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ORIGINAL RESEARCH PAPER

Effect of pretreatment chemicals on xylose fermentation by *Pichia stipitis*

Frank K. Agbogbo · Kevin S. Wenger

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Abstract Pretreatment of biomass with dilute H_2SO_4 results in residual acid which is neutralized with alkalis such as $Ca(OH)_2$, NaOH and NH_4OH. The salt produced after neutralization has an effect on the fermentation of *Pichia stipitis*. Synthetic media of xylose (60 g total sugar/l) was fermented to ethanol in the presence and absence of the salts using *P. stipitis* CBS 6054. $CaSO_4$ enhanced growth and xylitol production, but produced the lowest ethanol concentration and yield after 140 h. Na_2SO_4 inhibited xylitol production, slightly enhanced growth towards the end of fermentation but had no significant effect on xylose consumption and ethanol concentration. $(NH_4)_2SO_4$ inhibited growth, had no effect on xylitol production, and enhanced xylose consumption and ethanol production.

Keywords Ammonium sulphate · Calcium sulphate · Ethanol · *Pichia stipitis* · Sodium sulphate · Xylose

F. K. Agbogbo (✉) · K. S. Wenger
Novozymes North America Inc., 77 Perry Chapel Church Road, P.O. Box 576, Franklinton, NC 27525, USA
e-mail: fkag@novozymes.com

Introduction

Lignocelluloses of plant cell walls are composed of cellulose, hemicellulose, pectin and lignin. The major sugars produced after hydrolysis of lignocellulosic biomass are glucose, galactose, mannose, xylose, and arabinose. Efficient conversion of biomass to ethanol requires microorganisms with the ability to ferment the sugars generated after hydrolysis. The yeast, *Pichia stipitis*, produces ethanol from glucose, galactose, mannose, xylose, and cellobiose with high ethanol yields and low amounts of xylitol (Dellweg et al. 1984; du Preez et al. 1986).

One promising technology for converting lignocellulosic biomass to ethanol is the enzyme-based process, where enzymes are used to hydrolyze the fibers after pretreatment. Dilute sulphuric acid pretreatment at high temperatures extensively hydrolyzes the hemicellulose to soluble sugars (Schell et al. 2003; Fenske et al. 1998). The residual acid after pretreatment is neutralized with alkalis such as $Ca(OH)_2$ (van Zyl et al. 1988; Eken-Saracoglu and Arslan 2000), NH_4OH (Alriksson et al. 2005; Persson et al. 2002) and NaOH (Nilvebrant et al. 2005). In some cases, these alkalis are added in excess to reduce inhibitor concentrations in dilute acid pretreated biomass (Tran and Chambers 1986; Nigam 2001a, b).

Although the inhibitors generated after dilute acid pretreatment can decrease ethanol

production (Delgenes et al. 1996; Tran and Chambers 1986), Persson et al. (2002) have shown that the improved fermentability after alkali treatment are difficult to explain by the removal of inhibitors only. Therefore, the salts produced after neutralizing the excess H_2SO_4 with alkalis could play a role in the fermentation. The purpose of this study was to determine how $CaSO_4$, Na_2SO_4, and $(NH_4)_2SO_4$ salts affect the cell growth, xylose consumption, ethanol production, ethanol yield, and xylitol production in *Pichia stipitis*.

Materials and methods

Microorganism

Pichia stipitis CBS 6054 was generously supplied by Dr. Thomas Jeffries of the Forest Products Laboratory, USDA. The cells were grown overnight in a filter-sterilized fermentation medium containing per liter: 1.7 g yeast nitrogen base (without amino acid or ammonium sulfate), 2.27 g urea, 6.56 g peptone, and 20 g xylose. The cells were centrifuged at 3,000*g* for 5 min and resuspended in 5 ml sterile water to serve as inoculum.

