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Production of Ethanol from Pulp Mill Hardwood and Softwood Spent Sulfite Liquors by Genetically Engineered *E. coli*

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ABSTRACT

Although lignocellulosic biomass and wastes are targeted as an attractive alternative fermentation feedstock for the production of fuel ethanol, cellulosic ethanol is not yet an industrial reality because of problems in bioconversion technologies relating both to depolymerization and fermentation. In the production of wood pulp by the sulfite process, about 50% of the wood (hemicellulose and lignin) is dissolved to produce cellulose pulp, and the pulp mill effluent ("spent sulfite liquor" SSL) represents the only lignocellulosic hydrolysate available today in large quantities (about 90 billion liters annually worldwide). Although softwoods have been the traditional feedstock for pulping operations, hardwood pulping is becoming more popular, and the pentose sugars in hardwood SSL (principally xylose) are not fermented by the yeasts currently being used in the production of ethanol from softwood SSL.

This study assessed the fermentation performance characteristics of a patented (US Pat. 5,000,000), recombinant *Escherichia coli* B (ATCC 11303 pLOI297) in anaerobic batch fermentations of both nutrient-supplemented soft and hardwood SSL (30–35 g/L total reducing sugars). The pH was controlled at 7.0 to maximize tolerance to acetic acid. In contrast to the high-performance characteristics exhibited in synthetic media, formulated to mimic the composition of softwood and hardwood SSL (yield approaching theoretical maximum), performance in SSL media was variable with conversion efficiencies in the range of 67–84% for hardwood SSL and 53–76% for softwood SSL. Overlimiting treatment of HSSL, using $Ca(OH)_2$, improved overall volumetric productivity two- to sevenfold to a max of 0.42 g/L/h at an initial cell loading of

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0.5 g dry wt/L. A conversion efficiency of 92% (6.1 g/L ethanol) was achieved using diluted $Ca(OH)_2$-treated hardwood SSL. The variable behavior of this particular genetic construct is viewed as a major detractant regarding its candidacy as a biocatalyst for SSL fermentations.

Index Entries: Ethanol; hemicellulose fermentation; spent sulfite liquor; pentose; recombinant *E. coli*.

INTRODUCTION

The search to find alternative, renewable sources of energy and chemicals has fostered research and development relating to the design of efficient economical processes for converting forestry, agricultural, and municipal wastes into fuels and chemicals (*1–3*). Lignocellulosic biomass is considered an appropriate alternative fermentation feedstock for fuel ethanol production, because it is inexpensive, plentiful, and renewable (*4*). The structure of lignocellulosic biomass is complex and consists primarily of three different polymeric substances:

1. Lignin;
2. Hemicellulose; and
3. Cellulose

the latter two being carbohydrates and the potential source of fermentable sugars. However, depolymerization of these carbohydrates is a necessary prerequisite of fermentation. Depolymerization can be effected either by thermochemical (''hydrolysis'') or enzyme-catalyzed (''digestion'') processing. Unlike hemicellulose, which is efficiently hydrolyzed under relatively mild conditions with dilute acid, cellulose is much more resiliant and resistant to depolymerization (*5*). Woody biomass is strongly resistant to enzymic digestion unless it is ''pretreated'' to remove the impediments to enzyme digestion that are caused by lignin and the acetylated pentosan comprising the hemicellulose fraction of biomass (*6–8*). Hence, pretreatment (''prehydrolysis'') not only yields the monomeric sugar components of hemicellulose, but also produces acetic acid, which is commercially exploited as an antimicrobial agent (*9*). Other byproducts of thermochemical processing are known to be toxic to both yeasts and bacteria (*10–13*), and various procedures have been investigated for minimizing toxicity of lignocellulosic hydrolysates to ethanologenic microorganisms (*14–18*).

Several different prehydrolysis processes have been designed and tested experimentally (for a review, *see 19*), but none is yet operational on an industrial scale. The only lignocellulosic hydrolysate available today in large quantities is spent sulfite liquor (SSL), a byproduct of the sulfite pulp process (*20*). In 1981, the annual world production of pulp using the

sulfite process was 11.1 million tons (dry wt) with almost one-quarter of that amount being produced in Canada (*21*). Canada is the world's largest exporter of wood pulp, 60% of which is produced by chemical pulping. About 73% of that is processed by the Kraft process and about 20% by the sulfite process.

SSL is essentially a hemicellulose hydrolysate from wood, containing hexose and pentose sugars. About 2200 US gallons of SSL (11–14% solids) are produced/ton of pulp produced, which represents up to 50% of the wood raw material used in the pulping process (*22*). For each ton of pulp produced, there is approx 1 ton on solid waste in the wash waters. The composition of the sugar fraction in SSL depends on the type of wood used for pulping. Coniferous (''soft'') woods yield a high proportion of hexose sugars (predominantly mannose and glucose), whereas deciduous (''hard'') woods yield a liquor containing a high proportion of pentose sugars (predominantly xylose) (*22*).

The sulfite pulping process involves the delignification of wood in an aqueous solution, containing alkaline-earth bisulfites and an excess of SO_2, whereby the lignin is solubilized by combining with either the SO_2 or HSO_3^- (*22*). The wood cellulose remains undegraded, whereas the less resistant hemicellulose is hydrolyzed to its constituent monomeric sugars.

