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MINI-REVIEW

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Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass

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Abstract An overview of the different inhibitors formed by pre-treatment of lignocellulosic materials and their inhibition of ethanol production in yeast and bacteria is given. Different high temperature physical pre-treatment methods are available to render the carbohydrates in lignocellulose accessible for ethanol fermentation. The resulting hydrolyzsates contain substances inhibitory to fermentation—depending on both the raw material (biomass) and the pre-treatment applied. An overview of the inhibitory effect on ethanol production by yeast and bacteria is presented. Apart from furans formed by sugar degradation, phenol monomers from lignin degradation are important co-factors in hydrolysate inhibition, and inhibitory effects of these aromatic compounds on different ethanol producing microorganisms is reviewed. The furans and phenols generally inhibited growth and ethanol production rate (Q_{EtOH}) but not the ethanol yields (Y_{EtOH}) in *Saccharomyces cerevisiae*. Within the same phenol functional group (aldehyde, ketone, and acid) the inhibition of volumetric ethanol productivity was found to depend on the amount of methoxyl substituents and hence hydrophobicity (log P). Many pentose-utilizing strains *Escherichia coli*, *Pichia stipititis*, and *Zymomonas mobilis* produce ethanol in concentrated hemicellulose liquors but detoxification by overliming is needed. *Thermoanaerobacter mathranii* A3M3 can grow on pentoses and produce ethanol in hydrolysate without any need for detoxification.

Introduction

Fuel ethanol from biomass has been the subject of intensive research in the US, where it is widely used in gasoline blends up to 10%. European legislation has now stipulated increased use of biomass for electricity and fuel, and a new European directive has mandated the use of biofuels in the transportation sector corresponding to 2% in 2005 and 5.75% by 2010. Growing crops for bioethanol production will be expensive and have only limited energy benefits. Lignocellulosic plant residues containing up to 70% carbohydrates (as cellulose and hemicellulose) are prominent substrates for cheap ethanol production, however, due to the close association to lignin in the plant cell wall, pre-treatment is necessary to make the carbohydrates available for enzymatic hydrolysis and fermentation. Aqueous pre-treatment at elevated temperature result in an insoluble cellulose-rich fraction and a soluble fraction, containing hemicellulose sugars and degradation products (Klinke et al. 2002). The degradation products formed (e.g., phenols, furans and carboxylic acids) can be considered potential fermentation inhibitors (Fig. 1). Due to their inhibitory effects on productivity and end-product formation, the inhibitors can be a limiting factor in the feasibility of biotechnological conversions of lignocellulosics to ethanol.

In order to obtain an economically feasible conversion process, reduction in the content and/or inhibitory effect of the degradation products are necessary. Recirculation of the stillage water in the process will minimize water consumption, but detoxicification prior to recirculation is required (Larsson et al. 1997; Wilkie et al. 2000) unless the inhibitory compounds are removed (e.g., by anaerobic purification (Torry-Smith et al. 2003)). Also detoxification of pre-treated hydrolysates have been shown to improve their fermentability, however, the cost is often higher than the benefits achieved (Palmqvist and Hahn-Hägerdal 2000b; von Sivers and Zacchi 1996; von Sivers et al. 1994). Furthermore, separating the liquid hemicellulose fraction containing the inhibiting compounds from the cellulose fraction prior to enzymatic hydrolysis and

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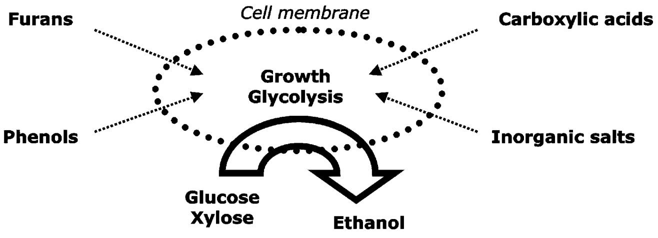


Fig. 1 Phenols, furans, carboxylic acids, and salts, are potential fermentation inhibitors that have a negative effect on cell membrane function, growth, and glycolysis in ethanol-producing yeast and bacteria

fermentation can reduce the content of inhibitory compounds. In this review, we present an overview of the different potential inhibitors formed by pre-treatment of lignocellulosics and their inhibition of ethanol production.

Degradation products from pre-treatment of lignocellulose

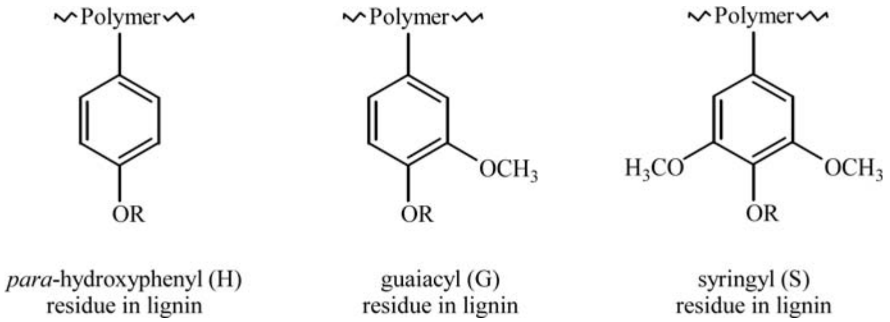
Biomass composition

Plants consisting of lignocellulose can be divided in three types according to plant taxonomy: softwood (gymnosperms), hardwood (woody angiosperms) and annual plants, e.g., crops (herbaceous angiosperms). The main component in lignocellulose is holocellulose approx. 60–70%, and is composed of the polysaccharides cellulose and hemicellulose. Cellulose is a high molecular weight glucose polymer, while hemicellulose is composed of various sugars, such as xylose, arabinose, mannose, galactose, and glucose, dependent on the plant material (Bobleter 1994; Fan et al. 1982). Lignin is a co-polycondensate of dehydrogenated products obtained from the lignin monomers (*p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol). The terms *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively, are used to denote the three types of aromatic rings in monomer residues (Fig. 2), and the ratio of H/G/S units in the lignin is highly dependent on plant taxonomy (Table 1). Softwood lignins are mainly formed from coniferyl alcohol (G), together with small proportions of *p*-coumaryl alcohol. Hardwood lignin generally results from coniferyl (G) and sinapyl alcohols (S) in roughly equal amounts as well as small quantities of *p*-coumaryl alcohol (H). Apart from the monolignols (H, G, and S), herbaceous plants (e.g., grasses) also contain *p*-hydroxycinnamic acids (*p*-coumaric acid, ferulic acid, and sinapic acids) integrated into their lignin (Campbell and Sederoff 1996; Lawther et al. 1996b). Wood materials have similar cellulose content, but the lignin content in softwood is generally higher than in hardwoods.

In addition to holocellulose and lignin, plant materials are composed of extractive (soluble in water or organic solvent) and non-extractive non-cell wall materials (NCWM) (Fan et al. 1982). The non-extractives are mainly inorganic ash components such as silica and alkali salts, but also includes pectin, proteins, and starch. Herbaceous material, especially straw has high non-extractive NCWM with ash contents up to 10%. Ca, Mg, and K are the major inorganic constituents in wood (Saka 1997). In addition, Si, Cl, and Na are abundant in herbaceous materials (Fan et al. 1982). Wood materials have low ash contents (<1%) and contain variable amounts of extractives or secondary metabolites such as resins, terpenes, phenols, quinones, and tannins (Umezawa and Higuchi 1991). The extractives often have protective biological and anti-microbial activities and aid in the chemotaxonomic division of plant species by their specific biosynthetic pathways (Torssell 1997).

