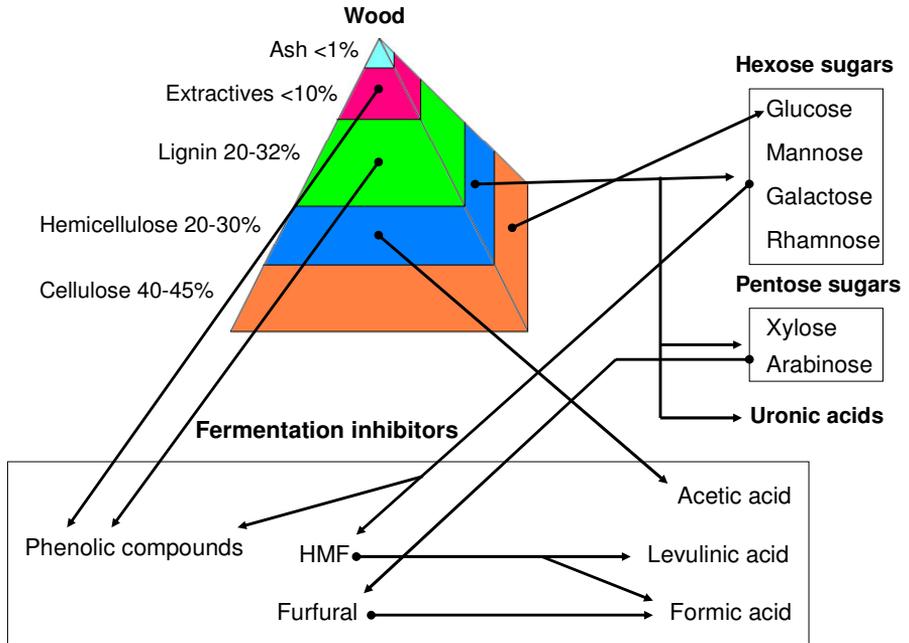

Figure 2. Composition of wood and compounds generated during dilute-acid hydrolysis.

Phenolic compounds

There are a large number of different phenolic compounds that are formed from lignin during dilute-acid hydrolysis (Clark and Mackie, 1984). Phenolic compounds can also be formed from sugars (Popoff and Theander, 1976). In addition, some of the wood extractives are phenolic compounds (Rowell *et al.*, 2005; Sjöström, 1993). Some of the phenolics are strongly inhibitory even at relatively low concentrations, while much higher concentrations are required for other phenolics to obtain an inhibitory effect (Ando *et al.*, 1986; Larsson *et al.*, 2000). The large number and diversity of the phenolics found in different lignocellulose hydrolysates make the identification and quantification complicated. Common phenolic compounds found in dilute-acid hydrolysates of spruce are vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, vanillic acid, hydroquinone, catechol, acetoguaiacone, homovanillic acid, and 4-hydroxybenzoic acid (Larsson *et al.*,

1999b). In most cases, the mechanism of toxicity has not been elucidated. One suggested mechanism is that the phenolics will interfere with the cell membrane, which will influence its function and change its protein-to-lipid ratio (Keweloh *et al.*, 1990). Various phenolic compounds have been found to inhibit both cell growth and ethanol productivity (Larsson *et al.*, 2000). *S. cerevisiae* can convert some of the phenolic compounds to less inhibitory compounds. During the fermentation vanillin and coniferyl aldehyde can be reduced to the corresponding alcohols. Furthermore, coniferyl alcohol can be converted to dihydroconiferyl alcohol (Larsson *et al.*, 2000).

Aliphatic acids

The three most common aliphatic acids in lignocellulose hydrolysates are acetic acid, formic acid, and levulinic acid. The aliphatic acids are formed from polysaccharides or from degradation products of polysaccharides during acid hydrolysis of lignocellulosic materials (Fig. 2). Acetic acid is formed by deacetylation of hemicellulose. Formic acid is a degradation product of HMF (Ulbricht *et al.*, 1984). Levulinic acid is formed during degradation of furfural and HMF (Dunlop, 1948; Ulbricht *et al.*, 1984). The most inhibitory of these three acids is formic acid followed by levulinic and acetic acid (Zaldivar and Ingram, 1999). Undissociated acids may enter the cell through diffusion over the cell membrane and become dissociated in the cytosol due to the neutral cytosolic pH (Pampulha and Loureiro-Dias, 1989). The dissociation of the acid leads to a decrease in the intracellular pH, which may lead to cell death (Verduyn *et al.*, 1990).

Small amounts of weak acids can, however, affect the metabolism so that the cell produces more ethanol. The mechanism behind this phenomenon is believed to be the cell's attempt to maintain a constant intracellular pH by pumping out protons through the plasma membrane ATPase. This process requires additional ATP, which is accomplished by an enhanced ethanol production and reduced biomass and by-product formation (Verduyn *et al.*, 1990; Verduyn *et al.*, 1992; Viegas and Sá-Correia, 1991). Concentrations of up to 3.3 g/L of undissociated acetic acid have been found to increase the ethanol yield and decrease the biomass and the glycerol yields in experiments with *S. cerevisiae* (Taherzadeh *et al.*, 1996). Larsson *et al.* (1999a) showed that concentrations of acetic acid, formic acid and levulinic acid over 100 mmol/L decrease the ethanol yield of *S. cerevisiae*, while concentrations lower than 100 mmol/L instead increase the ethanol yield.