The high level of soluble organics in SSL (primarily the fermentable sugars) results in an inherently high BOD (25,000–40,000 ppm) (*22*), and the increasingly more stringent environmental protection regulations require some form of waste-water treatment prior to discharge of mill effluent into local lakes and rivers. Fermentation of SSL is a means of reducing BOD while producing value-added byproducts. Production of fermentation alcohol from SSL dates back to 1907, when the first experimental plant was built in Sweden (*23*). During World War II, SSL was fermented to produce both alcohol for use as an alternative transportation fuel and yeast as a source of protein and vitamins (*22*). Prior to the discovery of ethanologenic organisms capable of utilizing pentose sugars, it had not been possible to produce ethanol from the pentose sugars in SSL. The recent shift to increased hardwood pulping in Canada exacerbates the problem of waste-water treatment because of the inability of the currently employed biocatalyst *Saccharomyces cerevisiae* to ferment pentose sugars (*24*).

Tembec Inc., a major Canadian producer of chemical-grade pulps, has recently begun to produce ethanol from its waste SSL. Because the Tembec plant (Temiscaming, Quebec) alternates between softwood and hardwood feedstocks, operating about one-third of the time on hardwood, the SSL produced by this facility presented a unique opportunity to compare fermentations of both softwood SSL (SSSL) and hardwood SSL (HSSL) from the same mill. At the present time, this plant uses an SSL-acclimated *S. cerevisiae* yeast (R. Benson—personal communication) and, hence, operates with the limited capacity to effectively ferment only softwood

SSL. However, initial feasibility studies, made prior to commissioning of the ethanol production plant, included fermentation performance testing with a pentose-utilizing yeast, *Pichia stipitis*, and the published results of several independent investigations (*24–26*) were available for the purpose of making a direct comparative fermentation performance assessment using a new, and potentially more efficient, biocatalyst.

For the past two years, the authors have been assessing the fermentation performance of a patented (*27*), genetically engineered ethanologen, *E. coli* B. (ATCC 11303 carrying the so-called PET plasmid designated pLOI297) (*28–31*) using both synthetic lab media and prehydrolysates prepared by different thermochemical processes from a variety of lignocellulosic feedstocks, including both hardwood (aspen) and softwood (pine) (*32–35*). The objective of this investigation was to compare the fermentation efficiency of two patented pentose ethanologens, namely recombinant *E. coli* B. (pLOI 297) (*27*) and *P. stipitis* (*36*), in the search for a more economic alternative to acclimated *S. cerevisiae* yeast.

MATERIALS AND METHODS

Organisms

Escherichia coli B. (ATCC 11303 carrying the "PET" plasmid pLOI297) (*28*) was a gift from L. O. Ingram (University of Florida, Gainsville, FL). Inocula were prepared in buffered Luria broth (LB) (*37*) containing 40 μg ampicillin and 10 μg tetracycline/mL medium, but antibiotics were absent from all fermentation media. Cultures were stored (−10°C) in LB/glycerol-citrate and were plated on selective media (LB + 20 g/L agar + 10 mg/L tetracycline and 40 mg/L ampicillin).

Spent Sulfite Liquors

Ammonia-based spent sulfite liquors (about 20–22% solids) were supplied to Temeco Enterprises Inc., a subsidary of Tembec Inc. (Temiscaming, Quebec), and were from either softwood (SSSL) or hardwood (HSSL) sulfite pulping.

Synthetic and SSL Culture Media

Hardwood SSL synthetic medium consisted of the following (g/L): 2.5, yeast extract (Difco); 0.25, $(NH_4)_2PO_4$; 0.0125, $MgSO_4$; 14.2, Na_2HPO_4; 12.0, NaH_2PO_4; 6.8, potassium acetate; 5.5, glucose, 29.8, xylose; 8.1, arabinose. The xylose and arabinose were obtained from Sigma Chemical Co. (St. Louis, MO). Softwood SSL synthetic media consisted of the following (g/L): 5.0, yeast extract; 10.0, tryptone (Difco); 5.0, NaCl; 23.2, mannose; 13.6, xylose; 8.9, glucose. The mannose was obtained from

Sigma Chemical Co. (St. Louis, MO). SSL media were prepared by supplementing the SSL, as received, with tryptone (10 g/L) and yeast extract (5 g/L), and adjusting the pH to 7 with KOH.

Overliming Treatment of SSL Media

Where indicated, the nutrient-supplemented SSL medium was treated as follows: powdered $Ca(OH)_2$ was added with stirring to pH 10 followed by neutralization (pH 7) with 1*N* H_2SO_4 and centrifugation to remove insolubles.

Fermentation Equipment

Batch fermentations were conducted in MultiGen™ (model F2000) stirred-tank bioreactors having agitation, pH (2*N* KOH), and temperature control (30°C) (New Brunswick Scientific Co., Edison, NJ).

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1-cm light path), and culture dry wt was measured by microfiltration—washing and drying the filter to constant weight under an infrared heat lamp. Compositional analyses of fermentation media, cell-free spent media, and SSL were determined using an HPLC equipped with an RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs). Separations were performed at 65 and 85°C on HPX-87H and HPX-87P columns, respectively (Bio-Rad Labs) (injection vol=0.02 mL).