Pre-treatment

Different pre-treatment methods are available to render the carbohydrates in lignocellulose accessible for enzymatic hydrolysis and ethanol fermentation, including acid hydrolysis, steaming or steam explosion (STEX), ammonia freeze explosion (AFEX) and wet oxidation (WO). Acid or alkaline catalysts are often applied in pre-treatments at 121–200°C, and when no catalysts are added, autohydrolysis occurs by the release of carboxylic acids (primarily acetic acid) from the lignocellulose during pre-treatment. Hydrosulphuric acid (added as H_2SO_4 or SO_2) and NaOH are widely used catalysts, however also NH_3 (Holtzapple et al. 1991), Na_2CO_3 (Bjerre et al. 1996),



para-hydroxyphenyl (H) residue in lignin

guaiacyl (G) residue in lignin

syringyl (S) residue in lignin

Fig. 2 The three main phenol building blocks in lignin, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), differs in the methoxy-groups *ortho* to the phenol group. The R-function indicates that cross-linking via ester or ether bonds can occur at these positions. R=H represents the free phenol. At the polymer site, many possible types of bonding on the propanoic sidechain are possible

Table 1 Chemical composition of the three main types of lignocellulosic biomass. The H/G/S ratio is the relative lignin composition of 4-hydroxybenzyl (H), guaiacyl (G) and syringyl (S) units

	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Lignin H/G/S-ratio (%)
Softwood	41–50	11—33	19–30	2–18/82–98/trace
Hardwood	39–53	19—36	17–24	0/22–66/44–86
Herbaceous plants	24–50	12—38	6–29	5–26/27–54/23–67

and other catalysts have been applied (Bobleter 1994; Fan 1992). Comparison of the pre-treatments and the mechanisms of lignocellulose fractionation and degradation have been reviewed recently (Bobleter 1994; Galbe and Zacchi 2002; Garrote et al. 1999; McMillan 1994b; Sun and Cheng 2002). Lignocellulose degradation is a heterogeneous process with initial solution and hydrolysis reactions followed by high temperature chemical reactions of the soluble components. During pre-treatment, lignin and hemicellulose are solubilized and/or decomposed in the aqueous phase, while cellulose remain in the solid fraction. Aromatic compounds like furans and phenols are prone to undergo condensation reactions at room temperature (Burtscher et al. 1987) and at high temperatures (Klinke et al. 2002; Shevchenko et al. 2000), resulting in dark humic or tannin-like precipitates.

Formation of degradation products

The degradation products formed by pre-treatment of lignocellulose depend on both the biomass and the pre-treatment conditions such as temperature, time, pressure, pH, redox conditions, and addition of catalysts. In high temperature pre-treatments, the formation of fermentable carbohydrates and degradation products is dependent on a combined severity factor, including reaction temperature, time, and pH (Chum et al. 1990; Tengborg et al. 1998).

Sugar degradation products—i.e., furfural (from pentoses) and hydroxymethyl furfural (HMF) (from hexoses) —are formed in high concentrations during severe acidic pre-treatment conditions (Dunlop 1948; Taherzadeh et al. 1997a). Acetic acid is ubiquitous in hemicellulose hydrolysates from all lignocellulosics, where the hemicellulose and to some extent lignin is acetylated (Fengel and Wegener 1989; Sarkanen and Ludwig 1971; Torssell 1997). Hydroxycarboxylic acids such as glycolic acid and lactic acid are common degradation products from alkaline carbohydrate degradation (Alén et al. 1990; Sjöström 1991). Formic acid is a product from sugar and lignin degradation (Klinke et al. 2002), while levulinic acid is formed by 5-HMF degradation (Palmqvist and Hahn-Hägerdal 2000b). Other carboxylic acids can also be found in hemicellulose hydrolysates, including aromatic acids as reviewed by McMillan (1994a).

Aromatic degradation products from sugar degradation are predominantly furans: 2-furfural, 5-HMF, 2-furoic acid and to a minor extent phenols formed by solubilization and hydrolytic or oxidative cleavage of lignin. The aromatic compounds present in hydrolysates are dependent on the type of pre-treatment and the H/G/S ratio of the lignin contained in the biomass material. An overview of the phenols identified from pre-treatment of lignocellulosic materials is presented in Table 2. The most versatile phenols found were 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic acid. Phenol monomers have been quantified in lignocellulosic hydrolysates from pine (Clark and Mackie 1984; Tran and Chambers 1986), oak (Buchert et al. 1990; Tran and Chambers 1985), willow (Jönsson et al. 1998), spruce (Larsson et al. 1999a), wheat straw (Klinke et al. 2002), bagasse (Martin and Jonsson 2003), poplar (Ando et al. 1986), corn stover and switch grass (Fenske et al. 1998). An overview of quantified phenols found in hemicellulose fractions of pre-treated lignocellulose is provided in Table 3. The phenols were divided into three groups by their degree of methoxylation (H, G, S) and their functionality (aldehydes, ketones, acids, other). Softwood materials almost exclusively produce G (guaiacyl) phenols, while hardwoods and herbaceous materials produce H, G and S phenols consistent to the biomass composition (Table 1). The H (hydroxy) phenol concentration in hardwood acid hydrolysates was high for willow due to benzenediols (Jönsson et al. 1998) and for poplar due to 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid (Ando et al. 1986). These compounds are thought to be extractive components rather than lignin components (Baeza and Freer 1997; Jönsson et al. 1998). Aspen and willow belong to the same family *Salicaceae*, and aspen lignin is esterified with 4-hydroxybenzoic acid, which is the main phenol monomer produced by steam pre-treatment (Bardet and Robert 1985). Phenol oligomers (polyphenols) were formed by steaming of aspen (Bardet and Robert 1985) or acid hydrolyzed spruce (Larsson et al. 1999b) and alkaline wet oxidation of wheat straw (Klinke et al. 2002). Phenol monomer concentrations in hydrolysates were lower than expected from the amount of lignin removed from the solid fraction by wet oxidation of wheat straw, due to further oxidation to carboxylic acids (Klinke et al. 2002). Syringyl lignin units have been shown to be more susceptible to hydrothermal degradation than guaiacyl lignin units (Jönsson et al. 1998). Thus, relative to untreated birch more syringyl type phenols than guaiacyl type phenols were found in hemicellulose hydrolysates (Buchert et al. 1990).

The hydrolysis conditions during the pre-treatment are important for the functionality of the degradation products, e.g., the formation of phenol aldehydes has been shown to be favored at oxidative acidic conditions (Klinke et al. 2002). Phenylpropane derivatives are generally formed by acid hydrolysis of biomass. Dihydroconiferyl alcohol has

Table 2 Phenolic compounds identified in hemicellulose fractions from pre-treated lignocellulosics

