

ETHANOL PRODUCTION FROM A MEMBRANE PURIFIED HEMICELLULOSIC HYDROLYSATE DERIVED FROM SUGAR MAPLE BY *PICHTIA STIPITIS* NRRL Y-7124

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In an effort to devise inexpensive and sustainable production of ethanol fuel, experiments were conducted to establish conditions for *Pichia stipitis* NRRL Y-7124 to ferment a membrane treated wood hydrolysate derived from sugar maple to produce ethanol. The degree of aeration required to effectively utilize xylose, produce ethanol, and minimize xylitol formation as well as the optimal hydrolysate concentration were the conditions examined. *P. stipitis* produced the highest concentrations of ethanol in shake flasks at 150 rpm (14.3 g/L in 71 h), and 50% hydrolysate maximized ethanol yield (12.4 g/L in 51.5 h). In the 50% hydrolysate cultures, *P. stipitis* produced ethanol at a rate of 0.24 g/L-h with a yield of 0.41 g ethanol/g wood-derived carbohydrate.

Keywords: Ethanol; Wood Hydrolysate; Hydrolysate; *Pichia stipitis*; Lignocellulose

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INTRODUCTION

Methods for inexpensive biofuel production are critical to developing alternatives to petroleum-based fuels. Corn-ethanol is currently the dominant biofuel produced in the United States, but it may not represent the best return on the energy invested (Hammerschlag 2006). An under-utilized resource with the potential to replace corn as a feedstock for ethanol production is lignocellulosic biomass. Current quantities of residual lignocellulose have the potential to produce an estimated 50 billion gallons of ethanol per year (Slininger *et al.* 2006). In order to employ this resource, methods must be devised to optimize ethanol yields and reduce production costs. Table 1 summarizes previous studies on ethanol yields from various lignocellulosic feedstocks.

Pichia stipitis NRRL Y-7124 is capable of fermenting glucose, galactose, mannose, xylose, lactose, and cellobiose to ethanol (Agbogbo and Wenger 2006). This versatility qualifies *P. stipitis* as an excellent candidate for conversion of lignocellulosic biomass to ethanol, since the hemicellulosic component of wood contains a variety of sugars with xylose as the major carbohydrate (Dominguez *et al.* 2000).

Tests were conducted to evaluate the feasibility of converting a hemicellulosic hydrolysate from sugar maple, using a membrane system, to ethanol by *P. stipitis* and to optimize yield. D-xylose was used to determine how aeration affected ethanol production. The effect of hydrolysate concentration was examined in an attempt to minimize inhibition by compounds such as acetic acid, furans, and phenolics typically present in wood hydrolysates and to maximize carbohydrate utilization (Delgenes *et al.* 1996).

Table 1. Summary of Ethanol Yields from Previous Studies using Hydrolysate Feedstocks

Microorganism	Max. [ethanol] (g/L)	Time (h)	Biomass source	Reference
Yeast				
<i>Saccharomyces cerevisiae</i>	24.3	9	Willow	Jönsson <i>et al.</i> 1998
<i>Pachysolen tannophilus</i> DW06	19	~30	Sugarcane bagasse	Cheng <i>et al.</i> 2008
<i>Pichia stipitis</i> NRRL Y-7124	41.0	48	Aspen	Parekh <i>et al.</i> 1986
	20.2	~20	Red oak	Nigam 2001b
	14.5	~100	Red oak	Nigam 2001a
	12.4	51.5	Sugar Maple	This study
	12.9	12.9	Wheat straw	Nigam 2001c
	12.6	75	Eucalyptus	Ferrari <i>et al.</i> 1992
	10.4	96	Corn cob	Eken-Saraçoğlu and Arslan 2000
	9.7	~58	Sunflower seed hull	Telli-Okur and Eken-Saraçoğlu 2006
Recombinant Yeast				
<i>Saccharomyces cerevisiae</i> MT8-1/Xyl	30	36	Wood chips	Katahira <i>et al.</i> 2006
<i>Saccharomyces</i> strain 1400	21	100	Corn fibre	Moniruzzaman <i>et al.</i> 1997
Recombinant bacteria				
<i>Escherichia coli</i> KO11	46	72	Rice hull	Moniruzzaman and Ingram 1998
	38	48	Corn hull	Bell <i>et al.</i> 1992
<i>Escherichia coli</i> FBR5	18.9	48	Wheat straw	Saha and Cotta 2006
<i>Escherichia coli</i> B (ATCC 11303)	16.9	22	Aspen	Lawford and Rousseau 1991