RESULTS AND DISCUSSION

The Sulfite Pulping Process

Figure 1 is a process flow diagram that illustrates the integration of the fermentation of the SSL with the pulping process and the production of other byproducts. The Tembec pulp mill uses an ammonium sulfite process. The SSL is pasteurized in processing through the cooker/digesters. Aldonic acid formation during cooking leads to a loss in sugar yield in the SSL and the amounts available for fermentation are reduced to about 200 kg/ton of strong paper pulp and 280 kg/ton for rayon pulp cooking (as in Tembec plant) (*24*). The corresponding maximum theoretical yield of 95% ethanol would be 137 and 190 L/t of pulp, respectively, but these yields assume not only 100% conversion efficiency by the biocatalyst, but also 100% product recovery efficiency. The amount of acetic acid varies with the type of wood used and typically ranges from 30–90Kg/t of pulp for soft and hardwoods, respectively (*24*). About 15,000 L of SSL (''weak red liquor'' at about 12% solids) is produced/t of pulp, which is reduced

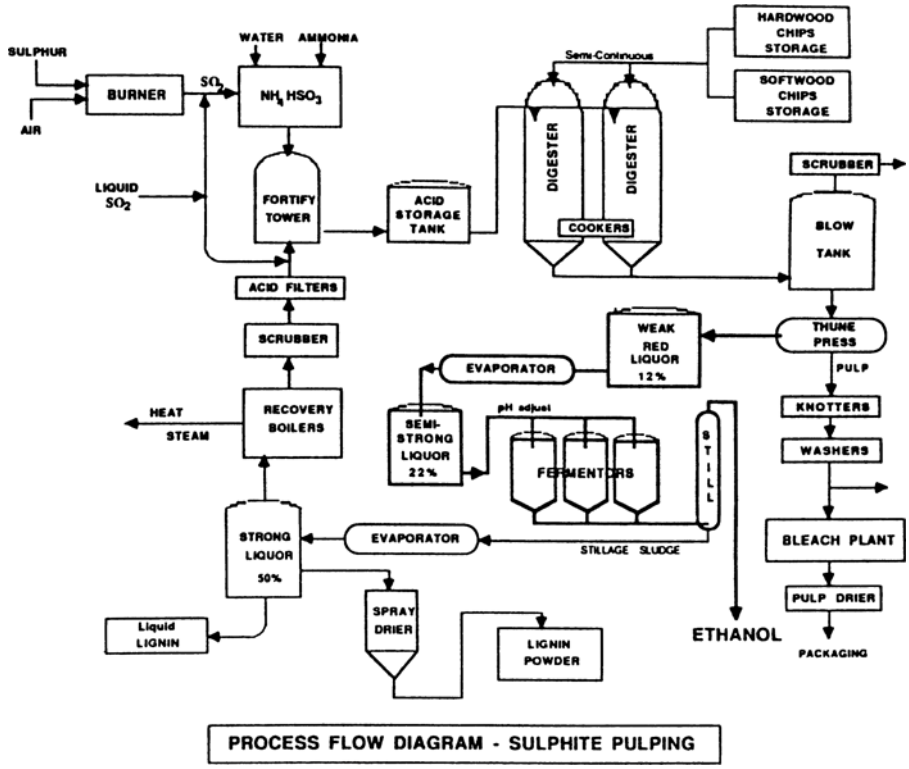

Fig. 1. Process flow diagram of wood pulping by an ammonia-based sulfite process.

to 8200 L ("semi-strong liquor" at about 22% solids) by an evaporator situated upstream of the train of fermentors (Fig. 1). The evaporator is important, because it not only increases the concentration of the fermentable sugars, but it also reduces the amounts of potentially toxic volatile sulfur compounds (SO_2). The concentration of acetic acid, however, is not altered by the evaporation process. Before entering the fermentors, the "semi-strong liquor" (about 22% solids) is supplemented with ammonium sulfate and superphosphate, and the pH adjusted to about 4.5 (Fig. 1). The fermentors are operated as a continuous-flow cascade with a residence time of about 24 h. With SSL-adapted *S. cerevisiae*, the overall process (i.e., fermentation and distillation) sugar-to-ethanol conversion efficiency in the production of rayon pulp (based on softwood SSL-reducing sugars) is in the range of 53–68% of theoretical maximum (24). Sulfonated lignin can be recovered as a marketable byproduct. The still bottoms and unfermented residual sugars (especially in the case of hardwood SSL) are concentrated by evaporation, and used as boiler feed or sold as animal feed (24).

Table 1
Compositional Analysis of Hardwood and Softwood Spent Sulfite Liquors[a,c]

Feedstock and Source	Pentose, g/L		Hexose, g/L			TRS g/L	Acetic acid, g/L
	Xylose	Arabinose	Mannose	Glucose	Galactose		
Hardwood SSL							
Sample No. 1	20.8	-	7.1	1.8	-	29.7	9.3
Sample No. 2	24.0	-	6.5	3.0	-	33.5	7.6
Softwood SSL	8.3	-	17.1	6.3	3.7	35.4	2.7

[a] Analyses were performed by HPLC (*see* Materials and Methods section for details).
[b] SSL = spent sulfite liquor.
[c] All SSL samples (ammonium based and approx 20% solids) were obtained from TEMBEC Inc., Temiscaming, Quebec, Canada.