The amount of aliphatic acids in lignocellulose hydrolysates is strongly dependent on the raw material. Taherzadeh *et al.* (1997) produced several hydrolysates from different types of wood using identical hydrolysis conditions. The amount of acetic acid was about 9 g/L (approx. 150 mmol/L) in hydrolysates made of alder, aspen, or birch, whereas

hydrolysates of pine or spruce only contained about 3 g/L (approx. 50 mmol/L) of acetic acid. In conclusion, the amount of aliphatic acids of many softwood hydrolysates does not reach the inhibitory level and can instead stimulate the ethanol production. The level of aliphatic acids in hardwood hydrolysates is often high and inhibition due to aliphatic acids can therefore be considered as a potential problem for fermentation of many hardwood hydrolysates and hydrolysates made from agricultural residues.

Furan aldehydes

Furfural and 5-hydroxymethyl furfural (HMF) are the predominant furan aldehydes in lignocellulose hydrolysates. Furfural is a degradation product of pentose sugars and HMF is formed by degradation of hexose sugars (Saeman, 1945).

Furfural has been shown to decrease the ethanol productivity of yeast (Chung and Lee, 1984; Larsson *et al.*, 1999a). Banerjee *et al.* (1981a) and Modig *et al.* (2002) suggested that furfural inhibits several glycolytic enzymes. However, the inhibition mechanism of furfural is not fully clear. HMF may affect microorganisms in a similar way but is more toxic than furfural when compared at equimolar concentrations (Larsson *et al.*, 1999a). Under anaerobic conditions, *S. cerevisiae* can convert furfural to furfuryl alcohol (Diaz De Villegas *et al.*, 1992; Taherzadeh *et al.*, 1999a) and HMF to 5-hydroxymethyl furfuryl alcohol (Taherzadeh *et al.*, 2000). The enzymatic reduction of furfural requires the cofactor NADH, while the enzymatic reduction of HMF requires NADPH as the main cofactor (Wahlbom and Hahn-Hägerdal, 2002). This suggests that the reduction of furfural and HMF is carried out by different enzymes.

Wahlbom and Hahn-Hägerdal (2002) also showed that a moderate addition of furfural to the growth medium could lead to increased ethanol yields for recombinant xylose-utilizing *S. cerevisiae* strains. This can be explained by the reduction of furfural to furfuryl alcohol, which will lead to a decreased formation of the undesirable by-product xylitol and an increased formation of ethanol.

In a study by Taherzadeh *et al.* (1999b), the levels of furfural, HMF, and acetic acid in an inhibitory spruce hydrolysate were found to be 2.2 g/L, 7.3 g/L, and 3.2 g/L, respectively. The same concentrations of these inhibitors were added to a synthetic hydrolysate. The synthetic hydrolysate was, however, easily fermented by *S. cerevisiae*. Furthermore, Larsson *et al.* (1999a) performed model fermentations with *S. cerevisiae* using two synthetic media containing 4.6 g/L furfural or 6.1 g/L HMF. The ethanol production was, however, not particularly retarded. Martinez *et al.* (2000) detoxified a bagasse hydrolysate, containing about 0.7 g/L HMF and 0.6 g/L furfural, by $Ca(OH)_2$ treatment. *Escherichia coli* was used

as the fermenting microorganism and the treatment resulted in a less inhibitory hydrolysate with reduced levels of furfural and HMF. However, an attempt to restore the toxicity of the hydrolysate by addition of furfural and HMF to the initial concentrations failed. It took an addition of three times the original concentrations of the furan aldehydes to restore inhibition. This suggests that the inhibition might be due to other inhibitors present in the hydrolysate, for example phenolic compounds, other yet unidentified compounds, or perhaps synergistic effects in which all of the mentioned substances could be involved.

Inorganic ions

Inorganic ions that are present in lignocellulose hydrolysates come from the raw material, from chemicals added during pretreatment and hydrolysis, and from the hydrolysis equipment. Another source is the chemicals that are added for pH adjustment prior and during fermentation. The addition of salts results in a higher osmotic pressure and the ions can give an inhibitory effect if the concentrations are high enough (Helle *et al.*, 2003). The mechanism behind osmotic stress is the buildup of an osmotic pressure gradient over the cell membrane. If the ions cannot cross the cell membrane to equalize the osmotic pressure, the cell will shrink due to an outflow of water. However, at moderate concentrations one could consider the possibility that inorganic ions enhance the ethanol production in a similar way as aliphatic acids do at moderate concentrations (see above). The mechanism proposed is an increase in ATP demand due to the cell's increased effort to import solutes over the membrane. The extra ATP is acquired by an increased ethanol production at the expense of biomass formation. For ions that are able to cross the cell membrane, the same mechanism can be applied with the exception that the ATP demand of the cell comes from the process of active transport of the ions out of the cell (Helle *et al.*, 2003).