EXPERIMENTAL

Microorganism

Pichia stipitis NRRL Y-7124 was obtained from the Northern Regional Research Laboratory USDA-ARS (Peoria, IL) and was maintained on 2% malt extract slants at room temperature. Cultures were transferred monthly to maintain viability.

Wood Hydrolysate

Sugar maple hydrolysate was generously supplied by the SUNY-ESF Paper and Bioprocess Engineering Department (Amidon *et al.* 2008; Liu *et al.* 2008). Debarked wood chips, which were screened (1.25 sq. in screen) to isolate smaller chips, were loaded into a 1.84 m³ digester and extracted with water at 160 °C for 120 minutes. The resulting liquid solution was then subjected to two stages of filtration and an acid hydrolysis. The first stage membrane filtration was performed with a molecular-weight limit of 200 Da. to remove lignin. The delignified solution was then subjected to an acid hydrolysis (2% sulfuric acid, 105 °C, 20 min.) to convert sugar oligomers to monosaccharides. The solution containing the monosaccharides was subjected to a second-stage membrane filtration with a 100 Da. molecular-weight limit. This membrane retained the monosaccharides and allowed acetic acid, furfural, and hydroxymethylfurfural (HMF) to pass. The retentate from the first filtration was reconstituted to its original volume and filtered a second time to remove more undesirable compounds and concentrate the monosaccharides. See Table 2 for typical component concentrations in the purified sugar maple wood hydrolysate. The final carbohydrate concentration was 58.99 ± 0.66 g/L and contained 29.17 ± 1.36 g/L xylose.

Table 2: Sugar Maple Wood Hydrolysate Carbohydrate and Organic Component Concentrations.

Carbohydrate Components ^a	Concentration (g/L)
Xylose	29.17 ± 1.36
Glucose	9.91 ± 0.29
Other Sugars ^b	19.91 ± 1.41
Other Organic Components ^c	Concentration (g/L)
Acetate	0.25
Acetate Equivalents ^d	11.49
Formic Acid	0.31
Furfural	4 x 10 ⁻⁴
Hydroxymethylfurfural	0.01

^a Determined by HPLC (this study)

^b Includes galactose, arabinose, and mannose, which have similar retention times

^c (Amidon *et al.* 2008)

^d Acetate equivalents are acetyls bonded with polysaccharides

Shake Flasks

Erlenmeyer flasks (250 mL) with 100 mL of medium, containing 4% D-xylose were prepared with growth medium containing (in g/L): urea (6.4), 1.2 KH₂PO₄ (1.2), Na₂HPO₄ (0.18), yeast extract (10), and 1 mL of a trace element solution (Eken-Saraçoğlu and Arslan 2000). The trace element solution, in grams per liter, consisted of: CaO (1.1), ZnO (0.4), FeCl₃ · 6H₂O (5.4), MgO (0.36), CuSO₄·5H₂O (0.25), CoCl₂·6H₂O (0.24), H₃BO₃ (0.06), and 13.0 mL concentrated HCl (Slininger *et al.* 1982). Shake flasks were stoppered with cotton plugs covered with aluminum foil. The medium was adjusted to pH

5.7, autoclaved, and subsequently inoculated with 5 mL from a 200 mL stationary culture of *P. stipitis* grown in an identical medium. All cultures were maintained at 30 °C and replicated in triplicate with agitation rates of 0, 50, 100, 150, 200, and 250 rpm.

