Journal of Industrial Microbiology & Biotechnology (2000) 25, 104–108

www.nature.com/jim

Acetaldehyde stimulation of the growth of *Saccharomyces cerevisiae* in the presence of inhibitors found in lignocellulose-to-ethanol fermentations

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The addition of small quantities of acetaldehyde to fermentations containing inhibitory concentrations of furfural, acetate and other compounds typically present in lignocellulosic hydrolyzates significantly reduced the lag phase of yeast growth and stimulated ethanol production. Similar effects were observed when acetaldehyde (0.06 g l^{-1}) was added to fermentations of a birch wood hydrolyzate produced by steam/acid pretreatment. Acetaldehyde addition appears to have potential as a low-cost alternative (or adjunct) to current procedures for medium detoxification in lignocellulose-to-ethanol fermentations, particularly those in which high inhibitor concentrations are generated through recycling of the culture broth. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 104–108.

Keywords: acetaldehyde; detoxification; lignocellulose hydrolyzates; *Saccharomyces cerevisiae*; inhibition; furfural

Introduction

The sugars present in lignocellulosic materials such as wood and crop residues can be fermented by yeasts or ethanologenic bacteria to produce ethanol for use as a motor fuel. Before fermentation, the substrate is either hydrolyzed completely with mineral acids [24] or given a milder pretreatment or prehydrolysis — often with steam and mild acid — to produce a hydrolyzate containing mainly the hemicellulose sugars and solubilised lignin; the residual insoluble cellulose is then hydrolyzed enzymatically [11]. Whichever route is chosen, lignocellulosic hydrolyzates contain, in addition to sugars, a wide range of breakdown products inhibitory to microbial growth, so that some form of detoxification of the medium is usually necessary to permit satisfactory rates of ethanol production.

The inhibitory substances in lignocellulose hydrolyzates may be broadly classified as furans, aromatic lignin degradation products, salts and acetic acid [2,10]. Furfural and other furans are formed by the dehydration of pentose and hexose sugars [16,22]. Lignocellulosic hydrolyzates may contain up to 5–7 g l^{-1} each of furfural and 5-hydroxymethylfurfural [21]. Furfural is a potent inhibitor of yeast fermentations, particularly at the low cell concentrations prevailing at the time of inoculation [9,16] while the toxicity of 5-hydroxymethylfurfural is somewhat less [12,15]. Lignocellulosic hydrolyzates also contain many phenolic lignin breakdown products including as much as 1 g l^{-1} hydroxybenzaldehyde [11], as well as hydroxybenzoic acid, syringaldehyde and syringic acid [1,15]. These exert their inhibitory effect predominantly by dissolving in the cell membrane and diminishing its integrity [4]. Acetic acid, derived from the acetyl substituents of hemicellulose sugars, is present in lignocellulosic hydrolyzates in concentrations of the order of 3–14 g l^{-1} [13,15]; undissociated acetic acid diffuses across the plasma membrane, dissociating in the cytoplasm and reducing the intracellular pH [20].

Lignocellulosic hydrolyzates can be partially detoxified by a variety of methods, including treatment with lime, sodium hydroxide or enzyme preparations, adsorption onto activated charcoal, ion exchange, solvent extraction, electrodialysis, and filtration through molecular sieves [8,11,17]. The extent of detoxification achieved varies considerably, with the cost of treatment contributing significantly to the overall cost of ethanol production. Overliming, one of the most widely used methods [11], raised the total production costs of a wood-to-ethanol conversion process by more than 22%, sufficient to make the overall process uneconomic [23].

An alternative to the above detoxification methods may be the addition to the pretreatment liquor of very small quantities of substances which stimulate growth and fermentation in the presence of toxic materials. In previous works, we showed that small quantities of acetaldehyde — a low-cost organic chemical — substantially relieved the toxic effects of ethanol on the growth of *Saccharomyces cerevisiae* and *Zymomonas mobilis* in complex media [5,18,19]. The lag phases of both organisms were reduced by 70–100% by the addition of less than 80 mg l^{-1} acetaldehyde, while the maximum specific growth rates increased by 15–60%.