Media and fermentation

Three different salt media were prepared by adding 3.3 ml H_2SO_4 (7.6 M) to 25 ml $Ca(OH)_2$ (1 M), 50 ml NH_4OH (1 M) or 50 ml NaOH (1 M). The salts were diluted with distilled water to a total liquid volume of 200 ml and a final pH of 6.0. The control was 200 ml distilled water at the initial pH of 6.1. Xylose, 12 g, was dissolved in each solution to give 60 g/l. Each sugar solution was filter-sterilized using a 0.2 μm filter. Nutrient solution (50× the concentration used) was prepared by dissolving 1.7 g yeast nitrogen base, 2.27 g urea and 6.56 g peptone in 20 ml water. Fermentations were performed in sterile 125 ml Erlenmeyer flasks (with 0.2 μm vent cap) in at 30°C and shaken at 100 rev/min. Each Erlenmeyer flask contained 50 ml sugar media, 1 ml nutrient solution, and 2 ml inoculum. All these experiments were performed in triplicate at the same initial cell concentration of 1.5 g/l.

Analytical methods

Samples, 1 ml, were periodically removed for analyses. The concentrations of xylose, xylitol, and ethanol were determined using an Agilent HPLC System with an analytical Bio-rad Aminex HPX–87H column and a Bio-rad Cation H refill guard column. The cell concentrations were determined as OD_{600} values; an OD of 1 = 0.23 g of dry cells/l.

Results and discussion

Effect of the salts on cell growth

The growth of *Pichia stipitis* on different salt media is shown in Fig. 1 and the pH during fermentation is shown in Fig. 2. Xylose consumption is shown in Fig. 3 and ethanol production in Fig. 4. A synopsis of the key fermentation data is given in Table 1. *Pichia stipitis* reached its final cell concentration after 90 h (Fig. 1). An initial cell concentration of 1.5 g/l grew to different final cell concentrations on the different salt media after 118 h of fermentation (Fig. 1). Inhibition by $(NH_4)_2SO_4$ on the growth of *Pichia stipitis* is in agreement with observations by Guebel et al. (1992). The medium containing $(NH_4)_2SO_4$ had the lowest cell concentration. Cell concentration on Na_2SO_4 medium was lower than the control initially ($t < 40$ h), and became higher towards the end (Fig. 1). The initial pH for all the salts and control was 5.5–6.0 and the final pH was 4.0–4.5. Studies on *Saccharomyces cerevisiae* suggest that the initial response of cells to saline conditions is the efflux of water, which leads to cell shrinkage (Blomberg 2000). *Pichia stipitis* might also respond similarly to *Saccharomyces cerevisiae* on Na_2SO_4 medium, since the cells did not grow well initially (compare to control). The highest cell growth was on $CaSO_4$ medium. Guebel and Nudel (1994) obtained maximum cell growth rates at low Ca^{2+} (0.34 mM) concentrations and low growth at high Ca^{2+} (1 mM) concentrations. The high cell growth of *Pichia stipitis* in our study might be because of low Ca^{2+} in solution due to the poor solubility of $CaSO_4$.

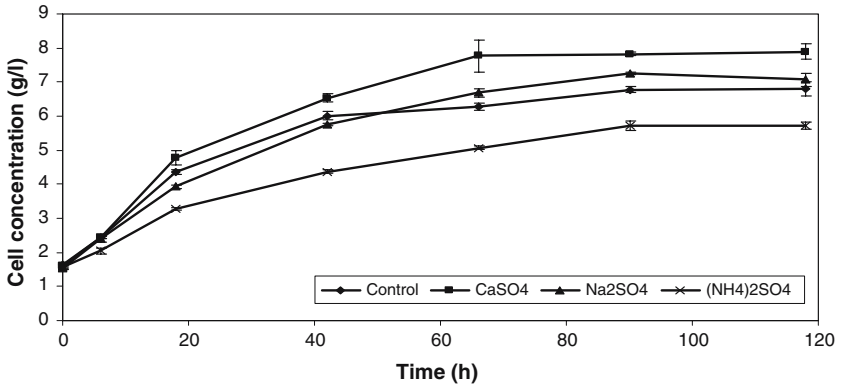

Fig. 1 The effect of salts on cell growth of *Pichia stipitis*. The control was fermentation with no salts added. $CaSO_4$, Na_2SO_4, and $(NH_4)_2SO_4$ were produced by adding 3.3 ml H_2SO_4 (7.6 M) to 25 ml $Ca(OH)_2$ (1 M), 50 ml NH_4OH (1 M), and 50 ml NaOH (1 M) respectively, and adding distilled water to a final liquid volume of 200 ml. *Pichia stipitis* fermentations in 60 g xylose/l at an initial cell concentration of 1.5 g/l in a shake flask incubator at 30°C. Cell concentrations were determined from OD_{600} values using Cary 3C UV-Visible spectrophotometer
*Error bars are ± 1 std