S. cerevisiae is relatively salt tolerant compared to other yeasts, such as *Schizosaccharomyces pombe* and *Pichia stipitis*, but less tolerant than several *Candida* species (reviewed by Wadskog and Adler, 2003). *S. cerevisiae* can grow on glucose in a 1.5 M solution of NaCl. However, a more important factor than the absolute concentration of sodium is the intracellular ratio of Na^+/K^+, which should be kept low. K^+ might be preferred by the yeast cell as intracellular cation because it is more compatible with cellular functions than Na^+. Na^+ can inhibit certain enzymes at lower concentrations than K^+. However, at growth-limiting potassium levels Na^+ or H^+ can be used by the cell as a substitute for K^+, but H^+ appears to be more inhibitory than Na^+. The effect of different intracellular anions on enzymes is not well known. However, the type of counter ion to K^+ or Na^+ has been shown to influence the cation tolerance in some yeasts (reviewed by Wadskog and Adler,

2003). Maiorella *et al.* (1984) investigated the inhibition of *S. cerevisiae* by different salts and found that inhibition decreased in the following order: $CaCl_2$, $(NH_4)_2SO_4$ > NaCl, NH_4Cl > KH_2PO_4 > $MgCl_2$ > $MgSO_4$ > KCl. Ranatunga *et al.* (2000) compared the toxicity of the anions sulfate and acetate in cultures of *Zymomonas mobilis* and concluded that the acetate ions were more inhibitory than the sulfate ions. Vriesekoop *et al.* (2002) examined the effects of Na^+ and Cl^- on *Z. mobilis*. They showed that both Na^+ and Cl^- inhibited growth, glucose consumption, and ethanol production, but the Na^+ ion displayed more severe inhibitory effect. However, Cl^- caused filamentous growth, which did not occur with SO_4^{2-}.

In the present study (Paper 2), the addition of extra $CaCl_2$ or NH_4Cl to the hydrolysate after alkali detoxification clearly resulted in a decrease in the ethanol productivity of *S. cerevisiae*. Nevertheless, our study suggests that alkali detoxification is much more important to achieve good fermentability than keeping the salt level low.

Synergistic inhibition

It is not unlikely that a mixture of different substances can have an increased inhibitory effect on a microorganism compared to the added effect of the seperate substances that are present in the mixture. Interaction between acetic acid and furfural has been shown to inhibit the biomass yield, the ethanol yield, and the growth rate of yeast synergistically (Palmqvist *et al.*, 1999). Zaldivar *et al.* (1999) and Zaldivar and Ingram (1999) showed that furfural in binary combinations with other aldehydes or acetic acid gave synergistic inhibition effects on *E. coli*. Klinke *et al.* (2003) tested nine different phenolic compounds for synergistic inhibition effects with substances in a wheat straw hydrolysate in which no furan aldehydes were present. The experiment resulted in inhibition of the yeast for all nine compounds tested and synergistic inhibition effects for two of the phenolic compounds, namely acetovanillone and syringaldehyde.

Solutions to inhibitor problems

To avoid problems caused by inhibitors during bioethanol production, there are several alternative measures that can be taken. One possibility is to try to reduce the formation of inhibitors during the hydrolysis. The concentrations of inhibitors and sugars in hydrolysates depend on the hydrolysis condition. A hydrolysate with a high yield of sugars will not necessarily give a higher ethanol yield than a hydrolysate with lower sugar content because they can be more or less inhibitory to the fermenting microorganism. However, high sugar yield without inhibitor formation can be hard to achieve, especially if dilute-acid hydrolysis is used. It is hardly reasonable to accept a poor sugar yield and

consequently a poor overall ethanol yield only to avoid inhibition problems. To reduce the negative effects of the inhibitors, one could consider special design of the fermentation process, selection of highly resistant microorganisms, strain adaptation, genetic engineering for strain improvement, or detoxification of the lignocellulose hydrolysate prior to fermentation.

The design of the fermentation process

To overcome the problem with inhibitory hydrolysates, the fermentation process can be designed in different ways. In batch-mode fermentations, all medium nutrients are available from the starting point when the yeast is inoculated. The fermention is continued until the sugar is depleted. The advantages of the batch-mode operations are the simplicity of the process, the low risk of contamination, and the possibility to utilize the sugars efficiently. The disadvantage is that the ethanol production is discontinuous because of the fermentation lag phase and the interruption when fresh medium should be added to the fermentation vessel.

Another possibility is to perform a fed-batch fermentation. The fed-batch mode is very similar to the batch mode but with the exception that fresh medium is fed into the fermentation vessel continuously. An advantage with this technique is that the inhibitors will be added in small doses continuously. The disadvantages include that the maximum working volume of the fermentation vessel is not utilized all the time and that the interruption due to refilling still remains.

Dilution of the hydrolysate will lead to improved fermentability but it will also lead to a larger hydrolysate volume that has to be fermented. Ethanol production from diluted hydrolysates with low sugar content is also associated with a high operating cost due to a more expensive distillation process (Lin and Tanaka, 2006).