To date, there have been no studies to indicate whether the toxic effects of chemicals other than ethanol can be relieved by acetaldehyde addition. Many components of lignocellulosic hydrolyzates (particularly the phenolic compounds) exert their primary inhibitory effect on the cell membrane, a mechanism similar to that seen with ethanol inhibition. It thus appeared possible that acetaldehyde addition might also be effective in overcoming inhibition due to these compounds. In this paper, we show for the first time the ability of acetaldehyde to alleviate inhibition of yeast due to furans, lignin-derived aromatics and acetic acid. In dilutions of a native hydrolyzate derived from steam/acid pretreatment of birch wood, acetaldehyde reduced the lag phase, stimulated ethanol production, and reduced the rate of cell death.

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Received 18 March 2000; accepted 2 June 2000

Materials and methods

Microorganism and media

S. cerevisiae strain X2180-1A (ATCC 26786) was obtained from the Yeast Genetic Stock Centre, University of California, Berkeley and maintained in a lyophilised condition. Fermentations with individual inhibitors were carried out in a defined medium [14] with glucose (5 g l^{-1}) as the carbon source. For fermentations in birch wood hydrolyzate, the liquor was supplemented with the ingredients of a modified Luria broth (MLB) containing (g l^{-1}): glucose, 20; tryptone, 10; yeast extract, 5 and sodium chloride, 9. Inhibitors used in the defined medium were of analytical grade and were obtained from Sigma (St. Louis, MO, USA) (furfural and 5-hydroxymethylfurfural), Koch Light Laboratories, UK (hydroxybenzaldehyde), Hopkin and Williams, UK (hydroxybenzoic acid) and Merck (Kilsyth, Victoria, Australia) (sodium acetate). Lignocellulosic hydrolyzate (prepared by Dr. R. Eklund, Department of Industrial Technology, Mid-Sweden University, Örnsköldsvik, Sweden) was obtained by steam pretreatment of birch wood (*Betula* sp.) using the method of Eklund [3]. The hydrolyzate was assayed by HPLC according to the method of Larsson *et al.* [7] and contained (g l^{-1}): glucose, 5.7; xylose, 39.4; galactose, 3.1 arabinose, 1.7; mannose, 3.5; acetic acid, 10.8; furfural, 0.70 and 5-hydroxymethylfurfural, 0.16. The initial pH of all media was 5.2.

Fermentation conditions and inoculum preparation

All cultures were grown in Erlenmeyer flasks in a gyrotory shaker at 100 rpm and 30°C. For experiments with individual inhibitors, inocula were grown in the defined medium; inocula for the experiments with birch hydrolyzate were grown in distilled water containing MLB ingredients as above. After three serial subcultures, exponential phase cells were washed twice in sterile medium and collected by centrifugation at 3840×*g* for use as the inoculum. The defined medium was sterilised by filtration and the complex medium by autoclaving it. Media were initially aerobic but became self-anaerobic during fermentation.

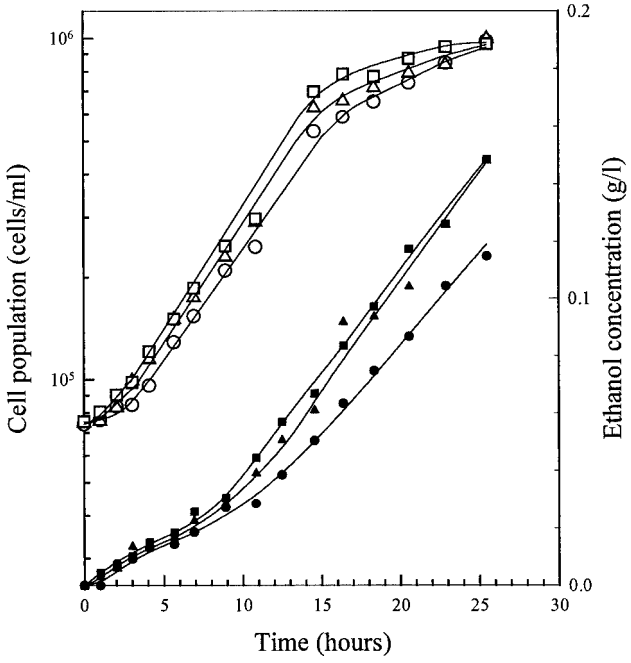

Figure 2 Effect of acetaldehyde addition on growth and ethanol production by *S. cerevisiae* X2180-1A in defined medium containing 1.5 g l^{-1} 5-hydroxymethylfurfural. Initial acetaldehyde concentrations (g l^{-1}): (○, ●) 0, (□, ■) 0.01, (△, ▲) 0.1.