Hydrolysate Concentration

Four treatments (in triplicate) of 25%, 50%, 75%, and 100% hydrolysate were prepared in Erlenmeyer flasks (250 mL) with 100 mL of autoclaved medium adjusted to pH 5.5. Hydrolysate was supplemented with 10 g/L yeast extract and 2 g/L KH_2PO_4 since previous experiments demonstrated comparable results to the shake flask experiment on simpler media (data not shown) (Saez-Miranda *et al.* 2006). Treatments were inoculated with 10 mL of a *P. stipitis* culture grown on 20 g/L xylose, 20 g/L glucose, 10 g/L yeast extract, and 2 g/L KH_2PO_4 (Saez-Miranda *et al.* 2006). Inocula and cultures were maintained at 30 °C and 150 rpm.

Sample Analyses

Samples of 3 mL were taken aseptically throughout the experiments and stored at -20 °C until analysis. Ethanol concentrations were determined by gas chromatography analysis using a Thermo Focus gas chromatograph (Waltham, MA) with H_2 as a carrier gas at a rate of 1.5 mL/min and a Thermo TR-Max (Waltham, MA) column (30 m x 0.25 mm ID x 0.25 μm film) at 50 °C for 3 minutes. Autosampler GC vials were prepared with 100 μL *tert*-butyl ether standard (5.5 g/L), 800 μL distilled water, and 100 μL centrifuged sample supernatant.

Carbohydrate concentrations were analyzed using a Waters (Milford, MA) Carbohydrate Analysis HPLC column with acetonitrile:water (80:20) as the mobile phase at a flow rate of 1 mL/min. Detection utilized a Waters 2414 refractive index detector.

Standard error bars in figures represent ± 1 standard deviation. Rates of ethanol production (g/L·h) were determined using the slope of linear trend lines.

RESULTS AND DISCUSSION

Shake Flasks

Agitation at 150 rpm resulted in higher ethanol concentrations, increased utilization of xylose, decreased xylitol production, and higher cell concentrations (data not shown), in comparison to treatments with less aeration (Fig. 1). The treatment at 150 rpm produced the highest concentration of ethanol (14.3 ± 0.98 g/L), required less time to reach maximum ethanol (71 h) and deplete available xylose (71 h), and produced less xylitol byproduct (3.0 ± 0.28 g/L) than treatments at a lower agitation rate. Maximum ethanol concentration at 150 rpm coincided with exhaustion of xylose and declined thereafter. Stationary and 50 rpm cultures exhibited an overall gradual change in all factors including ethanol, xylose, and xylitol concentrations. These conditions were not conducive to rapid xylose utilization, as exhibited by the residual concentrations of xylose (5.7 ± 0.60 and 7.4 ± 1.47 g/L) at 250 h for stationary and 50 rpm treatments, respectively. An intermediate result was observed in the 100 rpm cultures with xylose