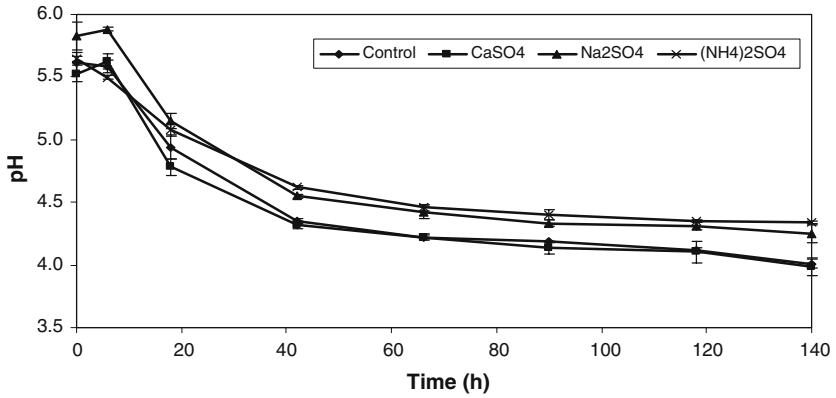

Fig. 2 Effect of salts on pH during fermentation using *Pichia stipitis*. This graph shows pH of samples taken at various time points using Orion portable pH meter
*Error bars are ±1 std

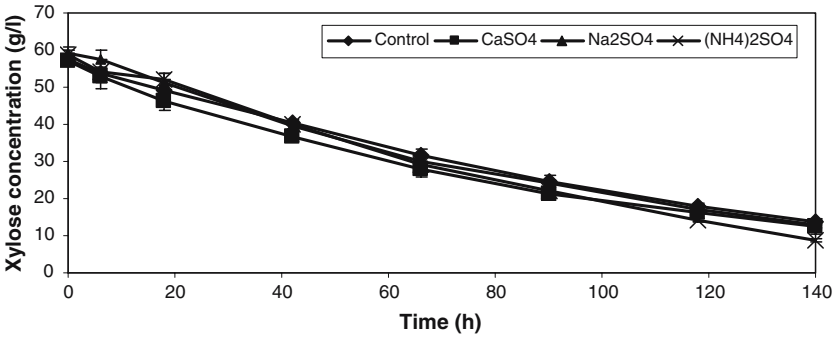

Fig. 3 The effect of salts on xylose consumption by *Pichia stipitis*
*Error bars are ±1 std

Effect of the salts on xylose consumption and ethanol production

The xylose consumption was initially (t < 60 h) faster on the $CaSO_4$ medium but towards the end, xylose consumption was faster in the $(NH_4)_2SO_4$ medium (Fig. 3). The fast rate of xylose consumption initially in $CaSO_4$ medium can be attributed the high cell growth in Fig. 1. However, towards the end of the fermentation (t > 90 h), xylose consumption slowed down in all the treatments apart from the $(NH_4)_2SO_4$ medium

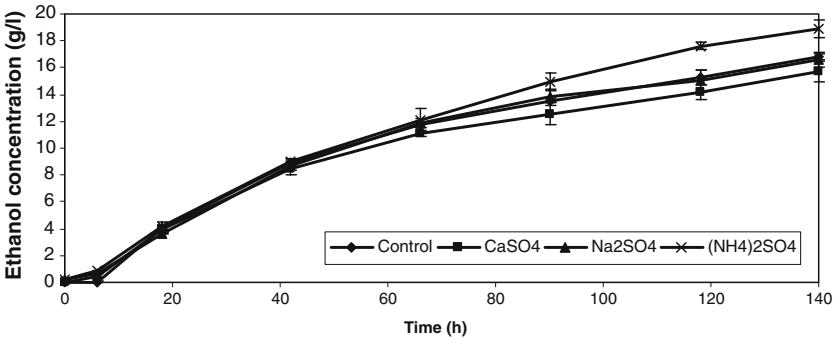

Fig. 4 The effect of salts on ethanol production by *Pichia stipitis**Error bars are ±1 std

Table 1 Average fermentation parameters on the effect of salts on xylose fermentation by *P. stipitis* after 140 h of fermentation