Hydroxyl (H) compounds	MW	CAS-RN	Reference	Guaiacyl (G) compounds	MW	CAS-RN	Reference	Syringyl (S) compounds	MW	CAS-RN	Reference
Phenol	94	108-95-2	Clark and Mackie 1984; Klinke et al. 2002	Guaiacol	124	90-05-1	Buchert et al. 1990; Jönsson et al. 1998; Klinke et al. 2002	Syringol	154	91-10-1	Buchert et al. 1990; Klinke et al. 2002
4-Hydroxybenzaldehyde	122	123-08-0	Ando et al. 1986; Buchert et al. 1990; Jönsson et al. 1998; Klinke et al. 2002; Barquinero et al. 1980	Vanillin	152	121-33-5	Ando et al. 1986; Buchert et al. 1990; Clark and Mackie 1984; Jönsson et al. 1998; Klinke et al. 2002; Larsson et al. 1999b; Tran and Chambers 1985; Tran and Chambers 1986; Barquinero et al. 1980; Fenske et al. 1999	Syringaldehyde	182	134-96-3	Buchert et al. 1990; Larsson et al. 1999b; Tran and Chambers 1985; Barquinero et al. 1980; Fenske et al. 1999
4-Hydroxybenzylalcohol	124	623-05-2	NA	Vanillylalcohol	154	498-00-0	Tran and Chambers 1986	Syringylalcohol	184	NA	NA
4-Hydroxybenzoic acid	138	99-96-7	Ando et al. 1986; Jönsson et al. 1998; Klinke et al. 2002; Larsson et al. 1999b; Barquinero et al. 1980; Fenske et al. 1999	Vanillic acid	168	121-34-6	Ando et al. 1986; Klinke et al. 2002; Tran and Chambers 1985	Syringic acid	198	530-57-4	Ando et al. 1986; Buchert et al. 1990; Jönsson et al. 1998; Klinke et al. 2002; Tran and Chambers 1985; Barquinero et al. 1980
4-Hydroxyacetophenone	136	99-93-4	Buchert et al. 1990; Klinke et al. 2002	Acetovanillone	166	498-02-2	Klinke et al. 2002; Larsson et al. 1999b	Acetosyringone	196	2478-38-8	Klinke et al. 2002; Tran and Chambers 1985; Fenske et al. 1999
–	–	–	–	Coniferyl alcohol	180	458-35-5	Buchert et al. 1990	Sinapyl alcohol	210	537-33-7	Buchert et al. 1990; Tran and Chambers 1985
–	–	–	–	Dihydroconiferyl alcohol	182	NA	Buchert et al. 1990; Larsson et al. 1999b; Tran and Chambers 1985; Tran and Chambers 1986	Dihydrosinapyl alcohol	212	NA	Buchert et al. 1990; Tran and Chambers 1985
–	–	–	–	Coniferyl aldehyde	178	458-36-6	Buchert et al. 1990; Clark and Mackie 1984; Larsson et al. 1999b; Tran and Chambers 1985; Tran and Chambers 1986; Fenske et al. 1999	Sinapyl aldehyde	208	NA	Buchert et al. 1990; Tran and Chambers 1985

Table 2 (continued)

Hydroxyl (H) compounds	MW	CAS-RN	Reference	Guaiacyl (G) compounds	MW	CAS-RN	Reference	Syringyl (S) compounds	MW	CAS-RN	Reference
4-Hydroxycinnamic acid (*p*-coumaric acid)	164	7400-08-0	Ando et al. 1986; Klinke et al. 2002; Barquinero et al. 1980; Fenske et al. 1999	Ferulic acid	194	1135-24-6	Klinke et al. 2002	Sinapic acid	224	NA	Barquinero et al. 1980
Not parasubstituted	–	–	–	Homovanillic acid	182	306-08-1	Larsson et al. 1999b	–	–	–	–
3-Hydroxybenzoic acid	138	99-06-9	Ando et al. 1986	4-propylguaiacol	166	2785-87-7	Jönsson et al. 1998	–	–	–	–
2-Hydroxybenzoic acid (salicylic acid)	138	69-72-7	Jönsson et al. 1998	2-methoxy-4-(2-propenyl) phenol (eugenol)	164	97-53-0	NA	4-propenylsyringol	178	NA	Buchert et al. 1990
2,5-Dihydroxybenzoic acid (gentisic acid)	154	490-79-9	Jönsson et al. 1998	2-methoxy-4-(1-propenyl) phenol (isoeugenol)	164	97-54-1	Jönsson et al. 1998	–	–	–	–
3,4-Dihydroxybenzoic acid (protocatechuic acid)	154	99-50-3	Jönsson et al. 1998	Guaiacylglycolic acid	182	NA	Buchert et al. 1990	Syringylglycolic acid	212	NA	Buchert et al. 1990
Hydroquinone	110	123-31-9	Larsson et al. 1999b	Guaiacylglycerol	198	NA	Buchert et al. 1990	Syringylglycerol (er-thr)	228	NA	Buchert et al. 1990
2,6-Dimethoxy-hydroquinone	178	NA	Buchert et al. 1990	1-guaiacylethanol	152	NA	Buchert et al. 1990	1-syringylethanol	182	NA	Buchert et al. 1990
o-Cresol(2-methylphenol)	108	95-48-7	Jönsson et al. 1998	2-guaiacylethanol	152	NA	Buchert et al. 1990	–	–	–	–
Catechol(1,2-benzenediol)	110	120-80-9	Jönsson et al. 1998; Larsson et al. 1999b	G–CH_2COCH_2OH (β-oxyconiferylalcohol)	180	NA	Clark and Mackie 1984; Larsson et al. 1999b; Tran and Chambers 1986	S-CH_2COCH_2OH (β-oxysinapylalcohol)	210	NA	Tran and Chambers 1985; Fenske et al. 1999
Ethylcatechol	138	933-99-3	Larsson et al. 1999b	G–$CH_2CHOHCH_3$	166	NA	Clark and Mackie 1984	–	–	–	–
–	–	–	–	G–CH_2COCH_3	164	NA	Clark and Mackie 1984; Larsson et al. 1999b	–	–	–	–
–	–	–	–	G–$COCHOHCH_3$ (α-hydroxypropiovanillone)	180	NA	Clark and Mackie 1984; Larsson et al. 1999b; Fenske et al. 1999	–	–	–	–
–	–	–	–	G–$CHOHCOCH_3$ (1-guaiacylacetol)	180	NA	Buchert et al. 1990; Clark and Mackie 1984; Larsson et al. 1999b	S–$CHOHCOCH_3$ (1-syringyl acetol)	210	NA	Buchert et al. 1990; Fenske et al. 1999
–	–	–	–	G–$COCOCH_3$	178	NA	Clark and Mackie 1984; Larsson et al. 1999b	S–$COCOCH_3$	208	NA	Fenske et al. 1999
–	–	–	–	G–$COCH_2OH$	182	NA	Clark and Mackie 1984	–	–	–	–

been found in hemicellulose fractions from acid hydrolysis of pine (Sears et al. 1971; Tran and Chambers 1986), spruce (Larsson et al. 1999b), switch grass (Fenske et al. 1998), oak (Tran and Chambers 1985) and birch (Buchert et al. 1990). Hibbert's ketones are phenylpropane derivatives with one or two keto groups on the 1 or 2 position of the propanyl group (Baeza and Freer 1997). Hibbert's ketones have been observed in hydrolysates from acidic pre-treatment conditions (Buchert et al. 1990; Fenske et al. 1998; Larsson et al. 1999b; Tran and Chambers 1986). From soda pulping of wheat straw the main phenols *p*-coumaric acid and ferulic acid are produced by hydrolysis of esterified hemicellulose and lignin (Lawther and Sun 1996a). Alkaline wet oxidation of wheat straw also produces these cinnamic acid derivatives. However due to oxidative cleavage of the conjugated double bonds, 4-hydroxybenzoic acid and vanillic acid are formed (Klinke et al. 2002). Phenol dimers were formed in low concentrations during steaming of willow (Jönsson et al. 1998), but in steamed and enzyme hydrolyzed birch the phenol dimers accounted for 61% of the total phenols formed (Buchert et al. 1990).

Removal of degradation products

Detoxification of the hemicellulose fraction from pre-treated high feed stock concentrations is needed in order to achieve reasonable fermentation of the soluble sugars to ethanol. Removal of inhibitory components can be done by extraction (Clark and Mackie 1984), ion exchange (Buchert et al. 1990), active coal (Gong et al. 1993), overliming (Larsson et al. 1999b; Martinez et al. 2001) or laccase and peroxidase treatment (Jönsson et al. 1998; Larsson et al. 1999b). Other methods have also been reported (McMillan 1994a; Palmqvist and Hahn-Hägerdal 2000a). Detoxification methods result in removal of different types of fermentation inhibitory components: steam stripping or evaporation at low pH remove volatile inhibitors such acetic acid and furans (Buchert et al. 1990; Dierssen et al. 1956). Over-liming (addition of $Ca(OH)_2$ to pH 11) removes the volatile and non-volatile inhibitors such as furans and phenols (Larsson et al. 1999b). The effectiveness of different detoxification procedures has been compared in spruce hydrolysates where over-liming and enzyme treatment with laccase produced the best results (Larsson et al. 1999b). The positive effect of detoxification on fermentation was primarily ascribed to lowered furfural and phenol concentrations in the hydrolysates.