A third fermentation mode is continuous fermentation. In a continuous fermentation, fresh medium is constantly fed to the fermentation vessel and there is also a constant out-take of product. When the feeding rate and product out-take rate are the same, the fermentation volume is kept constant. The advantage of continuous fermentation is that the ethanol is produced continuously without interruptions. Disadvantages associated with continuous fermentation are the risks of washout of the biomass at high dilution rates and of contamination due to the constant inflow of medium. Another disadvantage is that the sugar utilization will decrease at high dilution rates. The dilution rate can be reduced to increase the sugar utilization, but that will lead to a reduction in productivity.

Continuous fermentation with cell retention may be a way to improve the process. One way to perform cell retention is cell recirculation and another is cell immobilization.

A larger inoculum gives better performance in inhibitory hydrolysates. This is possible if the yeast can be recirculated and reused at a reasonable cost. However, if the used fermentation broth contains a lot of solids the separation of the yeast could become a tedious task. This is the case in SSF processes and as a consequence the use of fresh inocula is considered instead of recycling the microorganism (Wingren *et al.*, 2005). Irrespective of whether the yeast is bought from a yeast producer or if it is precultured at the ethanol plant, the use of a small inoculum size could be important for the entire process economy. The cost of the yeast in an SSF process has been estimated to be about 10% of the total ethanol production cost (Wingren *et al.*, 2003). Wingren *et al.* (2005) evaluated the effect of a reduction of the inoculum size from 5 g/L (D.W.) (dry weight) to 2 g/L (D.W.) for *S. cerevisiae* in an SSF process. Assuming that the inoculum is cultured on sugars from the hydrolysate, a reduction in inoculum size from 5 g/L (D.W.) to 2 g/L (D.W.) would be expected to lead to an increase in ethanol yield of about 7% at the same production cost (Wingren *et al.*, 2005). Sometimes an increase in the inoculum size is not sufficient to obtain reasonable fermentability. Cantarella *et al.* (2004) were not able ferment an undetoxified poplar hydrolysate with *S. cerevisiae* despite using an inoculum size as high as 10 g/L (D.W.). However, after $Ca(OH)_2$ detoxification the hydrolysate was readily fermented.

Selection of microorganism and strain

The choice of microorganism and strain is very important not only for high sugar utilization, ethanol tolerance, and ethanol producing properties, but also for the robustness and the ability to withstand inhibitors. Martín and Jönsson (2003) compared the resistance of 11 laboratory and industrial *S. cerevisiae* strains and two *Zygosaccharomyces* strains to an inhibitor cocktail consisting of lignocellulose-derived inhibitors. The resistance to the inhibitor cocktail proved to be very strain dependent. The study also showed that some strains can be highly resistant to certain inhibitors and more sensitive to other inhibitors.

The advantage of most *S. cerevisiae* strains is that they give high ethanol yields, exhibit relatively high ethanol tolerance, and have GRAS (generally regarded as safe) status. A disadvantage is that they cannot utilize pentose sugars (Lin and Tanaka, 2006). There are a lot of different microorganisms that could be used for ethanol production. *Z. mobilis* is a Gram-negative bacterium that may give higher ethanol yield than *S. cerevisiae*. It is relatively ethanol tolerant and it is a GRAS organism. However, it can only utilize glucose,

sucrose and fructose. Another drawback is that it is considered to be less robust than *S. cerevisiae* (Lin and Tanaka, 2006). Another bacterium that has been considered for ethanol production is *E. coli*. *E. coli* can be genetically engineered to produce more ethanol (see below). It can utilize both hexose and pentose sugars and it has been used for a long time in the industry for production of recombinant proteins. The disadvantages are that it lacks GRAS status, it is less robust and less ethanol tolerant than *S. cerevisiae*, and it requires a near neutral pH to grow (Lin and Tanaka, 2006). In conclusion, the choice of microorganism and strain for ethanol production from lignocellulose is highly dependent on the hydrolysate composition and the fermentation design.

The selection of microorganism may be influenced by its ability to ferment pentose sugars. The fraction of pentose sugars is generally low in softwood hydrolysates, but if hardwood or agricultural residues are considered, the ability to ferment pentoses becomes more important. There are several naturally occurring microorganisms that can utilize pentoses. *E. coli* is a natural pentose fermenter, but it gives low ethanol yields. This problem has been addressed by genetic engineering to increase the ethanol production (reviewed by Aristidou and Penttilä, 2000). *S. cerevisiae* and *Z. mobilis* are excellent ethanol producers, but they are unable to metabolize pentoses. Recombinant strains that are able to utilize pentose sugars have been developed for both *Z. mobilis* and *S. cerevisiae* (reviewed by Aristidou and Penttilä, 2000; Hahn-Hägerdal *et al.*, 2001). However, the genetically engineered pentose-fermenting *S. cerevisiae* strains have not yet been especially successful, which is due to problems like low pentose fermentation rates, low ethanol yields, and redox imbalances (Sonderegger *et al.*, 2004).