Treatments	Control	$CaSO_4$	Na_2SO_4	$(NH_4)_2SO_4$
Maximum ethanol concentration (g/l)	16.85 ± 0.34	15.74 ± 0.85	16.55 ± 0.52	18.89 ± 0.63
Ethanol production rate (g/l h)	0.12 ± 0.00	0.11 ± 0.01	0.12 ± 0.00	0.13 ± 0.00
Ethanol yield on xylose (g/g)	0.39 ± 0.00	0.35 ± 0.01	0.36 ± 0.01	0.38 ± 0.02
Ethanol yield on cells (g/g)	2.48 ± 0.05	1.97 ± 0.11	2.30 ± 0.07	3.26 ± 0.11
Xylitol yield on xylose (g/g)	0.01	0.02	0	0.01
Xylose consumption rate (g/l h)	0.31 ± 0.01	0.32 ± 0.02	0.33 ± 0.01	0.36 ± 0.01

Errors are ± 1 standard deviation of triplicate experiments

The control was fermentation with no salts added. $CaSO_4$, Na_2SO_4, and $(NH_4)_2SO_4$ were produced by adding 3.3 ml H_2SO_4 (7.6 M) to 25 ml $Ca(OH)_2$ (1 M), 50 ml NH_4OH (1 M), and 50 ml NaOH (1 M) respectively, and adding distilled water to a final liquid volume of 200 ml. The xylose concentration in each solution was 60 g/l. Each solution was filter sterilized

Ethanol production rate was calculated as final ethanol concentration (ethanol concentration at 140 h)/140 h. Xylose consumption rate was calculated as difference between initial xylose concentration and final xylose concentration/140 h

(Fig. 3). This makes the xylose consumption of the entire fermentation period (140 h) higher in the $(NH_4)_2SO_4$ medium compared to the other treatments.

The highest ethanol concentration was produced in the $(NH_4)_2SO_4$ medium and the lowest ethanol concentration was in the $CaSO_4$ medium (Fig. 4). The low ethanol concentration in $CaSO_4$ medium is due to high cell biomass production, because the xylose was used to produce cell mass instead of ethanol. Although $(NH_4)_2SO_4$ medium produced the highest ethanol concentration, the cell biomass produced was the lowest. The NH_3 produced from the dissociation of NH_4^+ stimulate ethanol production in *Pichia stipitis* (Guebel et al. 1992). Xylose consumption and ethanol production in the control and Na_2SO_4 medium were similar (Figs. 3 and 4).

The highest ethanol yield was in the control 0.39 g/g, which is not significantly different from 0.38 g/g for $(NH_4)_2SO_4$ medium, whereas the ethanol yield was 0.35 g/g and 0.36 g/g in $CaSO_4$ and Na_2SO_4 media respectively (Table 1). Ethanol yield per g of cell was higher on $(NH_4)_2SO_4$ compared to the other treatments because of stimulatory effect of ammonia on ethanol production in *Pichia stipitis* and the low cell concentration in $(NH_4)_2SO_4$ medium. Xylitol yield in $CaSO_4$ medium, 0.02 g/g was higher than all the other treatments. The control and $(NH_4)_2SO_4$ media had a xylitol yield of 0.01 g/g whereas there was no xylitol in Na_2SO_4 medium. $CaSO_4$ stimulated xylitol production, $(NH_4)_2SO_4$ had no effect on xylitol production and Na_2SO_4 inhibited xylitol production in *Pichia stipitis*.

Conclusion

Xylose consumed after 140 h of fermentation was highest in the $(NH_4)_2SO_4$ medium compared to the other treatments. The maximum ethanol

concentration after 140 h of fermentation was 18.9 g/l in the $(NH_4)_2SO_4$ medium whilst the lowest ethanol concentration was 15.7 g/l in the $CaSO_4$ medium. The production of ammonia from $(NH_4)_2SO_4$ enhanced ethanol production by *Pichia stipitis*, and therefore ethanol yield per g of cells was 3.3 g/g. The salts produced after neutralizing the excess H_2SO_4 with alkalis such as $Ca(OH)_2$, NaOH and NH_4OH have an effect on the cell growth, xylose consumption, ethanol production, ethanol yield, and xylitol production in *Pichia stipitis*.

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