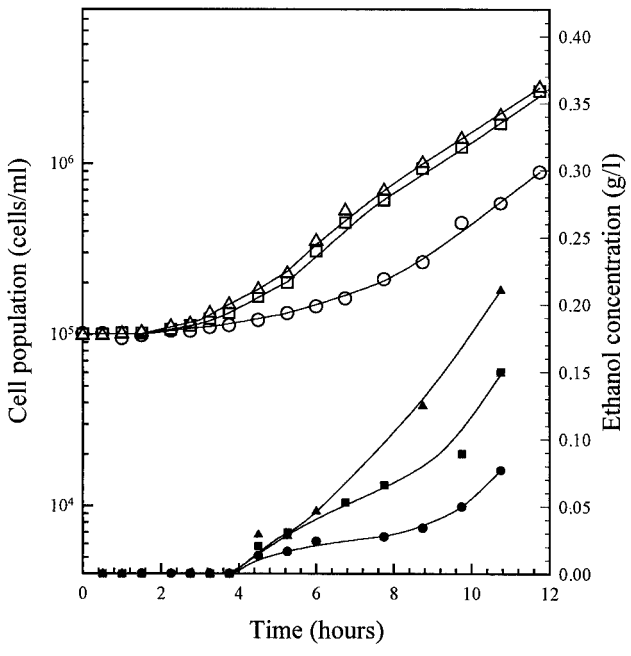

Figure 1 Effect of acetaldehyde addition on growth and ethanol production by *S. cerevisiae* X2180-1A in defined medium containing 0.2 g l^{-1} furfural. Open symbols, cell numbers; closed symbols, ethanol concentration. Initial acetaldehyde concentrations (g l^{-1}): (○, ●) 0, (□, ■) 0.005, (△, ▲) 0.08.

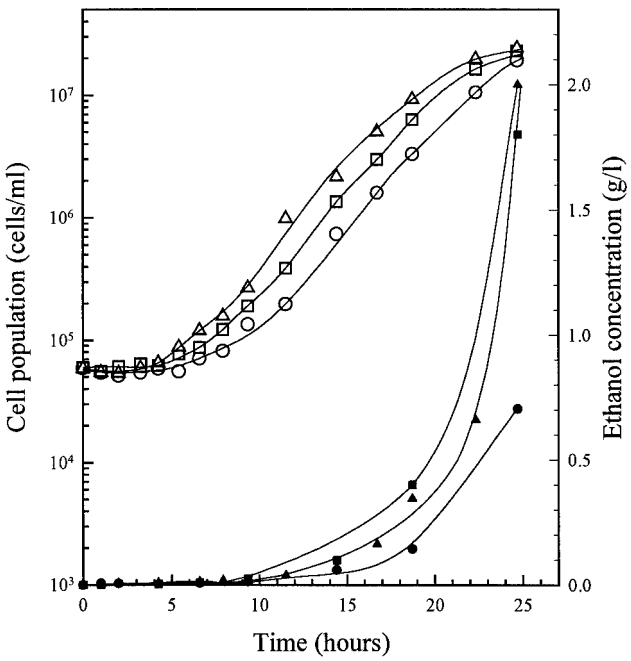

Figure 3 Effect of acetaldehyde addition on growth and ethanol production by *S. cerevisiae* X2180-1A in defined medium containing 0.9 g l^{-1} hydroxybenzoic acid. Initial acetaldehyde concentrations (g l^{-1}): (○, ●) 0, (□, ■) 0.005, (△, ▲) 0.05.

