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Comparison of industrial yeast strains for fermentation of spent sulphite pulping liquor fortified with wood hydrolysate

MT Smith[1], DR Cameron[2] and SJB Duff[1]

[1]*UBC Pulp and Paper Centre and Department of Chemical Engineering, University of British Columbia, 2216 Main Mall, Vancouver, BC V6T 1Z4;* [2]*Tembec Inc, PO Box 3000, Temiscaming, Quebec J0Z 3R0, Canada*

Ethanol production from spent sulphite pulping liquor (SSL) was compared for four different yeasts. A second strain of *S. cerevisiae* as well as a 2-deoxyglucose-resistant strain formed through protoplast fusions between *S. uvarum* and *S. diastaticus* produced up to 27% more ethanol from SSL fortified with hydrolysis sugars than was produced by *S. cerevisiae*. The incremental improvement in ethanol yield appeared to vary with the degree of fortification, ranging from 5.8% for unfortified SSL, to 27% for the highest level of fortification tested. Decreasing fermentation rates were observed for SSL fortified with glucose, mannose and galactose, respectively. Sugar uptake rates in SSL fortified with glucose, galactose and mannose were 6.8, 2.8 and 2.0 g L^{-1} h^{-1}, respectively. However, when these sugars were fermented along with a glucose cosubstrate, the rate at which the combined glucose/mannose medium was fermented was nearly identical to that of the glucose control.

Keywords: spent sulphite liquor; ethanol fermentation; cellulose hydrolysis; *S. cerevisiae; S. uvarum; S. diastaticus*

Introduction

Kraft-pulping technology has all but replaced low yield sulphite pulping due to the difficulty of recovering inorganic chemicals used in the sulphite-pulping process. The fact that low-yield acid sulphite mills often do not practise chemical recovery ensures that dissolved organics, which are typically burned to produce steam in the Kraft process, remain in the pulp mill effluent posing a significant environmental treatment challenge. Much of the biochemical oxygen demand (BOD) of such effluents is associated with wood sugars dissolved during the pulping process. Such sugars can be fermented to reduce the BOD load on (and therefore size of) downstream conventional biological effluent treatment operations, while at the same time producing a marketable product. Two such fermentation plants exist at low yield sulphite-pulping operations in North America: Tembec Inc (Temiscaming, Quebec, Canada) and Georgia Pacific (Bellingham, WA, USA).

Fermentation of spent sulphite liquor (SSL) presents a number of challenges: (1) most yeasts can use only hexose sugars; (2) the sugar content of the SSL is low (2–7%), resulting in prohibitively high costs for recovery of ethanol by distillation; and (3) acetic acid and other inhibitors limit the growth and fermentative capacity of the yeast. From a process perspective, insufficient cooling capacity often results in fermentations being carried out at temperatures well in excess of the optimum for *S. cerevisiae*. Considerable research activity has been focused on each of these problems.

Considerable effort has been directed toward the development of yeast- and bacteria-based pentose fermentation technologies. Using genetic techniques, advances have been made in yeast [3,4,9] and bacterial [5,10] fermentation technologies. Of particular note is the recent report of the introduction of pentose-utilizing capacity (xylose isomerase, xylulokinase, transketolase and transaldolase) into *Zymomonas mobilis* enabling the recombinant organism, CP4(pZB5), to simultaneously ferment glucose and xylose at 95% of theoretical yield [10]. Until these organisms are approved for, and proven under, industrial conditions, there will be an impetus to improve existing technologies for the production of ethanol from the hexose fraction of wood hydrolysates.

Chemical pulping operations produce 15–60 tonnes day^{-1} of waste cellulose fibre, much of which is recovered as primary clarifier sludge (PCS). Since this material is readily converted to fermentable sugars and ethanol using cellulase enzymes [1,2,7,8], it represents a potentially inexpensive way to increase the concentration of fermentable hexose sugars in SSL. Subsequent ethanol production from SSL and PCS-fortified SSL was studied in this investigation using *Saccharomyces cerevisiae* and three other commonly available industrial yeasts.

Materials and methods

Yeast

Conventional *S. cerevisiae* was obtained from the recycle yeast line at Tembec. The other three strains of yeast were obtained from Lallemand Inc (Montreal, Quebec, Canada). *Saccharomyces* strain L1400 was a product of protoplast fusion between *S. uvarum* and *S. diastaticus*, and was reported by the supplier to have enhanced thermotolerance and to be capable of using dextrins and other complex sugars. *Saccharomyces* strain L1491 was a 2-deoxyglucose-resistant derivative of *Saccharomyces* strain L1400, a mutation which rendered it capable of simultaneous uptake of other sugars in the presence of glucose. Strain ‘D’ was

Correspondence: SJB Duff, UBC Pulp and Paper Centre and Department of Chemical Engineering, University of British Columbia, 2216 Main Mall, Vancouver, BC V6T 1Z4, Canada
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a second strain of *S. cerevisiae* alleged to have enhanced competitive characteristics. Inocula for fermentation experiments were grown for 24 h prior to use in an enriched medium consisting of 2% glucose, 1% peptone and 0.5% yeast extract.