Composition of Hardwood and Softwood SSL

The results of the HPLC compositional analysis of the HSSL and SSSL are summarized in Table 1. The amount of reducing sugars (TRS) was about the same in all samples tested (3–3.5% w/v). However, whereas the HSSL was predominantly xylose (70%), the SSSL contained a much higher amount of hexose sugars (76%) of which about two-thirds was mannose (Table 1). Another notable difference was the three-times higher level of acetic acid in the HSSL (Table 1).

Fermentation of Hardwood SSL

Since the chemical composition of SSL is complex and could be expected to contain unknown, potentially toxic elements (*10–13,24*), the fermentation performance of the recombinant *E. coli* was first tested in a synthetic medium, the composition of which was designed and formulated to mimic the HSSL with respect to both reducing sugars and acetic acid. The time-course of the fermentation in the nutrient-supplemented model medium, with respect to both sugar utilization and ethanol production, is shown in Fig. 2. The fermentation parameters, for both yield and productivity, are summarized in Table 2. At an inoculation cell density of about 0.24 g dry wt/L, fermentation of the 4.5% TRS (75% xylose) was completed in 32 h, and the high degree of conversion efficiency (92% theoretical maximum) showed that acetic acid (4.2 g/L) was not inhibitory under the conditions employed (Fig. 2 and Table 2).

However, fermentation trials with nutrient-supplemented HSSL using the recombinant *E. coli* (inoculated at 0.5 g/L) were unexplainably variable with respect to both yield and productivity. The results of the first trial (expt. B1) were very encouraging, with a yield of 84% theoretical and a

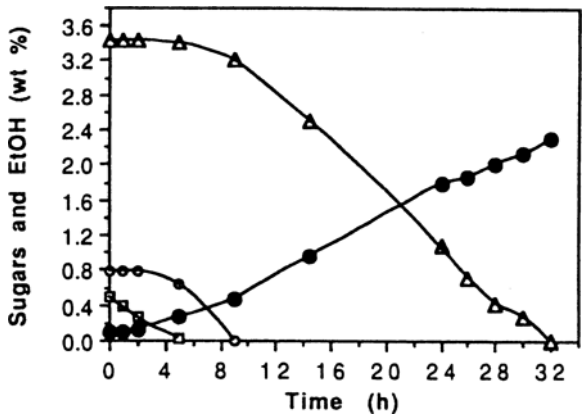

Fig. 2. Fermentation of a synthetic hardwood SSL by recombinant *E. coli* B ATCC 11303 (pLOI297). The inoculation cell density was 0.24 g dry wt/L, and the pH was controlled at 7.0 by 2*N* KOH addition. Data from expt. REC-B54T (*see* Table 2). Symbols (% by wt.): (△) xylose; (□) glucose; (○) arabinose; (●) ethanol.

fermentation time of 54 h (Fig. 3 and Table 2). However, the second experiment (B2) with the first sample of HSSL, was terminated after 80 h with only about half of the xylose having been consumed. After obtaining a second sample of HSSL from our supplier, the experiment was repeated a third time (expt. B5), and the results paralleled those of the second experiment (Fig. 3 and Table 2). It required 150 h to ferment the 3% sugars completely, and the ethanol conversion efficiency was only 70%.

The authors have previously demonstrated the efficacy of an overliming treatment (*see* Materials and Methods) in reducing the toxicity of sulfite-containing media to the recombinant *E. coli* (*19,33*). Calcium hydroxide treatment of the HSSL (expt. B6) resulted in a dramatic improvement in productivity (fermentation time decreased from 150 to 24 h). Calcium hydroxide treatment also resulted in a slight improvement in yield, from 0.36 to 0.40 g/g (Fig. 4 and Table 2). A similar, but modified $Ca(OH)_2$ procedure was used to improve the biocompatability of pinewood acid hydrolysates for another ethanologenic recombinant *E. coli*, strain KO11 (*38*).

The suspicion that there might be substances in the HSSL that negatively affect the fermentation performance of the recombinant *E. coli* was confirmed in experiments (B8 and B9) in which the medium was diluted approx 1:1 with water (Fig. 5). Figure 5 shows that all sugars are utilized simultaneously. The results are summarized in Table 2, and show that dilution of the HSSL medium resulted in an improvement in both yield and productivity to values more closely approximating those observed with the synthetic medium. In experiment B8 (Table 2), the ethanol yield was 0.43 g/g with 7.3 g/L ethanol being produced in 12 h. This can be compared to the example cited in the Alfa-Laval patent (*36*) relating to the use of pentose-fermenting yeasts in which *P. stipitis* CBS 6054 was used

Table 2
Production of Ethanol from Hardwood Spent Sulfite Liquor by Recombinant *Escherichia coli* B (pLOI297)