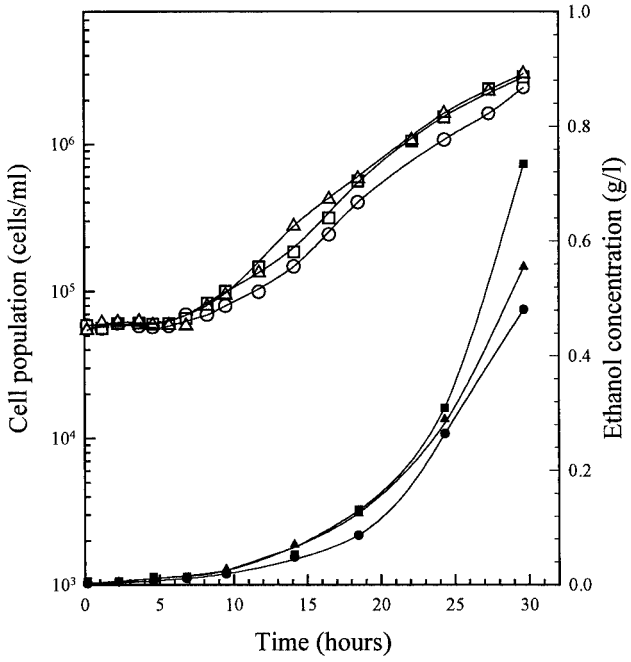

Figure 4 Effect of acetaldehyde addition on growth and ethanol production by *S. cerevisiae* X2180-1A in defined medium containing 0.9 g l^{-1} hydroxybenzaldehyde. Initial acetaldehyde concentrations (g l^{-1}): (○, ●) 0, (□, ■) 0.005, (△, ▲) 0.05.

Analytical methods

Cell numbers were monitored using a Coulter Counter Model Z_B. As the high particulate content of the birch hydrolyzate precluded use of the Coulter Counter, cell numbers in this medium were measured by spread plate counts on MLB agar. Ethanol and acetaldehyde concentrations were determined by head space gas chromatography as previously described [6].

Results

The effect of acetaldehyde addition on growth and ethanol production by *S. cerevisiae* was studied in media containing inhibitory concentrations of sugar degradation products (furfural and 5-hydroxymethylfurfural), phenolic lignin breakdown products (hydroxybenzoic acid and hydroxybenzaldehyde) or acetic acid. The inhibitor concentrations chosen were within the range measured in native lignocellulosic hydrolyzates. In medium containing 0.2 g l^{-1} furfural (Figure 1), the lag time was substantially reduced by the addition of as little as 0.005 g l^{-1} acetaldehyde, with a slight additional reduction at 0.08 g l^{-1} acetaldehyde. Ethanol production was also enhanced. In contrast to effects observed with ethanol-shocked cultures [18,19], acetaldehyde addition had no apparent effect on specific growth rate within the time period of the experiment. Cultures shocked with 5-hydroxymethylfurfural (1.5 g l^{-1}) were stimulated to a lesser extent by acetaldehyde addition (Figure 2), the effect being slightly greater for the culture containing the smaller amount of acetaldehyde, suggesting that the optimum concentration for stimulation lies between 0.01 and 0.1 g l^{-1}. Acetaldehyde is a hormetic substance, its stimulatory effect changing to inhibition with increasing concentration [18]. For the same yeast strain