Enzymes

Commercially available cellulase and β-glucosidase enzymes, sold under the trade names Celluclast 1.5L and Novozym 188 by Novo-Nordisk (Denmark), were used in all experiments. Celluclast 1.5L has endoglucanase and cellobiohydrolase activities and was found to contain 78 filter paper units (FPU) ml^{-1} of cellulase activity. Novozym 188 contained 780 international units (IU) ml^{-1} of β-glucosidase activity.

Spent sulphite pulping liquor

Tembec Inc operates a low yield, ammonia-based sulphite pulping operation in Temiscaming, Quebec, Canada. Weak red pulping liquor is concentrated from 10–20% solids (w/v) in multiple effect evaporators prior to entering the continuous fermentation train (three CSTRs in series). Concentrated SSL was shipped and stored at 4°C for use in the experiments. The composition of the SSL was (in g L^{-1}): total solids 248, glucose 10.4, mannose 21.8, galactose 2.1, xylose 7.5, acetic acid 2.5, BOD_5 65.

Preparation of hydrolysate

To prepare hydrolysis sugars, PCS from Tembec was hydrolysed using methods previously published [1,2,7,8]. After 48 h of hydrolysis, residual solids were removed by centrifugation ($6250 \times g$, 10 min) and the crude hydrolysate was concentrated using a Rotovap® until a final total reducing sugar (TRS) concentration of 72 g L^{-1} was achieved.

In some experiments, hydrolysis of varying amounts of PCS was carried out in a diluted SSL matrix, by direct addition of the sludge and cellulase enzymes. For these *in situ* hydrolysis runs, 10 FPU Celluclast and 100 IU Novozym were used per gram of PCS added. Hydrolyses were carried out at 50°C using methods previously described [1,2,7,8].

Fermentation experiments

Fermentation experiments were carried out at 33°C and 37°C, which correspond to the upper and lower limits of the temperature range observed in Tembec's fermentation process. Unless otherwise stated, fermentation was carried out in 250-ml Erlenmeyer shake flasks (100 ml working volume), shaken at 150 rpm. SSL was supplemented by the direct addition of 1% peptone and 0.5% yeast extract. To initiate fermentation, 10 vol% inoculum solution was added to flasks containing unfermented SSL in various amounts ranging from 25–100% diluted in water. Representative samples were withdrawn and centrifuged ($13000 \times g$, 5 min). The supernatant phase after centrifugation was analysed for reducing sugars and ethanol.

Analyses

The concentration of total reducing sugars (TRS) in each sample was determined colorimetrically using a dinitrosalicylic acid (DNS) reagent [6] as previously described [1,2,7,8]. For ethanol analysis, a Hewlett-Packard Gas Chromatograph (5880A Series) equipped with a flame ionization detector (FID) was used. Injection port and detector temperatures were 120°C and 200°C, respectively. A Carbowax 20M 30 m × 0.32 mm capillary column (Supelco Inc, Bellefonte, PA, USA) was used. External standards ranging from 0.1–1.0 g L^{-1} ethanol and containing 1.5 g L^{-1} isopropanol as an internal standard were used. The oven temperature was 65°C.

Results and discussion

Fortification of SSL by hydrolysis of PCS

SSL could be readily fortified by *in situ* hydrolysis of PCS (Table 1). There are a number of observations which can be made from these data. First, no increase in the concentration of reducing sugars was observed upon the addition of cellulase enzymes to SSL. This indicated that there is little carbohydrate present in the form of oligomers or residual fibre. Since extensive settling of fibrous material has been observed in the first fermenter at Tembec, this result was surprising, and may have been due to insufficient mixing of the SSL prior to sampling. Second, since we had previously observed a maximum of 66% conversion of the Tembec PCS to reducing sugars [8], it is clear that nearly complete hydrolysis occurred at the lowest PCS loading. Third, the degree of conversion of PCS decreased with increasing PCS loading. This is likely due to the more rapid accumulation of sugars in the flasks with higher PCS concentrations as well as the increased binding of enzyme to 'non-reactive' sites.