Strain adaptation
Increased resistance of *S. cerevisiae* by adaptation to separate inhibitors or lignocellulose hydrolysates has been concluded in several studies (Johnson and Harris, 1948; Banerjee *et al.*, 1981b; Azhar *et al.*, 1982). Lindén *et al.* (1992) isolated an *S. cerevisiae* strain (ATCC 96581) from a spent sulfite liquor fermentation plant. The strain had been recirculated and used for several years and had been adapted to the inhibitory compounds of the spent sulfite liquor. The strain has been used in several comparisons of the resistance of different strains and has performed favorable (Palmqvist *et al.*, 1999; Martín and Jönsson, 2003; Brandberg *et al.*, 2004). However, the strain has also been shown to be a less effective ethanol producer than ordinary baker's yeast when cultivated in the absence of acetic acid (Lindén *et al.*, 1992). A problem with adapted strains could be to maintain stability.

Genetic engineering for improved resistance

Another approach is to increase the resistance of the organism by genetic engineering. There are two general approaches to alter a microorganism by genetic engineering; either by homologous expression (overexpression of a gene that is already present in the genome of the organism) or heterologous expression (expression of a gene from another species). Heterologous expression of a laccase-encoding gene from the white-rot fungus *Trametes versicolor* in *S. cerevisiae* resulted in improved resistance to phenolic inhibitors (Larsson *et al.*, 2001a). In an attempt to try to improve the resistance of *S. cerevisiae* by homologous expression, a gene encoding the enzyme phenyl acrylic acid decarboxylase was overexpressed (Larsson *et al.*, 2001b). The overexpression of the gene resulted in a yeast that was more resistant to some aromatic acids.

Selection of highly resistant strains, strain adaptation and genetic engineering can be useful methods to overcome problems with moderately inhibitory hydrolysates. If strongly inhibitory hydrolysates are considered, a more powerful approach may be required, such as detoxification prior to fermentation.

Detoxification

Detoxification prior to fermentation can be performed in several different ways. The use of ion exchange for removing inhibitory substances has been reported to be a successful method for increasing the fermentability of various lignocellulose hydrolysates (Maddox and Murray, 1983; Buchert *et al.*, 1990; Rivard *et al.*, 1996; Larsson *et al.*, 1999b; Nilvebrant *et al.*, 2001). Sárvári Horváth *et al.* (2004) investigated the capability of six different ion-exchange resins to improve the fermentabilty of a dilute-acid spruce hydrolysate. They concluded that the use of a strong anion-exchange resin with a styrene-based matrix gave the best improvement in fermentability.

Treatments with resins without functional groups can also result in improved fermentability (Nilvebrant *et al.*, 2001). Björklund *et al.* (2002) showed that it is possible to use the lignin residue that is formed as a by-product during hydrolysis for removal of inhibitors in lignocellulose hydrolysates.

Treatment of lignocellulose hydrolysates with the enzyme laccase has been shown to increase the fermentability due to removal of phenolic compounds. This has been shown for hydrolysates made from hardwood, softwood, and agricultural residues (Jönsson *et al.*, 1998; Larsson *et al.*, 1999b; Martín *et al.*, 2002).

Other detoxification methods are detoxification with activated charcoal (Miyafuji *et al.*, 2003a; Mussatto *et al.*, 2004), evaporation (Palmqvist *et al.*, 1996; Larsson *et al.*, 1999b), wood ash treatment (Miyafuji *et al.*, 2003b), sulfite treatment (Larsson *et al.*, 1999b), alkali treatment (Leonard and Hajny, 1945), and treatment with the fungus *Trichoderma reseei* (Palmqvist *et al.*, 1997). Larsson *et al.* (1999b) performed a comparison of 12 different detoxification methods for treatment of a dilute-acid spruce hydrolysate prior to fermentation with *S. cerevisiae*. Two of the methods, $Ca(OH)_2$ treatment at pH 10 and anion exchange at pH 10, were superior to the other methods with regard to improvement of the ethanol productivity. All detoxification methods have advantages and drawbacks, but if the cost of the detoxification is considered, $Ca(OH)_2$ treatment (overliming) seems to be the most economical choice in many cases (Ranatunga *et al.*, 2000).

Alkali detoxification

Detoxification of lignocellulose hydrolysates by addition of $Ca(OH)_2$ is considered to be one of the best detoxification methods (Larsson *et al.*, 1999b; Martinez *et al.*, 2001). Already in 1945 Leonard and Hajny showed that the fermentability of a wood hydrolysate was improved by raising the pH of the hydrolysate to 9-10 with $Ca(OH)_2$ and then adjusting the pH with acid to obtain conditions suitable for fermentation. The mechanism of $Ca(OH)_2$ treatment is, however, after over 60 years still not completely elucidated. Van Zyl *et al.* (1988) compared $Ca(OH)_2$ treatment with KOH treatment at pH 10 and achieved better result with the $Ca(OH)_2$ treatment. The experiment was performed using a bagasse hemicellulose hydrolysate and *Pichia stipitis* was used as the biocatalyst. They suggested that the detoxification effect was due to precipitation of toxic substances. Persson *et al.* (2002) compared the use of $Ca(OH)_2$, KOH, NaOH and NH_3 at pH 10 for detoxification of a spruce hydrolysate with *S. cerevisiae* as the fermenting microorganism. The treatments with $Ca(OH)_2$ and NH_3 rendered the best fermentability. Precipitated material formed during the alkali detoxification treatments was collected by filtration, extracted by supercritical fluid extraction and analysed by HPLC. The amount of ten different inhibitors, including furan aldehydes and phenolic compounds, that were present in the precipitate was less than 1% of the concentrations initially found in the hydrolysate. This suggested that the detoxification effect was due to chemical conversion rather than removal of precipitated inhibitors. This result is in agreement with the present study (Paper 3) in which only a small amount of precipitate was formed during detoxification of a spruce hydrolysate with NH_4OH and no precipitate was formed when detoxification was performed with NaOH. Regardless of whether $Ca(OH)_2$, NH_4OH or NaOH was used, excellent fermentability was possible to achieve.