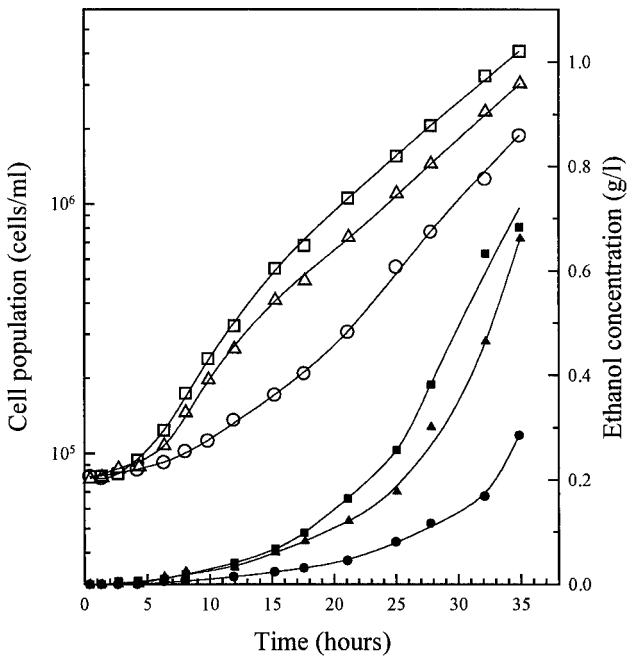

Figure 5 Effect of acetaldehyde on growth and ethanol production by *S. cerevisiae* X2180-1A in defined medium containing 8.05 g l^{-1} acetic acid. Initial acetaldehyde concentrations (g l^{-1}): (○, ●) 0, (□, ■) 0.005, (△, ▲) 0.08.

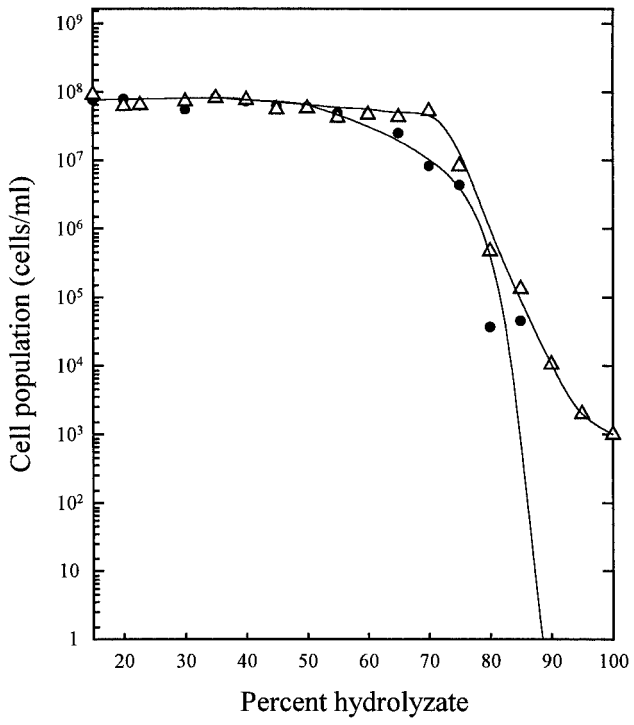

Figure 6 Effect of added acetaldehyde on viable cell numbers of *S. cerevisiae* X2180-1A after 48 h cultivation in various dilutions of birch wood hydrolyzate in water. All media contained MLB ingredients at the concentrations shown in the Materials and methods section. The initial acetaldehyde concentrations were (g l^{-1}): (●) 0, (△) 0.06. No viable cells were detected after 48 h in control cultures containing more than 85% (v/v) hydrolyzate.

exposed to 4% v/v ethanol in a complex medium, the growth rate relative to cultures lacking acetaldehyde is halved at 0.5 g l^{-1} acetaldehyde and growth stops completely at 0.8 g l^{-1} [18].

Cultures containing hydroxybenzoic acid or hydroxybenzaldehyde (both at 0.9 g l^{-1}) were also stimulated by acetaldehyde addition (Figures 3 and 4), the effect being slightly greater at the higher acetaldehyde concentration (a reduced maximum concentration, 0.05 g l^{-1}, was used in these experiments). All cultures containing added acetaldehyde showed reduced lag times relative to the control, and faster ethanol production.

Acetaldehyde had a strong stimulatory effect on cells shocked with 8.05 g l^{-1} acetic acid, a major source of inhibition in lignocellulosic hydrolyzates (Figure 5). As observed with 5-hydroxymethylfurfural (Figure 2), the reduction in lag time was greater for the culture containing the lesser amount of acetaldehyde, suggesting that the optimum stimulatory concentration lies between 0.005 and 0.08 g l^{-1} for inhibition by acetic acid at this concentration.