Fermentation inhibitors from hemicellulose hydrolysates

Microbial inhibition

Microorganisms differ in their ability to adapt and grow in the hydrolysates, and the fermentative performances of microorganisms in lignocellulosic hydrolysates also depend on raw material and pre-treatment (Olsson and Hahn-Hägerdal 1996). There are several measures of fermentability: growth, ethanol yield, ethanol productivity (rate), and specific ethanol productivity that should be taken into account when comparing data from literature (Hahn-Hägerdal et al. 1994). Ethanol yield can be reported as the ethanol produced relative to the initial fermentable sugar concentration (total ethanol yield) or relative to the consumed sugar concentration (ethanol yield). In testing microbial inhibition, the hydrolysate medium is supplemented with essential growth factors and sterilized prior to inhibition assays. Precautions must be taken when using autoclavation (121°C, 10–20 min) as sterilization method has been found to increase the inhibitory effect of hydrolysates compared to sterile filtration of wet oxidized wheat straw (Klinke et al. 2003) or low temperature sterilization of acid hydrolyzed oak (Lee et al. 1999). Concentrations of phenol acids, formic acid, glycolic acid and malic acid increased by 30–40% and of acetic acid by 75% during autoclavation of wheat straw hydrolysates (Klinke et al. 2003). Total inhibition of *Saccharomyces cerevisiae* occurred in autoclaved acid hydrolysates of birch (Barber et al. 2000) and mixed waste paper (Rivard et al. 1996).

Growth and ethanol production of *S. cerevisiae* was strongly inhibited by acid hemicellulose hydrolysates from spruce (Larsson et al. 1999b), bagasse (Martin et al. 2002b; Martin and Jonsson 2003), alder and aspen (Taherzadeh et al. 1997a). Hemicellulose hydrolysates from acid hydrolyzed bagasse inhibited growth and fermentation of *Escherichia coli* LY01 (Martinez et al. 2001) and *Candida* sp. (Gong et al. 1993). However, xylose and glucose fermenting adapted *Zymomonas mobilis* strains were able to produce ethanol in dilute acid hydrolysates from yellow poplar (McMillan et al. 1999).

The performance of *S. cerevisiae* in lignocellulosic hydrolysates was compared and correlated to the content of acetic acid, formic acid, furfural, 5-HMF and phenol monomers (Table 4). Poor fermentability of dilute acid wood hydrolysates by *S. cerevisiae* correlated to high concentrations of furfural, 5-hydroxymethylfurfural and acetic acid (Taherzadeh et al. 1997a). Phenol monomer content was also shown to be important for the fermentabilty of spruce hemicellulose hydrolysates (Larsson et al. 1999b). The inhibitory potential of hemicellulose fractions is due to the combined effects of acetic acid, furans and phenols. The hemicellulose fraction from alkaline wet oxidized wheat straw had similar phenol concentrations to acid pre-treated spruce, SO_2 steamed willow and steam exploded poplar. However, the ethanol productivity was at least 3.5 times higher in alkaline wet oxidized wheat straw hemicellulose fraction, resulting in reduced fermentation time. The better fermentability was probably due to the absence of furfural, which previously has been shown to inhibit fermentation synergistically with phenols in *E. coli* (Zaldivar et al. 1999, 2000) and acetic acid in *S. cerevisiae* (Palmqvist et al. 1999). Acid

Table 3 The distribution of monomeric and dimeric phenols in hydroxyl (H), guaiacyl (G) and syringyl (S) derivatives semiquantified in hemicellulose hydrolysates, calculated from data reported in literature. The distribution of phenol ketones, aldehydes, acids and other phenols is given as relative percentages of total phenols quantified

Feed stock	H (%)	G (%)	S (%)	Ketones[a] (%)	Aldehydes (%)	Acids (%)	Other[b] (%)	Total (g/l)/(%DM)	Pre-treatment	Reference
Softwood										
Spruce (*Picea abies*)	3	97	0	33 (32)	33	9	26	0.46/NA	Acid steaming	Larsson et al. 1999b
Pine (*Pinus radiata*)	0	100	0	65 (65)	20	10	5	3.35/NA	Acid hydrolysis	Clark and Mackie 1984
Hardwood										
Willow (*Salix caprea*)	29	51	0	0	17	0[c]	83(3)	2.56/NA	Acid (SO_2) steaming	Jönnson et al. 1998
Yellow poplar (*Populus* sp.)	4	32	63	72 (72)	21	4[d]	2	0.25/0.25	Acid steaming	Fenske et al. 1998
Poplar (*Populus* sp.)	67	5	27	0	25	75	0	1.35/NA	STEX	Ando et al. 1986
Birch (*Betula* sp.)	1	14	24	3 (2)	23	3	71(61)	0.29/NA	Steaming & enzyme hydrolysis	Buchert et al. 1998
Red oak *(Quercus falcata)*	0	27	73	24 (18)	40	16	20	1.13/0.39	Acid hydrolysis	Tran and Chambers 1986
Herbaceuos material										
Wheat straw *(Triticum aestivum)*	18	49	33	20	29	45	7	0.27/0.48	Alkaline WO	Klinke et al. 2002
Wheat straw (*Triticum aestivum*)	32	64	3	0	1	99	0	0.10/0.40	Soda pulping	Lawther et al. 1996b
Bagasse	39	18	43	0	10	90	0	1.06/NA	Soda pulping	Barquerino et al. 1980
Switch grass	5	68	27	69	12	5[d]	14	0.14/0.14	Acid steaming	Fenske et al. 1998
Corn stover	10	44	46	60 (56)	30	10[d]	0	0.11/0.11	Acid steaming	Fenske et al. 1998

DM origianl dry matter, STEX steam explosion, WO wet oxidation, NA not available.

[a] Percentage of Hibbert's ketones (phenylpropane with hydroxy and ketogroups) is given in parenthesis.

[b] Phenols, phenol alcohols etc. with percentage of total phenol dimers given in parenthesis.

[c] Present but not quantified.

[d] Content of aromatic acids probably higher, because of sub-optimal extraction at neutral pH.

Table 4 Performance of *S. cerevisiae* in different lignocellulosic hydrolysates in relation to content of degradation products

Feed stock	Pre-treatment	DM[a] (%)	Q_{EtOH}[b] (g/l h)	Y_{EtOH}[c] (g/g)	Time (h)	Inoculum (g/l)	Acetate (g/l)	Formate (g/l)	Furfural (g/l)	5-HMF (g/l)	Phenols (g/l)	Reference
Softwood												
Pine	Acid STEX	33	2.87	0.39	24	10	2	NA	0.7	1.7	NA	Taherzadeh et al. 1997a
Fir	SO_2 STEX	20[d]	NA	0.44	48	6	NA	NA	2.3	1.8	NA	Boussaid et al. 1999
Spruce	SO_2 STEAM	10[d]	NA	0.50	24	5	4.2	NA	1.3	2.0	NA	Stenberg et al. 2000
Spruce	Acid STEAM	NA	NA	0.47	64	1	2.8	0.7	1.4	2.3	2.9[e]	Larsson et al. 2001
Spruce	Acid STEAM	NA	0.04	0.32	36	2	2.4	1.6	1.0	5.9	0.64	Larsson et al. 1999a,b
Spruce	Acid STEX	33	2.16	0.40	24	10	2.4	NA	0.6	1.5	NA	Taherzadeh et al. 1997a
Spruce bark	Acid STEX	33	5.15	0.43	24	10	0.0	NA	0.7	0.4	NA	Taherzadeh et al. 1997a
Hardwood												
Alder	Acid STEX	33	0.35	0.04	24	10	10.7	NA	3.2	2.6	NA	Taherzadeh et al. 1997a
Aspen	Acid STEX	33	0.37	0.03	24	10	9.1	NA	3.5	2.2	NA	Taherzadeh et al. 1997a
Birch	Acid STEX	33	1.24	0.31	24	10	2	NA	0.5	0.2	NA	Taherzadeh et al. 1997a
Willow	Acid STEX	33	6.50	0.42	24	10	NA	NA	0.3	0.7	NA	Taherzadeh et al. 1997a
Willow	SO_2 STEAM	100[d]	0.30	0.06	8	10	5	NA	NA	NA	NA	Palmqvist et al. 1996
Willow	SO_2 STEAM	100[d]	0.80	0.14 (0.53)[f]	9	5	NA	NA	NA	NA	2.5	Jönsson et al. 1998
Poplar	STEX	13[d]	0.57	0.45	200	10^6 cells/l	NA	NA	NA	NA	1.8	Ando et al. 1986
Herbaceuos												
Wheat straw	Alkaline WO	33	2.0	0.46	8	2	5.8	6.5	0.0	0.0	2.7	Klinke et al. 2003