A problem associated with alkali detoxification is that not only inhibitors may be affected by the treatment, but also the sugars, which will lead to reduced ethanol yield (Nilvebrant *et al.*, 2003; Martinez *et al.*, 2001; Millati *et al.*, 2002; Nigam, 2001). Nilvebrant *et al.* (2003) studied the effect of treatment time, temperature, and pH during alkali treatment of a spruce hydrolysate. The degradation of sugars was extensive when harsh conditions (*i.e.* long treatment time, high temperature and high pH) were applied. Total degradation of all sugars present in the hydrolysate occurred at 80°C and pH 12, irrespective of the treatment time. Xylose was slightly more easily degraded than the other sugars (glucose, mannose, galactose and arabinose) during the alkali treatments. Martinez *et al.* (2001) treated a bagasse hydrolysate with various amounts of Ca(OH)$_2$ at 60°C. The ethanol production of *E. coli* LY01 increased when higher pH had been used during the treatment. However, treatments at pH higher than 9 resulted in decreased ethanol production due to an extensive degradation of sugars.

The goal of the alkali detoxification is to achieve maximum fermentability and minimum sugar degradation at a low cost. A convenient metric for evaluation of alkaline detoxification conditions is the balanced ethanol yield (Ψ_{EtOH}) (Papers 1, 2 and 3). The balanced ethanol yield is given as the amount of ethanol produced divided by the total amount of fermentable sugars present in the hydrolysate prior to the detoxification. The metric is given in percent of a reference fermentation of a sugar solution without inhibitors. Therefore, the balanced ethanol yield takes both the loss of sugars and the production of ethanol into account.

The effect of alkali detoxification depends on pH, temperature and treatment time. In the current study (Papers 1 and 3), the pH and the temperature were varied while the treatment time was kept constant.

Present study

Optimization of Ca(OH)$_2$ detoxification (Paper 1)

The optimization of detoxification with Ca(OH)$_2$ was made by varying the pH between 8 and 12 and the temperature between 5 and 80°C. Under harsh conditions (*i.e.* high pH and high temperature), there was a substantial conversion of furan aldehydes and sugars. The sugars were, however, converted to a lesser extent than the furan aldehydes, especially under mild conditions. This suggests that it is possible to find treatment conditions that combine efficient removal of inhibitors and high sugar yields. The concentrations of acetic acid and, especially, formic acid increased under harsh conditions. The levels of levulinic acid were relatively unaffected under most treatment conditions.

Formic and acetic acid are degradation products of monosaccharides under alkaline conditions and the increased levels can therefore be explained by the sugar degradation (De Bruijn *et al.*, 1986). The concentration of phenolic compounds decreased under most treatment conditions. However, there was a considerable increase in the concentration of phenolic compounds when harsh conditions were applied. The increased levels of phenolic compounds can possibly be explained by degradation of dissolved lignin fragments present in the hydrolysate.

All tested treatment conditions led to increased ethanol productivity compared to the untreated hydrolysate sample. However, all treatment conditions that led to a high productivity were not optimal because some of them resulted in substantial sugar degradation. A better evaluation of the treatment conditions could be obtained by comparison of the balanced ethanol yields. The balanced ethanol yields showed that low pH combined with high temperature and high pH combined with moderate temperatures gave the best result (see Paper 1, Fig. 2). The highest balanced ethanol yield was obtained for the sample treated at pH 11 and 30°C and corresponded to 120% of the reference fermentation. The untreated sample reached a balanced ethanol yield of 5%.

It might seem remarkable that the balanced ethanol yield could be higher than that of a sugar solution without inhibitors. However, one should keep in mind that a lignocellulose hydrolysate is very complex and might contain substances that can affect the yeast in a positive way. For example, it contains aliphatic acids, such as acetic, formic and levulinic acid, which can improve the ethanol yield when they are present in moderate concentrations. In conclusion, it is possible to find treatment conditions that combine extensive removal of inhibitors with low sugar degradation and therefore result in excellent fermentability. However, a disadvantage of using $Ca(OH)_2$ for alkali detoxification is the formation of a precipitate consisting of $CaSO_4$ (gypsum).

Detoxification with NH₄OH (Paper 2)

Detoxification with NH_4OH gave a favorable result in a comparison of treatments with $CaOH_2$, NaOH, $Mg(OH)_2$, and $Ba(OH)_2$. The reason for this was the alkaline treatment rather than the supplementation of extra nutrients (in the form of a nitrogen source). The possibility that inorganic salts would have a positive effect on the fermentability was dismissed after experiments in which NH_4Cl or $CaCl_2$ was added to detoxified and undetoxified hydrolysates. The addition of extra salts instead resulted in poorer fermentability, possibly due to salt stress of the yeast. Measurements of the conductivity suggested a connection between poor fermentability and high conductivity.