With these data, we are unable to resolve the influence of enzyme inhibition by sugars and other components of SSL from the impact of the time-dependent loss of enzyme activity associated with non-hydrolytic binding. However the total impact of these factors is evidenced by the 20% decrease in the yield of sugars when comparing the hydrolysis of 50 g L^{-1} PCS in the presence or absence of SSL.

Fermentation of glucose, PCS hydrolysates

When glucose was used as a model substrate, there was no discernible difference in the performance of any of the three yeast strains tested, with each strain able to ferment glucose to ethanol at 96% efficiency in 24 h (results not shown). Similarly, each strain was able to ferment PCS hydrolysate, which was predominantly glucose, with approximately

Table 1 *In situ* hydrolysis of primary clarifier sludge (PCS) in diluted spent sulphite liquor (SSL) (6.7% solids) after 72 h incubation

Medium	Total reducing sugars after 72 h (g L^{-1})	% Conversion of PCS to TRS in 72 h
SSL (Control, no enzymes)	21	–
SSL	21	–
50 g L^{-1} PCS	33	66
SSL + 20 g L^{-1} PCS	33	60
SSL + 50 g L^{-1} PCS	47.5	53
SSL + 80 g L^{-1} PCS	61	50

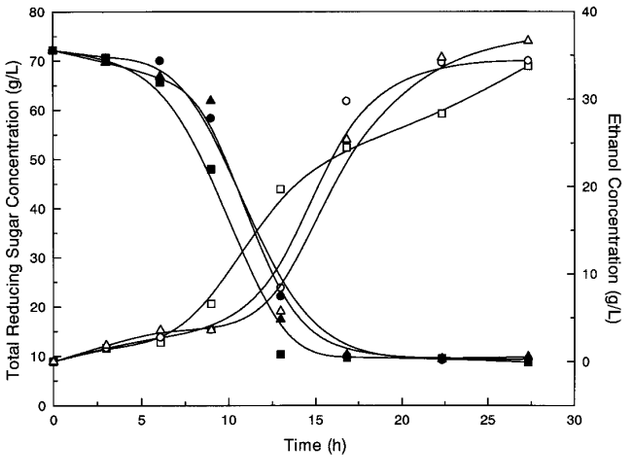

Figure 1 Sugar uptake (closed symbols) and ethanol production (open symbols) by three yeast strains in PCS hydrolysate. Symbols: ●/○ *Saccharomyces* strain 1400, ■/□ *Saccharomyces* strain 1491, ▲/△ *Saccharomyces* strain C.

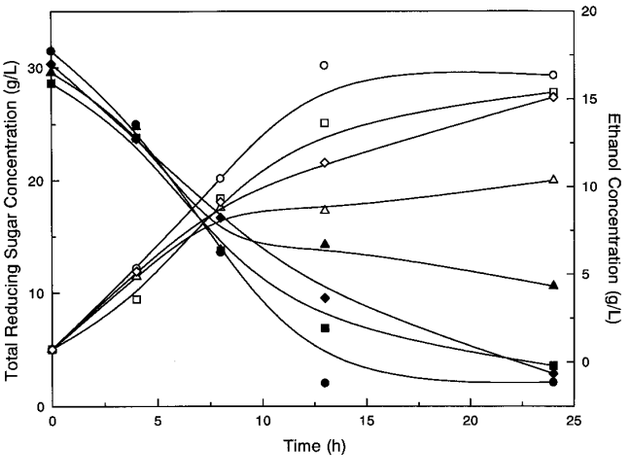

Figure 2 Sugar uptake (closed symbols) and ethanol production (open symbols) by four yeast strains in glucose/galactose media. Symbols: ●/○ *Saccharomyces* strain 1400, ■/□ *Saccharomyces* strain 1491, ▲/△ *Saccharomyces* strain C, and ◆/◇ *Saccharomyces* strain D.

equal efficiency (Figure 1). In all fermentations involving hydrolysate, approximately 13% of the TRS from the hydrolysate remained after fermentation (Figure 1). This non-fermentable TRS is likely comprised of pentose sugars such as xylose and arabinose, as well as small amounts of non-fermentable hexose sugars.

Fermentation of SSL

Trials with SSL showed that approximately 34% of the initial TRS was not fermented by any of the yeast strains tested (results not shown). It is suspected that this fraction consists primarily of 5-carbon sugars. *Saccharomyces* strain D produced more ethanol than did the original strain C in both fortified SSL runs. The incremental improvement in ethanol yield appeared to vary with the degree of fortification, ranging from 5.8% for unfortified SSL, to 27% for the highest level of fortification tested (Table 2).