NA not available.

[a] DM dry matter content of original feed stock *prior* to pre-treatment and filtration of the hydrolysate.

[b] Q_{EtOH} Volumetric ethanol productivity is dependent on the inoculum size, more cells provides higher ethanol production rate.

[c] Y_{EtOH} Ethanol yield from total fermentable sugars at terminated fermentation.

[d] Dry matter content of pre-treated material *prior* to filtration.

[e] Includes polymeric phenols determined by Prussian blue method.

[f] Ethanol yield from consumed sugars.

catalyzed hydrolysis done using SO_2 has shown similar efficiency as with H_2SO_4. However SO_2 pre-treated material shows better fermentability than H_2SO_4 pre-treated material (Martin et al. 2002a; Tengborg et al. 1998). Spruce hydrolysates from H_2SO_4 pre-treatment were fermented with higher ethanol yield in a genetically engineered *S. cerevisiae* strain expressing laccase to detoxify phenols (Larsson et al. 2001) than by Bakers yeast (Larsson et al. 1999b) (Table 4). Addition of acetaldehyde has been shown to alleviate inhibition of acid pre-treated birch hemicellulose hydrolysates in *S. cerevisiae* with no prior detoxification step (Barber et al. 2000).

Pentose fermenting microorganisms are generally more inhibited by hemicellulose hydrolysates than hexose fermenting yeasts. Comparison of fermentability of acid hydrolysates from poplar, switchgrass and corn stover by *Pichia stipititis* was correlated to the phenol monomer content (Fenske et al. 1999). The performance of genetically engineered *S. cerevisiae*, *Z. mobilis*, and *E. coli* in lignocellulosic hydrolysates has been reviewed recently by Zaldivar et al. (2001) and Dien et al. (2003). In detoxified hydrolysates from corn fiber, all microorganisms were able to convert 80–98% of the fermentable sugars to ethanol. *E. coli* obtained a 85% ethanol yield from detoxified steam pre-treated pine hemicellulose sugars (Zaldivar et al. 2001). Thermophilic bacteria have also been shown to ferment lignocellulosic hydrolysates (Sommer 1998). An adapted xylanolytic anaerobic thermophilic bacterium *Thermoanaerobacter mathranii* ferment xylose in the hemicellulose fraction from alkaline wet oxidized wheat straw to ethanol with no *prior* detoxification (Ahring et al. 1996; Klinke et al. 2001).

Inhibitory effects of degradation products

Degradation products from chemical pre-treatment of biomass can be divided in the following classes: carboxylic acids, furans, phenols and inorganic salts—with phenols showing the most inhibitory effect to fermentations (McMillan 1994a). Low molecular weight (MW) organic compounds or salts are able to penetrate cell membranes, whereas fermentation inhibitors with high MW influence the expression and activity of sugar and ion transporters in the cell membrane. Mechanisms for inhibition on growth and ethanol production of weak acids, furans and phenols have been reviewed recently (Palmqvist and Hahn-Hägerdal 2000a). Low MW phenolics were shown to be more toxic to microorganisms than high MW polyphenolics (Clark and Mackie 1984; Sierra-Alvarez and Lettinga 1991) and also extractives (including phenolic components) were shown to inhibit fermentation (Ranatunga et al. 1997b; Tran and Chambers 1986). The furans and phenols are aromatic compounds that have different functional groups (e.g., acid, ketone, or aldehyde) and hence different potential inhibitory activity. The inhibitory effect of aromatic compounds on glucose fermentation and growth in hexose fermenting yeasts (*S. cerevisiae*, *Candida shehatae*) and bacterium (*Z. mobilis*) is shown in Table 5. The inhibitory effects of acids and aromatic compounds on xylose fermentation and growth in pentose fermenting yeasts (*C. shehatae*, *P. stipititis*) and bacteria (*E. coli*, *T. mathranii, Z. mobilis*) are shown in Table 6.

Effect of furans and phenols on glucose fermentation

The furans and phenols generally inhibited growth and ethanol production rate (Q_{EtOH}) but not the ethanol yields (Y_{EtOH}) in *S. cerevisiae* and *Z. mobilis* (Table 5). An exception was the lower ethanol yield of *S. cerevisiae* Hakken No. 1 because it was determined at the mid-exponential phase and not as the final fermentation yield (Ando et al. 1986). Caution should be taken when growth is monitored by optical density because additions of fermentation inhibitors can induce changes in cell morphology (Ranatunga et al. 1997a). In spite of different inocula and strains, there was a good coherence in the data reported from *S. cerevisiae* fermentations with the phenol acids. *S. cerevisiae* tolerance towards aldehydes was strain dependent and the CBS 1200 strain was less tolerant than both the ATCC 96581 strain and Bakers yeast. *Z. mobilis* had an intermediate aldehyde tolerance compared to the four *S. cerevisiae* strains. *C. shehateae* improved the ethanol production by 30% relative to the reference fermentation upon addition of furfural and 4-hydroxybenzoic acid. The inhibitory effect of 4-hydroxybenzoic acid and vanillic acid was about the same and syringic acid was not inhibitory at all. The phenylpropane unsaturated acids—4-hydroxycinnamic acid and ferulic acid—severely inhibited ethanol productivity at low concentrations in *S. cerevisiae*. The detoxification of phenol aldehydes by conversion to alcohols in anaerobic cultures have been shown in *S. cerevisiae* (de Wulf et al. 1986; Klinke et al. 2003; Larsson et al. 2000) and in *Klebsiella pneumoniae* (Nishikawa et al. 1988).

Effect of acids, furans and phenols on xylose fermentation

The tolerance towards inhibitors differed in the tested pentose fermenting strains (Table 6). The ethanol production of *Z. mobilis* CP4 (pZB5) was generally inhibited at much lower concentrations of acids and aldehydes than the other strains tested. *E. coli* and *T. mathranii* tolerated aliphatic and aromatic acids well, but ferulic acid inhibited ethanol production by *E. coli* at only 3 mM concentration. *E. coli* was very tolerant to furfural and 5-hydroxymethylfurfural compared to *Z. mobilis*, *P. stipititis*, and *C. shehateae*. *T. mathranii* tolerated phenol aldehydes better than *Z. mobilis*, *P. stipititis*, and *C. shehateae*, but not as well as *E. coli*. The acids inhibited growth more than ethanol production in *E. coli*, but this was not the case for the phenol aldehydes or other phenols.

The ethanol yields of both glucose and xylose fermenting microorganisms were generally improved by adding sub-inhibitory levels of phenols to the medium (Tables 5, 6), and this effect has also been shown for acetic acid and furfural due to reduced cell mass production (Palmqvist et al. 1999; Taherzadeh et al. 1997b). In addition, the capacity to metabolize monocyclic aromatic compounds as sole carbon source has been shown to be present in six genera of yeasts (Mills et al. 1971).