Medium treatment/expt.		Initial cell concentration, g dry wt/L	pH	Total sugar, g/L	Acetic acid, g/L	Fermentation time, h	Ethanol, g/L	Yield $Y_{p/s}$,[b] g/g	Productivity Q_p,[c] g/L/h	Productivity q_p,[d] g/g/h
Synthetic hardwood "prehydrolysate"										
REC-B54		0.2	7.0	43.4	4.2	36	21.6	0.49	0.59	
REC-54(T)		0.2	7.0	46.9	4.2	32	22.0	0.47	0.69	
Hardwood spent sulfite liquor										
Sample No. 1										
Fresh/untreated	B1	0.5	7.0	29.7	9.3	54	13.0	0.43	0.24	0.48
	B2	0.5	7.0	27.4	9.2	155[e]	9.6[e]	0.34[e]	0.06[e]	0.12[e]
Sample No. 2[a]										
Fresh/untreated	B5	0.5	7.0	30.3	7.2	150	10.9	0.36	0.07	0.13
$Ca(OH)_2$ treated	B6	0.5	7.0	25.1	5.7	24	10.0	0.40	0.42	0.84
Diluted/untreated	B8	0.5	7.0	16.9	3.8	12	7.3	0.43	0.61	1.22
$Ca(OH)_2$ treated	B9	0.5	7.0	13.0	3.2	11	6.1	0.47	0.55	1.10

[a]Expt. B7 (not shown) was "control" for Expt. B5 with added $CaSO_4$ (results were same as for Expt. B5).
[b]Yield ($Y_{p/s}$, g/g) was based on total reducing sugars.
[c]Volumetric productivity (Q_p, g/L/h) was determined as final ethanol concentration divided by time for complete sugar utilization.
[d]Specific productivity (q_p, g/g/h) was calculated by dividing Q_p by initial cell (dry wt) concentration.
[e]Values estimated from extrapolated fermentation time course post 80 h (see also Expt. B5).

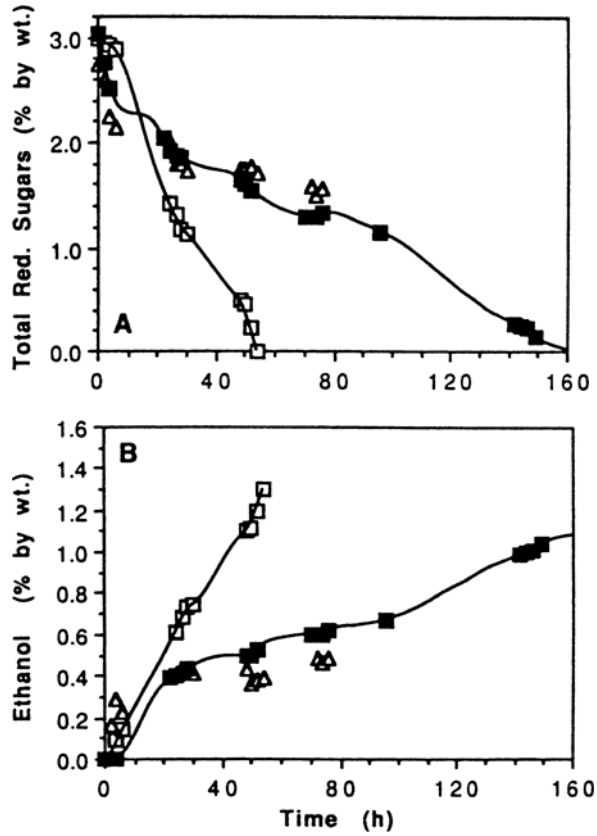

Fig. 3. Fermentation of hardwood SSL by recombinant *E. coli* B. (A) Sugar (as total reducing sugars), and (B) ethanol production. Inoculation cell density was 0.5 g dry wt/L, pH was controlled at 7.0, and temperature at 30°C. Fermentations B1 and B2 were performed on HSSL Sample No. 1, whereas B5 was performed with HSSL Sample No. 2 (*see* Table 1). Symbols (% by wt): (□) expt. B1; (△) expt. B2; (■) expt. B5.

to ferment SSL with a yield of 0.41 g/g or 9.9 g/L ethanol in a period of 24 h. With reference to the process flow diagram shown in Fig. 1, the improved fermentation performance with the diluted HSSL medium suggests that bypassing the first evaporator might prove an effective means of increasing productivity. However, the potential gain could be offset by the increase in energy required in distillation and recovery of a lower product concentration.

Fermentation of Softwood SSL

Although softwood hemicellulose contains much less pentose and therefore the economic impact might be anticipated to be less when softwood is utilized as feedstock, it is nevertheless important to assess the fermentation efficiency of the genetically engineered biocatalyst with SSSL