Cultures inoculated into full-strength birch wood hydrolyzate supplemented with MLB ingredients failed to grow within 48 h after inoculation, reflecting the presence in the hydrolyzate of furfural, acetate and perhaps other inhibitory substances (see Materials and methods section). Dilution of the hydrolyzate in MLB medium was necessary to obtain growth: in mixtures containing *ca.* 50% or more of the hydrolyzate by volume, supplementation with 0.06 g l^{-1} acetaldehyde significantly increased the number of viable cells obtained after 48 h of cultivation (Figure 6). In cultures containing 75% (v/v) hydrolyzate, acetaldehyde addition stimulated ethanol production and reduced the lag time by approximately 10 h. While cultures containing 90% v/v hydrolyzate failed to grow, acetaldehyde addition markedly slowed down the rate of decline in viable cell numbers (Figure 7).

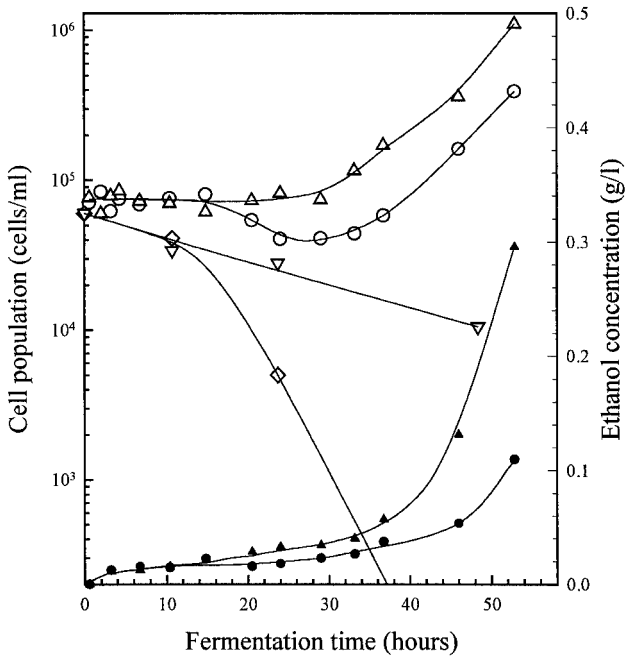

Figure 7 Effect of added acetaldehyde on the time course of viable cell numbers (open symbols) and ethanol production (closed symbols) for *S. cerevisiae* X2180-1A in diluted birch wood hydrolyzates (75% and 90% v/v) containing MLB ingredients. Hydrolyzate dilution factor (% v/v) and initial acetaldehyde concentrations (g l^{-1}): (○, ●) 75, 0; (△, ▲) 75, 0.06; (◇) 90, 0; (▽) 90, 0.06. Ethanol production was not detected in cultures containing 90% v/v hydrolyzate.

Discussion

Previously, we demonstrated the ability of added acetaldehyde to stimulate the growth of yeast in medium containing inhibitory concentrations of ethanol [5,18,19]. The data presented here extend this finding for the first time to a variety of other inhibitors of varying chemical structure and indicate that the acetaldehyde effect has a more general significance. The mechanism of acetaldehyde stimulation is insufficiently understood, but is likely to be at least in part attributable to the ability of acetaldehyde to restore the cellular redox balance in the presence of compounds which reduce glycolytic flux [19].

Acetaldehyde addition appears to have the potential for practical application as a means of reducing the inhibitory effects of substances present in lignocellulosic hydrolyzates. Further work is needed to assess the effects of acetaldehyde with other inhibitors, and with hydrolyzates produced using other raw materials and hydrolysis procedures. Its efficacy, when used in conjunction with other detoxification procedures such as overliming, also needs to be determined.

Acknowledgements

We are grateful to Prof. B. Hahn-Hägerdal and Dr. T.J. Hobley for the gift of the birch wood hydrolyzate and the analysis of its composition. We also thank Dr. G.J. Dumsday for microbiological assistance and the Australian Research Council for financial support.