Structure activity relationship for predicting inhibitory potential

The inhibitory potential of the aromatic compounds shown in Tables 5, 6, was found to be dependent on their chemical structure. However, this structure-activity relationship (SAR) is very complex and was shown to be dependent on the microbial strain due to different features of cell membranes and metabolism (McMillan 1994a; Mikulásova et al. 1990; Zemek et al. 1979). It is long known that in yeast the inhibitory effects of compounds from wood hydrolysates are closely related to their type: terpenes > aldehydes > polyhydroxy aromatics, and formic acid > acetic acid (Leonard and Hajny 1945). Furfural is more inhibitory than 5-HMF (Sanchez and Bautista 1988) and this was also concluded from model inhibition experiments (Tables 5, 6).

Model inhibition assays of 20 phenols produced detailed information on SAP affecting growth and ethanol production in *S. cerevisiae* (Larsson et al. 2000). In an extensive survey of genetic engineered ethanol-producing *E. coli*, the inhibitory activity of furans and phenols on the ethanol production was closely related to the functionality of the aliphatic sidechain, e.g., aldehydes > acids > alcohols (Zaldivar et al. 1999, 2000; Zaldivar and Ingram 1999). Typical fermentation pH of yeasts is slightly acidic (pH 4–5), while for bacteria it is neutral (pH 7). The pK_a value of the phenol hydroxyl group in phenol aldehydes and ketones (including Hibbert's ketones) is 7.3–8.2, phenol acids 9–11, other phenols 9.5–10.3. The pK_a value of the carboxyl-group in phenol acids is 3.4–4.6, and in alpha-oxy acids it is 1.6–2.6 (Maman et al. 1996; Ragnar et al. 2000). The pKa value of phenol acids is not dependent on methoxy group substituents on the aromatic ring; thus, there is not a significant difference between H, G, or S derivatives. The lower pKa value of the phenol hydroxyl group of aldehydes and the ketones mean that the phenolic proton is not completely dissociated at neutral pH. The inhibitory activity of fermentation inhibitors were also correlated to their partition coefficients in octanol-water (log *P*, e.g., hydrophobicity) in *E. coli* (Zaldivar et al. 2000). Hydrophobic parts of proteins, enzymes, or membrane transport systems are possible sites of inhibitory action. Methoxy substituents ortho to the phenol hydroxy group has been shown to decrease the toxicity of the phenols towards *S. cerevisiae*, e.g., the inhibitory activity of a phenol is H>G>S (Ando et al. 1986; Clark and Mackie 1984; Delgenes et al. 1996, Klinke et al. 2003). The introduction of methoxyl groups in the aromatic ring of a phenol drastically reduces the hydrophobicity (log *P*), which is less dependent of the functional group para to the phenol hydroxyl group (Table 7). There is very small difference in hydrophobicity when comparing different types of phenols with the same amount of methoxyl groups, hence the hydrophobicity can only be used as an indicative tool for comparison within a functional group. However, a correlation between the hydrophobicity and inhibition of volumetric ethanol productivity of *S. cerevisiae* was seen, when plotting series of separate functional groups of phenol aldehydes, ketones, and acids (Fig. 3). It can be concluded that the more hydrophobic the compound was, the more inhibition of the volumetric ethanol productivity Q_{EtOH} in *S. cerevisiae* was evident. The degree of inhibition increased almost linear as function of log *P*, and was higher in phenolic aldehydes and ketones compared to phenolic acids.

Additive or synergistic inhibition The biological effect of certain compounds can be enhanced by the presence of other compounds. In the case of microbial inhibition, the effect can be additive or synergistic if the inhibition increases significantly more than expected from individual measurements, respectively. When tested in combination with acetic acid, aromatic aldehydes and alcohols, 2-furfural and furfuryl alcohol has shown to increase the inhibitory potential for these compounds, resulting in synergistic inhibition of growth and ethanol yield in *E. coli* (Zaldivar et al. 1999, 2000; Zaldivar and Ingram 1999). Synergistic inhibition of 2-furfural with acetic acid was also shown for *S. cerevisiae* (Palmqvist et al. 1999).

Hemicellulose fractions from alkaline wet oxidation of wheat straw were added nine phenols and 2-furoic acid to test if hydrolysate components other than furfural or 5-hydroxymethylfurfural were present to display synergistic inhibition. Synergistic inhibitory effects of 10-mM syringaldehyde and acetovanillone with wheat straw hydrolysate components were shown in *S. cerevisiae* (Klinke et al. 2003). Growth and ethanol production in *T. mathranii* A3M3 was not inhibited at 2-mM phenol additions to hydrolysate, but by increasing the concentration to 10-mM, synergistic inhibitory effects with hydrolysate components were evident for all nine phenols and 2-furoic acid compared to synthetic medium (Klinke et al. 2001). Removal of phenols from hemicellulose hydrolysates with high concentrations of Hibbert's ketones improved fermentability (Buchert et al. 1990; Larsson et al. 1999b; Tran and Chambers 1986). The toxicity of Hibbert's ketones has not been demonstrated, but the inhibitory potentials of other phenol ketones (acetophenone-type) were comparable with phenol aldehydes towards *S. cerevisiae* (Table 5) and *T. mathranii* (Table 6) (Klinke et al. 2001, 2003).

Salt inhibition Alkali salts and heavy metal salts are present in lignocellulosic hemicellulose hydrolysates. The biomass, chemicals added during pre-treatment, and

Table 5 Effect of aromatic acids, aldehydes, ketones and other aromatics on fermentation by hexose-fermenting microorganisms. The percentages are given as the result in the fermentation with added compound relative to the reference fermentation with no compounds added

Compound	Conc. (Mm)	Q_{EtOH}[a] (%)	Y_{EtOH}[b] (%)	Growth[c] (%)	Microorganism[d]	Reference
Acids						
2-Furoic acid	10	85	100	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
4-Hydroxybenzoic acid	14	NA	131	NA	*C. shehateae* NJ23	Palmqvist et al. 1999
	7	69	70[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	10	89	102	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	14	NA	57	NA	*S. cerevisiae* ATTC 96581	Palmqvist et al. 1999
	14	NA	97	NA	*S. cerevisiae* Bakers yeast	Palmqvist et al. 1999
	56	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
Vanillic acid	6	96	98[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	10	87	102	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	22	50	NA	NA	*S. cerevisiae* Bakers yeast	Clark and Mackie 1984
	64	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
Syringic acid	5.1	112	117[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	10	95	102	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	253	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
4-Hydroxycinnamic acid	1	99	100	132	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	6	61	101	92	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Ferulic acid	1	45	102	92	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	5	20	100	39	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Aldehydes						
2-Furfural	10	NA	20[e]	19	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	10	NA	82[e]	81	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	21	NA	10[e]	11	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	21	NA	86	NA	*S. cerevisiae* ATTC 96581	Palmqvist et al. 1999
	21	NA	82	NA	*S. cerevisiae* Bakers yeast	Palmqvist et al. 1999
	21	NA	56[e]	44	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	21	NA	131	NA	*C. shehateae* NJ23	Palmqvist et al. 1999
	65	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
5-Hydroxymethylfurfural	8	NA	35[e]	29	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	24	NA	17[e]	17	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	24	NA	87[e]	69	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	40	NA	47[e]	33	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	57	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
4-Hydroxybenzaldehyde	4	NA	97[e]	75	*S.cerevisiae* CBS 1200	Delgenes et al. 1996
	4	NA	21[e]	16	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	6	NA	63[e]	47	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	8	52	28[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	10	36	112	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	12	NA	25[e]	13	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	12	NA	11[e]	8	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	17	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
Vanillin	1	106	99	132	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	3	NA	70	49	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	3	NA	86	62	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	6	NA	17	14	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	6	NA	74	37	*Z. mobilis* ATCC 10988	Delgenes et al. 1996