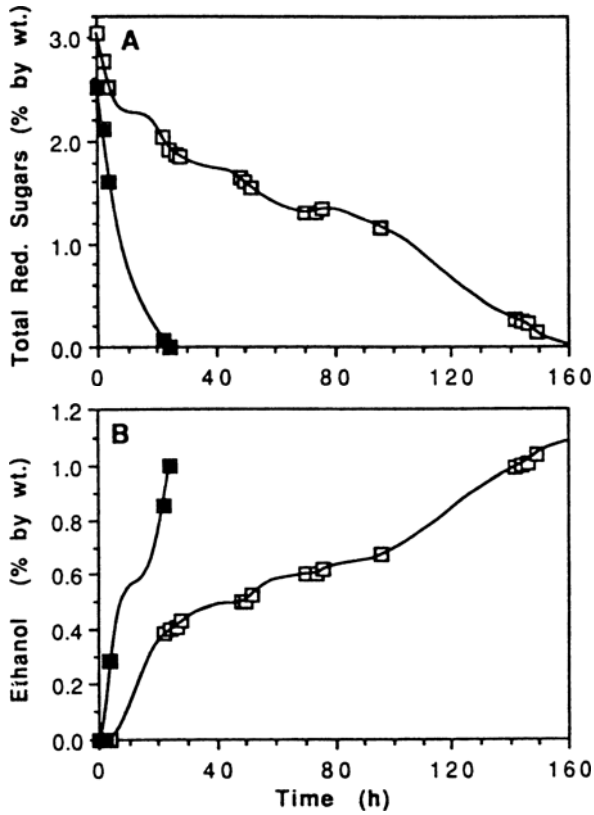

Fig. 4. Fermentation of hardwood SSL by recombinant *E. coli* B: Effect of $Ca(OH)_2$ treatment of medium. (A) Sugar (as total reducing sugars), and (B) ethanol production. Conditions were as described in Fig. 3. Symbols (% by wt.): (□) control expt. B5; (■) expt. B6—medium treated with calcium hydroxide as described in Materials and Methods.

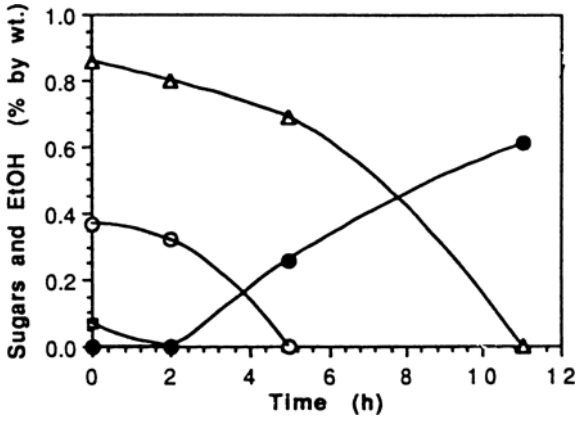

Fig. 5. Fermentation of hardwood SSL by recombinant *E. coli* B: Effect of dilution of the medium 1:1 with water. Conditions were as described for Fig. 3. Data from expt. B9 (*see* Table 2). Symbols (% by wt): (△) xylose; (○) mannose; (□) glucose; (●) ethanol.

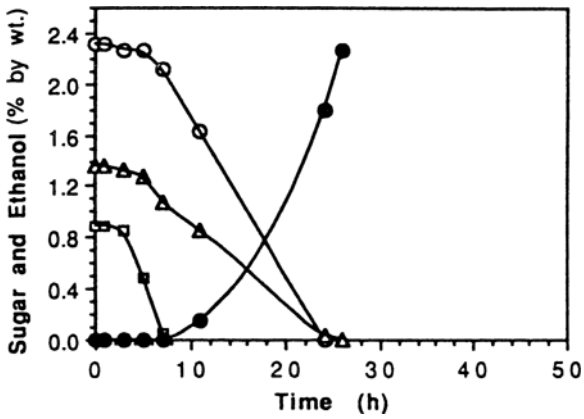

Fig. 6. Fermentation of a synthetic softwood SSL by recombinant *E. coli* B ATCC 11303 (pLOI297). The inoculation cell density was 0.05 g dry wt/L, and the pH was controlled at 7.0 by 2*N* KOH addition. Data from expt. REC-B39 (*see* Table 3). Symbols (% by wt): (○) mannose; (△) xylose; (□) glucose; (●) ethanol.

because (1) coniferous woods are the major raw material, and (2) the product yield exhibited by *S. cerevisiae* yeast (<60% conversion efficiency) (*24*) offers a good opportunity for improvement.

Fermentations with the softwood SSL were fashioned after those previously conducted using the HSSL and initially involved testing with a synthetic medium that was formulated to mimic the composition of SSSL (Fig. 6 and Table 3). With the nutrient-supplemented synthetic SSSL, both the conversion efficiency (96% theoretical) and productivity (4.5% TRS fermented in 26h) were excellent.

However, as was observed with the HSSL, fermentations of the softwood SSL were variable, most notably with respect to the product yield. Experiment B1 (Fig. 7 and Table 3) is typical of a series of batch fermentations that produced particularly poor yields (0.21–0.27 g/g), and in which lactic acid was produced in addition to ethanol and carbon dioxide. These three major fermentation end products accounted for >94% of the carbon in the reducing sugars. The recombinant is resistant to the antibiotics tetracycline and ampicillin, but inclusion of these in the fermentation medium did not improve the yield. Preliminary results of our investigation into the mechanism of the poor sugar-to-ethanol conversion efficiency exhibited by isolated antibiotic-resistant recombinant variants suggest the metabolic shift toward lactic acid production may relate to the low copy number of the PET (pLOI297) plasmid with selective pressure seemingly because of extended exposure to mannose as the predominant carbon source (Lawford and Rousseau, unpublished observations). The manner in which mannose may affect replication of pLOI297 in *E. coli* B (ATCC 11303) remains problematic (*19*). However, the issue of plasmid instability

Table 3
Production of Ethanol from Softwood Spent Sulfite Liquor by Recombinant *Escherichia coli* B (pLOI297)