Martinez *et al.* (2001) suggested that a decrease in the concentration of soluble salts due to the precipitation of $CaSO_4$ would be one of the benefits of using $Ca(OH)_2$ detoxification. In our study, the hydrolysate samples that were treated with $Ca(OH)_2$ displayed a lower conductivity than the NH_4OH-treated samples. The NH_4OH-treated samples displayed a better fermentability than the $Ca(OH)_2$-treated samples. However, within the same treatment series (*i.e.* the $Ca(OH)_2$-treated series or the NH_4OH-treated series) the fermentability followed the conductivity (see Paper 2, Fig. 4).

Optimization of detoxification with NH₄OH and NaOH (Paper 3)

NH₄OH treatment

The optimization of detoxification with NH_4OH was made by varying the pH between 8 and 10 and the temperature between 5 and 80°C. The conversion of furan aldehydes was extensive, especially under harsh conditions. The sugars were only degraded to a slight extent under the conditions studied, which also was reflected by the relatively small changes in the concentrations of acetic and formic acid. The concentration of phenolic compounds generally decreased slightly under most treatment conditions, but high temperatures instead resulted in an increase.

A brownish precipitate was formed during the treatments. The amount of precipitate increased when the conditions were harsher.

All treatment conditions tested led to a higher balanced ethanol yield than for the untreated sample. The most favorable conditions were a combination of intermediate pH and intermediate-high temperature (see Paper 3, Fig. 2A). The highest balanced ethanol yield was achieved at pH 9 and 55°C and reached 120% of the reference fermentation. The untreated sample reached a balanced ethanol yield of 7%.

In conclusion, the advantages of using NH_4OH for alkali detoxification are low sugar degradation, extensive removal of inhibitors, good buffering effect, and that the treatment can be performed under relatively mild conditions and still result in excellent fermentability. The disadvantages are the formation of a brownish precipitate, although the amounts of precipitate are lower than the amounts obtained after $Ca(OH)_2$ treatment, and that the dose of acid needed for the adjustment to a suitable fermentation pH after the detoxification is large due to the buffering capacity.

NaOH treatment

The optimization of detoxification with NaOH was made by varying the pH between 9 and 12 and the temperature between 30 and 80°C. The concentrations of furan aldehydes

and sugars decreased with increasing pH and temperature. The phenolic compounds increased under harsh conditions but decreased slightly under mild conditions. The concentrations of acetic acid and formic acid followed the sugar degradation, *i.e.* extensive sugar degradation gave increased concentrations of the acids. No significant amount of precipitate was formed under any of the treatment conditions tested.

All performed treatments resulted in higher balanced ethanol yield than that of the untreated sample. The most favorable conditions were moderate pH combined with high temperature or high pH combined with moderate temperature (see Paper 3, Fig. 2B). The best result was obtained at pH 9 and 80°C, which resulted in a balanced ethanol yield of 111%. The untreated sample reached a balanced ethanol yield of 6%.

An experiment in which different mixtures of NaOH and NH$_4$OH were used was also performed (Paper 3). The experiment showed that one advantage of using NH$_4$OH for detoxification is its buffering effect, which keeps the pH high and stable during the treatment. The pH of a sample treated with only NaOH decreased considerable, which in turn resulted in poorer fermentability than if the pH was kept high during the treatment. This implies that continuous pH adjustment during alkali treatments is important when NaOH is used.

In conclusion, the advantages of using NaOH for alkali detoxification are low sugar degradation, extensive removal of inhibitors, and no formation of precipitate. A disadvantage is the drop in pH under the treatment, if automatic pH adjustment is not used. Poor buffering capacity is on the other hand an advantage when the pH should be lowered to a suitable level prior to the fermentation.

Industrial implementation of alkali detoxification

A drawback with Ca(OH)$_2$ detoxification is the formation of a CaSO$_4$ (gypsum) precipitate. However, it has been suggested that the gypsum can be sold for use in concrete and wallboard manufacturing (Martinez *et al.*, 2001). If long-term processes are considered, for example continuous fermentations, the use of Ca(OH)$_2$ may also result in problems with calcium oxalate precipitation. Calcium oxalate precipitation is a well known problem in the pulp and paper industry where it causes clogging of pipework and filters, a phenomenon known as "scaling" (Nilvebrant *et al.*, 2002). The present study has shown that there are alternative forms of alkali that can be used for detoxification and that they will result in an excellent fermentability and will give rise to less or no precipitate in comparison with traditional overliming.