Table 5 (continued)

Compound	Conc. (Mm)	Q_{EtOH}[a] (%)	Y_{EtOH}[b] (%)	Growth[c] (%)	Microorganism[d]	Reference
Vanillin	6.6	84	101	92	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	6.6	90	75[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	9	50	NA	NA	*S. cerevisiae* Bakers yeast	Clark and Mackie 1984
	10	58	105	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	13	NA	20	12	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	18	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
	33	0	NA	NA	*S. cerevisiae* Bakers yeast	Clark and Mackie 1984
iso-Vanillin	1	110	101	141	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	6.6	91	101	118	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
ortho-Vanillin	0.1	45	100	118	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	1	0	0	0	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Syringaldehyde	1	NA	74[e]	100	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	4	NA	46[e]	39	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	5.5	93	85[e]	NA	*S. cerevisiae* Hakken No. 1	Ando et al. 1986
	8	NA	33[e]	19	*S. cerevisiae* CBS 1200	Delgenes et al. 1996
	8	NA	83	60	*Z. mobilis* ATCC 10988	Delgenes et al. 1996
	10	69	105	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
	25	NA	50	NA	*S. cerevisiae* Bakers yeast	Lee et al. 1999
Coniferyl aldehyde	1	35	90	92	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
	1	NA	116	100	*S. cerevisiae* pL^+S^s	Larsson et al. 2001
Ketones						
4-Hydroxyacetophenone	10	38	100	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
Acetovanillone	10	48	112	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
Acetosyringone	10	76	110	NA	*S. cerevisiae* ATTC 96581	Klinke et al. 2003
Phenols						
Cathecol	9	104	103	105	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Hydroquinone	9	103	103	105	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Coniferyl alcohol	5.5	100	102	145	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Eugenol	1	35	102	79	*S. cerevisiae* Bakers yeast	Larsson et al. 2000
Isoeugenol	1	35	106	79	*S. cerevisiae* Bakers yeast	Larsson et al. 2000

NA not available.

[a] Q_{EtOH} Volumetric ethanol productivity in exponential phase relative to reference fermentation.

[b] Y_{EtOH} Ethanol yield (g ethanol/g consumed glucose) relative to reference fermentation.

[c] Growth=0%=MIC (minimal inhibitory concentration).

[d] Fermentation conditions—*C. shehateae* NJ23: 30 g l^{-1} glucose, 30°C, pH 5.5, stirred flask (20 ml), 2.2 g l^{-1} inoculum (Palmqvist et al. 1999); *S. cerevisiae* ATTC 96581: 30 g l^{-1} glucose, 30°C, pH 5.5, stirred flask (40 ml), 2 g l^{-1} inoculum (Klinke et al. 2003) and 30 g l^{-1} glucose, 30°C, pH 5.5, stirred flask (20 ml), 3.2 g l^{-1} inoculum (Palmqvist et al. 1999); *S. cerevisiae* Bakers yeast: 90 g l^{-1} glucose, 30°C, pH 5, shake flask (50 ml), 10–20% (v/v) inoculum (Lee et al. 1999), 30 g l^{-1} glucose, 30°C, pH 5.5, stirred flask (20 ml), 3.7 g l^{-1} inoculum (Palmqvist et al. 1999), 20 g l^{-1} glucose, 30°C, pH 5.5, fermentor (140 ml), 0.16 g l^{-1} inoculum (Larsson et al. 2000) and 30 g l^{-1} glucose, 30°C, pH 5, tubes (10 ml), 0.6 g l^{-1} inoculum, reducing sugar consumption (Clark and Mackie 1984); *S. cerevisiae* CBS 1200: 20 g l^{-1} glucose, 30°C, pH 5.6, shake flask (50 ml), 3% (v/v) inoculum, 12 h (Delgenes et al. 1996); *S. cerevisiae* Hakken No. 1: 150 g l^{-1} glucose, 30°C, pH 5.5–6, standing flask (200 ml), 10^{6} cells ml^{-1} inoculum. EtOH yield at mid-exponential phase (Ando et al. 1986); *S. cerevisiae* pL^+S^s: 20 g l^{-1} glucose, 22°C, pH 5.5, fermentor (800 ml), 0.1 g l^{-1} inoculum (Larsson et al. 2001); *Z. mobilis* ATCC 10988: 20 g l^{-1} glucose, 30°C, pH 5.6, shake flask (50 ml), 3% (v/v) inoculum, 24 h (Delgenes et al. 1996).

[e] Ethanol produced relative to reference fermentation at a given time.

metals released from the walls of the pre-treatment equipment are the main sources of inorganic salts. Recirculation of process water in a bioethanol process will also result in higher salt concentrations. The varying quality of molasses or worts utilized by breweries, wineries or distilleries are due to different mineral concentrations and other organic components affecting fermentation yields. Much attention has been given to understand the effects of inorganic nutrients required for cell growth and ethanol production in yeast (Jones and Greenfield 1984; Jones 1986).

Trace minerals or metals are transported across the cell membrane by either active or passive mechanisms, and differences in toxicity have been demonstrated for the cations Ca^{2+}, Mg^{2+}, K^+, Na^+, NH_4^+, and anions Cl^-, SO_4^{2-}, HPO_4^- in *S. cerevisiae* (Maiorella et al. 1984). Chloride (Cl^-) has been shown to have detrimental effects in concentrations above 6 g/l in sugarcane molasses on ethanol formation in *Z. mobilis*(Doelle et al. 1990). Magnesium is important for many metabolic and physiological functions in yeast and bacteria (Alexandre and Charpentier 1998), while calcium has toxic effect when it

Table 6 Effect of aliphatic acids, aromatic acids, aldehydes, ketones and other aromatics on fermentation by pentose-fermenting microorganisms. The percentages are given as the result in the fermentation with added compound relative to the reference fermentation with no compounds added

Compound	Conc. (mM)	C_{EtOH}[a] (%)	Y_{EtOH}[b] (%)	Growth[c] (%)	Microorganism[d]	Reference
Acids						
Acetic acid	150	0	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	250	64	NA	79	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	283	78	NA	NA	*E. coli* LY01	Zaldivar et al. 1999,b
	435	110	119	74	*T. mathranii* A3	Sommer 1998
Formic acid	217	43	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Levulinic acid	172	15	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Caproic acid	0.6	57	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	17	27	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
2-Furoic acid	10	97	92	76	*T. mathranii* A3M4	Klinke et al. 2001
	22	10	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
4-Hydroxybenzoic acid	10	106	106	75	*T. mathranii* A3M4	Klinke et al. 2001
	18	21	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Vanillic acid	0.5	101	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	10	101	101	85	*T. mathranii* A3M4	Klinke et al. 2001
	24	17	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Syringic acid	0.5	95	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	10	104	105	91	*T. mathranii* A3M4	Klinke et al. 2001
	25	38	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Protocatechuic acid	0.3	72	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
Ferulic acid	3	36	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Gallic acid	1	77	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	147	48	NA	80	*E. coli* LY01	Zaldivar et al. 1999,b
Aldehydes						
2-Furfural	10	58	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	10	53	NA	62	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	10	29	NA	53	*P. stipititis* NRRL Y 7124	Delgenes et al. 1996
	39	116	NA	85	*E. coli* LY01	Zaldivar et al. 1999
5-Hydroxymethylfurfural	0.7	80	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	24	10	NA	32	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	24	10	NA	31	*P. stipititis* NRRL Y 7124	Delgenes et al. 1996
	36	106	NA	96	*E. coli* LY01	Zaldivar et al. 1999
4-Hydroxybenzaldehyde	6	17	NA	23	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	6	16	NA	30	*P. stipititis* NRRL Y 7124	Delgenes et al. 1996
	10	116	NA	100	*E. coli* LY01	Zaldivar et al. 1999
	10	15	15	15	*T. mathranii* A3M4	Klinke et al. 2001
Vanillin	0.3	65	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	6.6	6	NA	9	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	6.6	9	NA	1	*P. stipititis* NRRL Y 7124	Delgenes et al. 1996
	10	110	NA	96	*E. coli* LY01	Zaldivar et al. 1999
	10	20	21	14	*T. mathranii* A3M4	Klinke et al. 2001
Syringaldehyde	0.7	64	NA	NA	*Z. mobilis* CP4(pZB5)	Ranatunga et al. 1997a,b
	4	40	NA	40	*C. shehatae* ATCC 22984	Delgenes et al. 1996
	4	38	NA	20	*P. stipititis* NRRL Y 7124	Delgenes et al. 1996
	10	32	47	26	*T. mathranii* A3M4	Klinke et al. 2001
	14	66	NA	57	*E. coli* LY01	Zaldivar et al. 1999
Ketones						
4-Hydroxyacetophenone	10	48	75	62	*T. mathranii* A3M4	Klinke et al. 2001
Acetovanillone	10	80	97	52	*T. mathranii* A3M4	Klinke et al. 2001
Acetosyringone	10	72	89	56	*T. mathranii* A3M4	Klinke et al. 2001
Phenols						