Medium treatment/expt.	Initial cell concentration, g dry wt/L	pH	Total sugar, g/L	Acetic acid, g/L	Fermentation time, h	Ethanol, g/L	Yield $Y_{p/s}$,[b] g/g	Productivity Q_p,[c] g/L/h	Productivity q_p,[d] g/g/h
Synthetic softwood "prehydrolysate"									
REC-B39	0.05	7.0	45.7	–	26	22.6	0.49	0.87	
Softwood spent sulfite liquor									
Untreated B1	0.5	7.0	37.2	2.7	46	10.0	0.27	0.22	0.44
B2	0.5	7.0	35.2	2.7	41	13.7	0.39	0.33	0.67

[b] Yield ($Y_{p/s}$, g/g) was based on total reducing sugars.
[c] Volumetric productivity (Q_p, g/L/h) was determined as final ethanol concentration divided by time for complete sugar utilization.
[d] Specific productivity (q_p, g/g/h) was calculated by dividing Q_p by initial cell (dry wt) concentration.

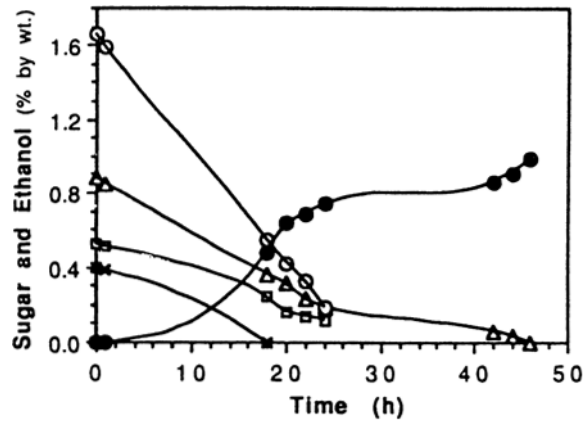

Fig. 7. Fermentation of softwood SSL by recombinant *E. coli* B. Inoculation cell density was 0.5 g dry wt/L, pH was controlled at 7.0, and temperature at 30°C. Data from expt. SSSL B1 (*see* Table 3). Symbols (% by wt): (○) mannose; (△) xylose; (□) glucose; (X) galactose; (●) ethanol.

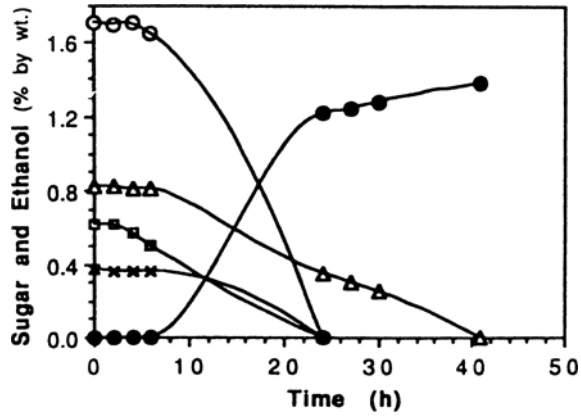

Fig. 8. Fermentation of softwood SSL by recombinant *E. coli* B. Conditions were as described for Fig. 7. Data from expt. SSSL B2 (*see* Table 3). Symbols (% by wt): (○) mannose; (△) xylose; (□) glucose; (X) galactose; (●) ethanol.

may be considered of less practical importance in light of the newest generation of a more stable, chromosomally integrated ethanologens (e.g., *E. coli* strain KO11) (*39*).

Experiment B2 (Fig. 8 and Table 3) is typical of a series of batch fermentations in which ethanol was the major end product, but in which the yield was only about 0.4 g/g (78% conversion efficiency). In these latter experiments, the medium was either microfilter sterilized or autoclaved in order to ensure pure culture fermentation.

Fermentation Performance of Recombinant *E. coli* Compared to *Pichia stipitis* and *Saccharomyces cerevisiae*

The results of independent fermentation assessments using different biocatalysts and spent sulfite liquors produced by the Tembec pulp mill are reported in the literature (*24–26*) and offer a rare opportunity to make direct comparisons among different ethanologenic organisms. Table 4 summarizes the fermentation parameters from these independent investigations in which both softwood and hardwood SSL were employed. Because different amounts of biocatalyst were used in these studies, comparisons with respect to fermentation time (i.e., volumetric productivity Q_p and specific productivity q_p) are difficult and perhaps not very meaningful. Another important element that prevents direct across-the-board comparisons is the different levels of acetic acid and/or pH that were used. Both *S. cerevisiae* and *P. stipitis* (at their optimal pH of 4.5 and 5.5, respectively) are severely inhibited by the levels of acetic acid present in HSSL (*17,20,40–42*). With the recombinant *E. coli*, sensitivity to acetic acid can be reduced by operating at pH 7 (*19,35*). This approach has also been used with *P. stipitis* CBS 5776 in which the pH was elevated to 6.5; however, at the higher pH, there is an increased opportunity for bacterial contamination (*24*). The acetic acid concentration can be lowered by steam-stripping (*26,41*), but the cost of this extra operation would have too high a negative economic impact on the cost of ethanol production (*24*). Ultimately, it was the acetic acid sensitivity of *P. stipitis* that caused it to fail in Tembec's feasibility study as an alternative process organism to *S. cerevisiae* for ethanol production from mixed feedstock SSL (*24*).