While the thermotolerance of *Saccharomyces cerevisiae* is purported to be limited, we saw only a slight (6%) decrease in ethanol concentration when the fermentation temperature was increased to 37°C. *Saccharomyces* strains L1400, L1491 and D each performed as well at 37°C as at 33°C.

Table 2 Ethanol production in 33% SSL with and without fortification with hydrolysis sugars (HS)

Saccharomyces strain	Ethanol (g L^{-1}) in 33% SSL medium containing		
	No fortification	8 g L^{-1} HS	13 g L^{-1} HS
C	5.2	10.0	15
1400	5.8	10.1	16
1491	5.15	12.6	18.7
D	5.6	12.6	19

Fermentation of component sugars

Batch experiments were performed to characterize the ability of each yeast strain to ferment individual 5- and 6-carbon sugars alone, or with SSL. For each of the yeast strains tested, glucose and mannose were fermented rapidly, galactose was metabolized more slowly, and arabinose, a 5-carbon sugar, was not metabolized. Of the sugars tested, the uptake rate of galactose varied most dramatically (Figure 2). Strain C was least capable of utilizing galactose as a substrate. Of the three strains which were able to use galactose as a substrate, the maximum galactose uptake rate was observed with *Saccharomyces* strain 1400, at approximately 3.1 g L^{-1} h^{-1}.

The addition of SSL also influenced the metabolism of sugars by the yeasts. For example, the uptake rate of mannose, which was approximately equal to that of glucose in pure sugar growth media (Figure 3) was considerably

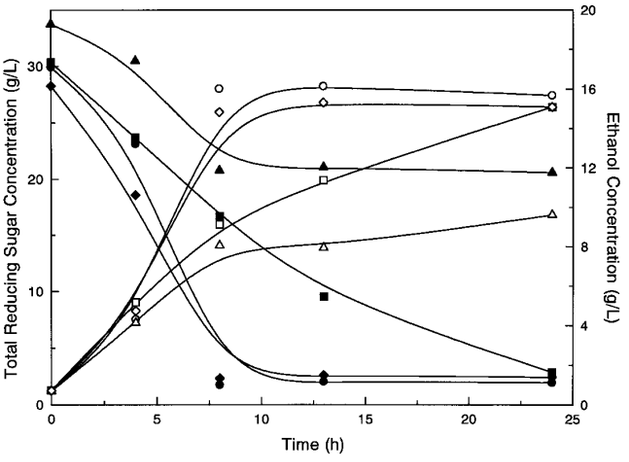

Figure 3 Sugar uptake (closed symbols) and ethanol production (open symbols) by *Saccharomyces* strain D in: ●/○ glucose, ■/□ glucose and galactose, ◆/◇ glucose and mannose, and ▲/△ glucose and arabinose.

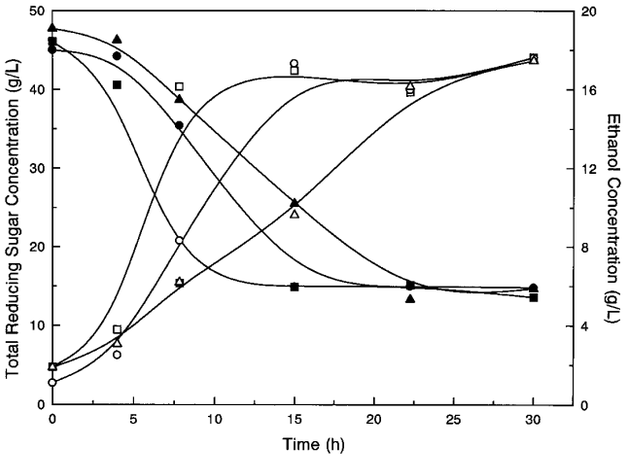

Figure 4 Sugar uptake (closed symbols) and ethanol production (open symbols) by *Saccharomyces* strain D in SSL fortified with: ■/□ glucose, ▲/△ galactose, or ●/○ mannose.

retarded in the presence of SSL (Figure 4). Sugar uptake rates in SSL fortified with glucose, mannose, and galactose were 6.8, 2.8 and 2.0 g L^{-1} h^{-1}, respectively.

Conclusions

Ethanol production can be increased up to 20% through preliminary attempts at improved yeast strain selection. Using carbon sources other than glucose, *Saccharomyces* strains L1400, L1491 and D all performed consistently better than the control *S. cerevisiae*. Differing rates of fermentation of individual wood sugars were observed: with glucose and mannose fermented at approximately the same rate, galactose fermented more slowly, and arabinose was not fermented.

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