References

1 Ando S, I Arai, K Kiyoto and S Hanai. 1986. Identification of aromatic monomers in steam-exploded poplar and their influences on ethanol fermentation by *Saccharomyces cerevisiae*. *J Ferment Technol* 64: 567–570.
2 Delgenes JP, R Moletta and JM Navarro. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis* and *Candida shehatae*. *Enzyme Microb Technol* 19: 220–225.
3 Eklund R. 1994. Ethanol from wood. An experimental study of steam pretreatment, enzymatic hydrolysis and enzyme recovery. PhD Thesis, Department of Chemical Engineering I, Lund University, Sweden.
4 Heipieper HJ, FJ Weber, J Sikkema, H Keweloh and JAM de Bont. 1994. Mechanisms of resistance of whole cells to toxic organic solvents. *Trends Biotechnol* 12: 409–415.
5 Hobley TJ and NB Pamment. 1994. Differences in response of *Zymomonas mobilis* and *Saccharomyces cerevisiae* to changes in extracellular ethanol concentration. *Biotechnol Bioeng* 43: 155–158.
6 Hobley TJ and NB Pamment. 1997. Liquid injection gas chromatography and the ALDH assay overestimate free acetaldehyde in complex fermentation media. *Biotechnol Tech* 11: 39–42.
7 Larsson S, E Palmqvist, B Hahn-Hägerdal, C Tengborg, K Stenberg, G Zacchi and N-O Nilverbrant. 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb Technol* 24: 151–159.
8 Larsson S, A Reimann, N-O Nilvebrant and LJ Jönsson. 1999. Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. *Appl Biochem Biotechnol* 77/79: 91–103.

9 Navarro AR. 1994. Effect of furfural on ethanol fermentation by *Saccharomyces cerevisiae*: mathematical models. *Curr Microbiol* 29: 87–90.
10 Olsson L and B Hahn-Hägerdal. 1993. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Process Biochem* 28: 249–257.
11 Olsson L and B Hahn-Hägerdal. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb Technol* 18: 312–331.
12 Palmqvist E, B Hahn-Hägerdal, M Galbe and G Zacchi. 1996. The effect of water soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme Microb Technol* 19: 470–476.
13 Palmqvist E, B Hahn-Hägerdal, Z Szengyel, G Zacchi and K Reczey. 1997. Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. *Enzyme Microb Technol* 20: 286–293.
14 Pamment NB and RJ Hall. 1978. Absence of external causes in lag in *Saccharomyces cerevisiae*. *J Gen Microbiol* 105: 297–304.
15 Ranatunga TD, J Jervis, RF Helm, JD McMillan and C Hatzis. 1997. Identification of inhibitory components toxic toward *Zymomonas mobilis* CP4(pZB5) xylose fermentation. *Appl Biochem Biotechnol* 67: 185–198.
16 Sanchez B and J Bautista. 1988. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. *Enzyme Microb Technol* 10: 315–318.
17 Sreenath HK and TW Jeffries. 2000. Production of ethanol from wood hydrolyzates by yeasts. *Bioresour Technol* 72: 253–260.
18 Stanley GA, NG Douglas, EJ Every, T Tzanatos and NB Pamment. 1993. Inhibition and stimulation of yeast growth by acetaldehyde. *Biotechnol Lett* 15: 1199–1204.
19 Stanley GA, TJ Hobley and NB Pamment. 1997. Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomonas mobilis* subjected to environmental shocks. *Biotechnol Bioeng* 53: 71–78.
20 Stratford M and PA Anslow. 1998. Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. *Lett Appl Microbiol* 27: 203–206.
21 Taherzadeh MJ, C Niklasson and G Liden. 1999. Conversion of dilute acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. *Bioresour Technol* 69: 59–66.
22 Torget R and T-A Hsu. 1994. Two-temperature dilute acid prehydrolysis of hardwood xylan using a percolation process. *Appl Biochem Biotechnol* 45/46: 5–22.
23 von Sivers M, L Olsson, B Hahn-Hägerdal and G Zacchi. 1994. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Biotechnol Prog* 10: 555–560.
24 Wright JD. 1988. Ethanol from lignocellulose: an overview. *Bioenergy* 8: 71–78.