Proper control of the rate at which oxygen is delivered to the fermentation medium is very important in producing good yields with *P. stipitis* (*43–46*). Insufficient aeration leads to slow xylose utilization, whereas excessive aeration reduces the ethanol yield because of either product oxidation or cell growth. The recombinant *E. coli*, on the other hand, performs better under anaerobic conditions, and oxygen is not an issue for concern (*32*).

There are several reports in the literature of softwood-based SSL ethanol fermentations using *S. cerevisiae* (*20–22,36,46–47*). Dahlgren (*48*) quotes an ethanol yield from softwood SSL using *S. cerevisiae* as equivalent to 72 L/ton of paper pulp. According to Wilson et al. (*24*), the ethanol yield varies from 50–100 L of 95% ethanol/ton of paper pulp and 100–130 L/ton of rayon pulp. In comparing the sugar-to-ethanol conversion efficiencies observed in this study to those reported by others using different ethanologens (Table 4), it would appear that, in the case of softwood SSL, replacement of *S. cerevisiae* by one of the alternative organisms could be justified in terms of the 20–35% potential increase in product yield, and an increase in ethanol yield also means a further reduction in the BOD of the effluent.

Table 4
Comparative Fermentations of Hardwood and Softwood Spent Sulfite Liquors Using Different Ethanologens

Organism	Initial cell concentration, g dry wt/L	pH	Total sugar, g/L	Acetic acid, g/L	Fermentation time, h	Ethanol, g/L	Yield[a] $Y_{p/s}$, g/g	Productivity[a] Q_p, g/L/h	Productivity[a] q_p,[b] g/g/h	Ref.
Softwood spent sulfite										
Saccharomyces cerevisiae (baker's)	0.3	4.1	28.0		80	8.0	0.29	0.10		(21)
Saccharomyces cerevisiae NRCC 202001	17.4	4.5	38.0	1.1	6	10.9	0.29	1.82	0.10	(24)
S. cerevisiae + xylose isomerase (azide) (baker's)		6.0	41.0		45	16.8	0.41	0.37		(47)
Saccharomyces cerevisiae (SSL acclimatized)	12	5.5	43.2	0.4[c]	18	13.9	0.32	0.58	0.05	(26)
Pichia stipitis (R-strain) CBS 5776	12	5.5	46.6	4.1	48	15.7	0.34	0.35	0.03	(26)
E. coli ATCC11303 with pLOI297	0.5	7.0	35.4	2.7	41	13.8	0.39	0.34	0.68	this work
Hardwood spent sulfite liquor										
Saccharomyces cerevisiae (SSL acclimatized)	12	5.5	50.0	0.5[c]	24	5.3	0.11	0.29	0.02	(26)
Pichia stipitis CBS 5776	14.9	6.5	17.0	3.3	17	6.8	0.40	0.40	0.03	(24)
	10.7	6.5	27.8	6.1	51	10.3	0.37	0.19	0.02	
Pichia stipitis (R-strain) CBS 5776	12	5.5	54.0	8.3	72	12.3	0.23	0.19	0.02	(26)
	12	5.5	50.0	0.5[c]	72	18.0	0.36	0.25	0.02	
Pichia stipitis CBS 5773	5	6.0	43.0	3.0	20	16.3	0.38	0.82	0.16	(20)
E. coli ATCC11303 with pLOI297 + $Ca(OH)_2$	0.5	7.0	25.1	7.0	24	10.0	0.40	0.42	0.84	this work

[a] Fermentation parameters were determined as described in the legend to Table 2.
[b] Based on initial cell density.
[c] Level of acetic acid lowered by steam-stripping.

However, plasmid instability, presumably induced by the nonbiocompatability of the SSL environment, caused recombinant *E. coli* B (pLOI297) to exhibit variable fermentation performance characteristics. Consequently, the inconsistent behavior of this particular plasmid-bearing genetic construct (Table 3) seriously compromises its attractiveness as a viable alternative to acclimatized *S. cerevisiae* for softwood SSL fermentations.

In the case of hardwood SSL, since *S. cerevisiae* converts only about 20% of the reducing sugars into ethanol (Table 4), there exists a much greater opportunity for yield improvement through the use of a more efficient biocatalyst. More complete fermentation reduces the BOD, thereby reducing alternative obligatory waste treatment. Tembec Inc. did not consider the use of calcium hydroxide in conditioning the fermentation medium a viable option because of the increase in ash load in the boilers (R. Benson—personal communication). However, if the overliming treatment were a permissible option, the improved acetic acid tolerance of the *E. coli* combined with improved productivity would make it the superior alternative to *P. stipitis*.

The technoeconomic issues relating to choice of process organism are:

1. Conversion efficiency reliability;
2. Acetic acid tolerance;
3. Aeration requirements;
4. Need for asepsis;
5. Regulatory approval; and
6. Technology licensing.

Public apprehension about genetically engineered microorganisms relating to concerns of perceived safety and the insistence on containment to prevent release into the environment remain unresolved issues. The prevailing public attitude regarding "human altered" organisms appears to be "presumed guilt [hazardous] until proven innocent [safe]". Hence, with all else being about equal between these patented biocatalysts (*27,36*), the present nature of the regulatory climate concerning the use of genetically engineered organisms probably gives the advantage to the pentose-utilizing yeast.

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