Alkali detoxification adds an extra process step to the ethanol production, which will lead to an increased production cost. However, the results from the current study could be used to reduce the cost of the detoxification treatment. One way to reduce the cost would be to minimize the amount of alkali used during the treatment. The optimization experiments showed that it is possible to perform excellent detoxification at low pH if the temperature is high. To avoid heating costs, it would be an attractive option to perform the detoxification treatment directly after the dilute-acid hydrolysis when the hydrolysate is still hot. Another cost to consider is the amount of acid added for adjustment to a suitable fermentation pH. If NH_4OH is used, a larger amount of acid would be required to reach a suitable fermentation pH compared to when NaOH is used due to the different buffering capacity. The price of different types of alkali should also be taken into account as well as the possibility to recycle the alkali.

The biorefinery concept – a future outlook

A biorefinery can be defined as a facility that converts biomass to fuels, chemicals and power through integrated processes (Ragauskas *et al.*, 2006a). A way to improve the economy of the production of ethanol from lignocellulose as well as increasing the net energy gain would be to manufacture co-products and important chemicals during the process. In the petroleum industry about 5% of the total output from an ordinary refinery goes into chemical products, while the remaining 95% is used for energy and transportation fuels (Ragauskas *et al.*, 2006b). In the case of corn ethanol production, an important co-product is distiller's grain, which is used as cattle feed (Wheals *et al.*, 1999). With regard to cellulosic ethanol, a major residual product is lignin. The lignin as well as other solids left after hydrolysis can be burned for generating heat and electricity (Wyman, 2003).

There is a wide range of valuable chemicals and materials that can be produced from lignocellulose. Two interesting products that can be made from the hemicellulose and cellulose fraction are furfural and HMF. Furfural can be used as the starting material in the production of Nylon 6,6 and Nylon 6 (Kamm and Kamm, 2004). There is also a wide range of valuable products that can be produced from the lignocellulose-derived sugars by microbial conversion. Potential products include hydrogen, methane, propanol, acetone, butanol, butanediol, succinic acid, itaconic acid, acetic acid, levulinic acid, butyraldehyde, ascorbic acid, adipic acid, propylene glycol, acrylic acid, acetaldehyde, sorbitol, glycerol, and malic acid (Kamm and Kamm, 2004; Wyman, 2003). Another very interesting product that can be made from carbohydrates by microorganisms is lactic acid. Polymeric materials derived from lactic acid, for example polylactide, which is a very versatile

thermoplastic, can replace some of the plastics made from petroleum. Polylactide is especially interesting in the food packaging industry because it is fully compostable and biodegradable (Kamm and Kamm, 2004). Other plastics, such as polyvinylacetate and polyethylene, can be produced with ethanol as the starting material. The ethanol must, however, first be converted into ethene by chemical methods (Kamm and Kamm, 2004). An advantage with chemicals produced by microbial catalysis rather than by using petrochemical methods is that the products from the microorganisms are typically stereo- and regiochemically pure. There is no need for expensive chiral catalysts and complex syntheses, which is the case in the production of many petrochemicals (Ragauskas *et al.*, 2006b).

Even if the major part of the biomass can be utilized in an efficient way in a biorefinery, there will nevertheless probably be some waste products that are uneconomical to convert further to valuable chemicals or materials. As mentioned above, residual materials, such as lignin, can be burned to generate power, but another possibility would be thermochemical conversion of the residues to syngas. The produced syngas can then be used for production of methanol, ammonia and Fisher-Tropsch hydrocarbons (Ragauskas *et al.*, 2006b)

Studies have shown that a cellulosic refinery plant that combines the production of fuels, chemicals and power can generate these products with a lower cost than if just one of them is produced (Wyman, 2003). Another possibility is to transform the pulp mills of today into biorefineries (Ragauskas *et al.*, 2006a).

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Ethanol from lignocellulose

Alkali detoxification of dilute-acid spruce hydrolysates

Detoxification of dilute-acid lignocellulose hydrolysates by treatment with $Ca(OH)_2$ (overliming) efficiently improves the production of fuel ethanol, but is associated with drawbacks like sugar degradation and $CaSO_4$ precipitation. In factorial designed experiments, in which pH and temperature were varied, dilute-acid spruce hydrolysates were treated with $Ca(OH)_2$, NH_4OH or $NaOH$. The concentrations of sugars and inhibitory compounds were measured before and after the treatments. The fermentability was examined using the yeast *Saccharomyces cerevisiae* and compared with reference fermentations of synthetic medium without inhibitors. The treatment conditions were evaluated by comparing the balanced ethanol yield, which takes both the degradation of sugars and the ethanol production into account. Treatment conditions resulting in excellent fermentability and minimal sugar degradation were possible to find regardless of whether $Ca(OH)_2$, NH_4OH or $NaOH$ was used. Balanced ethanol yields higher than those of the reference fermentations were achieved for hydrolysates treated with all three types of alkali. As expected, treatment with $Ca(OH)_2$ gave rise to precipitated $CaSO_4$. The NH_4OH treatments gave rise to a brownish precipitate but the amounts of precipitate formed were relatively small. No precipitate was observed in treatments with $NaOH$. The possibility that the ammonium ions from the NH_4OH treatments gave a positive effect as an extra source of nitrogen during the fermentations was excluded after experiments in which NH_4Cl was added to the medium. The findings presented can be used to improve the effectiveness of alkali detoxification of lignocellulose hydrolysates and to minimize problems with sugar degradation and formation of precipitates.