Table 6 (continued)

Compound	Conc. (mM)	C_{EtOH}[a] (%)	Y_{EtOH}[b] (%)	Growth[c] (%)	Microorganism[d]	Reference
Catechol	27	2	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Coniferyl alcohol	17	3	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Furfuryl alcohol	204	5	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Guaiacol	24	6	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Hydroquinone	27	11	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Methylcatechol	12	0	NA	0	*E. coli* LY01	Zaldivar et al. 2000
Vanillyl alcohol	58	26	NA	0	*E. coli* LY01	Zaldivar et al. 2000

NA not available.

[a] C_{EtOH} Ethanol produced relative to reference fermentation at a given time.

[b] Y_{EtOH} Ethanol yield (g ethanol/g consumed xylose) relative to reference fermentation.

[c] Growth=0%=MIC (minimal inhibitory concentration)

[d] Fermentation conditions—*C. shehatae* ATCC 22984: 20 g l^{-1} xylose, 30°C, pH 5.6, shake flask (50 ml), 3% (v/v) inoculum, 32 h (Delgenes et al. 1996); *E. coli* LY01: 100 g l^{-1} xylose, 30°C, pH 7, inoculum OD$_{550\ \mathrm{nm}}$ 0.025. Growth: Standing tubes (4 ml), 24 h (acids and alcohols) or shake flasks 48 h (aldehydes). Ethanol: Shake flasks 24 h (acids) or 48 h (aldehydes and alcohols) (Zaldivar et al. 2000); *P. stipititis* NRRL Y 7124: 20 g l^{-1} xylose, 30°C, pH 5.6, shake flask (50 ml), 3% (v/v) inoculum, 32 h (Delgenes et al. 1996); *T. mathranii* A3: 5 g l^{-1} xylose, 70°C, pH 6.8, standing tubes (10 ml), inoculum OD$_{578\ \mathrm{nm}}$ 0.05, 48 h. Growth measured as OD (Sommer 1998); *T. mathranii* A3M4: 4 g l^{-1} xylose, 70°C, pH 7, standing tubes (10 ml), 3% inoculum OD$_{578\ \mathrm{nm}}$ 0.05, 24 h. Growth measured as pressure increase (Klinke et al. 2001).

Table 7 The partition coefficients in octanol-water (log *P*), e.g., hydrophobicity in *para*-phenol (Ph) acids, aldehydes and ketones and the number of methoxyl groups (OCH_3) *ortho* to the phenol hydroxyl group

	Aldehyde (Ph-CHO) (log *P*)	Ketone (Ph-COCH3) (log *P*)	Acid (Ph-COOH) (log *P*)
H (0 *ortho*-OCH_3)	1.35	1.45	1.58
G (1 *ortho*-OCH_3)	1.21	1.27	1.48
S (2 *ortho*-OCH_3)	0.99	0.98	1.04

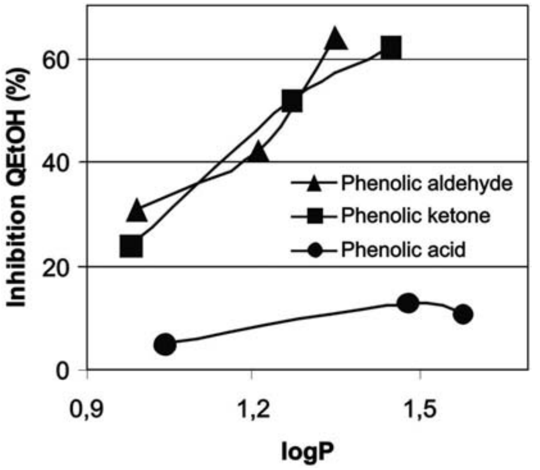


Fig. 3 Inhibition of *S. cerevisiae*'s volumetric ethanol productivity by *para*-phenol (Ph) aldehydes, ketones and acids (10 mM), as function of the partition coefficients in octanol-water (log *P*), e.g., hydrophobicity (Klinke et al. 2003)

is present in high amount (Maiorella et al. 1984). High concentrations of calcium in molasses have been shown to be the main inhibitory salt to *S. cerevisiae* (Tajima et al. 1966). If inorganic salts with the same valence are present in wrong ratios (e.g., Ca/Mg-ratio), they slow down fermentation. Fermentation was improved by addition of magnesium to beet molasses (Wolniewicz et al. 1988) and to barley wort (Bromberg et al. 1997). Heavy metals Zn, Cu, Fe, Co are micronutrients with a relatively narrow optimum concentration range for the organisms, and at higher concentrations they have toxic effects. Sluggish fermentation was correlated to higher cupper content in raisin (Akrida-Demertzi et al. 1988). Recently, it was shown that high mineral salt concentration rather than high ethanol concentration is the main inhibitor of xylose fermentation by the thermophilic bacterium *T. thermosaccharolyticum* (Lynd 2001). Generally, thermophiles show lower tolerance towards high sugar and ethanol concentrations than mesophiles. This may be due to the 30–40°C higher growth temperature where inorganic salts and other organic components have higher solubility and osmotic pressure (Herrero and Gomez 1980; Lynd 1989).

Conclusion

The aromatic compounds formed by pre-treatment display different inhibitory potential according to their structure. Formation of certain degradation products such as 2-furfural should be minimized because of its synergistic inhibitory effects with other degradation products present in lignocellulosic hydrolysates. Aromatic and carboxylic acids are generally not inhibitory to either pentose or hexose fermenting microorganisms, but phenols, phenol aldehydes and phenol ketones (Hibbert's ketones) are potent inhibitors and their formation during pre-treatment should be minimized.

In addition to high ethanol, sugar and salt tolerance, ethanol-producing strains only perform well in hemicellulose hydrolysates if they are tolerant towards inhibitors.

Screening for inhibitor resistance will be needed for future selection and development of microbial strains. Genetically engineered microorganisms that can utilize all sugars in the hemicellulose hydrolysates are being developed. These organisms have not yet been reported to ferment lignocellulosic hydrolysates without prior detoxification. From model inhibition experiments *E. coli* and *T. mathranii* seems to be potential candidates for future work. *S. cerevisiae* has recently been genetically modified to produce ethanol in severely inhibiting spruce hemicellulose hydrolysates by heterologous expression of laccase that detoxifies the phenolic inhibitors during fermentation. However, at the present wild-type adapted microbial strains perform better in lignocellulosic hydrolysates.

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