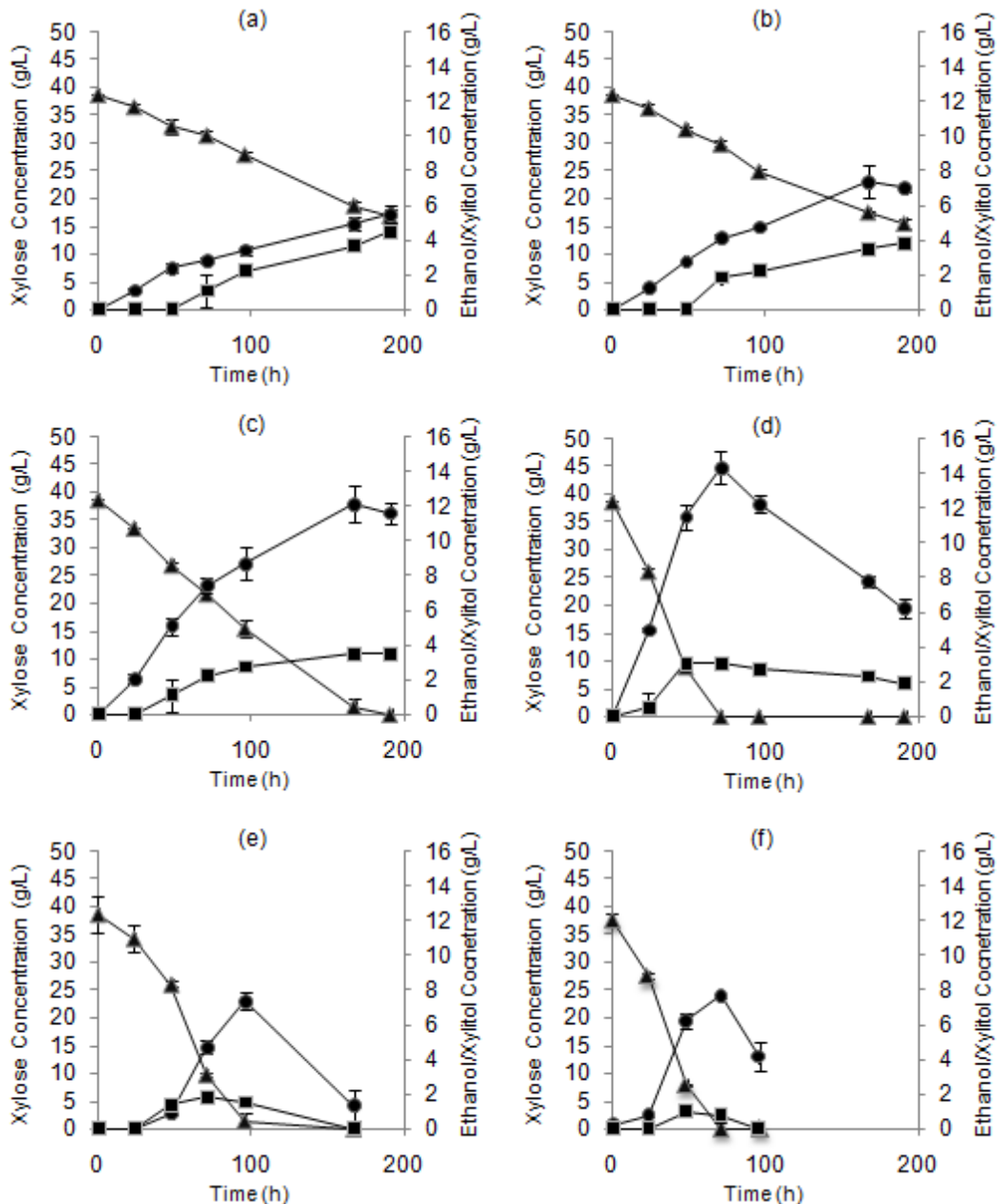


Fig. 1. Shake flasks (250 mL) were analyzed at (a) 0 rpm, (b) 50 rpm, (c) 100 rpm, (d) 150 rpm, (e) 200 rpm, and (f) 250 rpm with *P. stipitis* on 4% D-xylose. Ethanol concentration (—●—), D-xylose concentration (—▲—), and xylitol concentration (—■—) are shown for the first 191 h of the experiment, with the exception of 200 and 250 rpm treatments, because they were terminated earlier due to xylose exhaustion. Some standard error bars are smaller than data point symbols.

depletion at 191 h, ethanol maximum of 12.1 ± 1.04 g/L at 167.5 h, and xylitol concentration maximum of 3.5 ± 0.17 g/L. At higher agitation rates, 200 and 250 rpm, conditions were not conducive for maximum ethanol production, since peak concentrations were 7.40 ± 0.50 g/L and 7.70 ± 0.12 g/L, respectively. With greater aeration, the rate of xylose consumption increased and xylitol production decreased.

The rate and efficiency of ethanol production were also greater with increased agitation up to 150 rpm (Fig. 2). In theory, 0.50 g of ethanol can be produced per 1 g of pentose and hexose carbohydrate consumed (Parekh *et al.* 1986); however, the highest yield achieved in this experiment (150 rpm) was $0.37 \pm 0.03 \text{ g g}^{-1}$. The rate of ethanol production was three times greater at 150 rpm than at 100 rpm (0.21 ± 0.009 and $0.07 \pm 0.007 \text{ g/L}\cdot\text{h}$, respectively). At 200 rpm and 250 rpm, yield, rate of production, and conversion efficiency were significantly reduced.

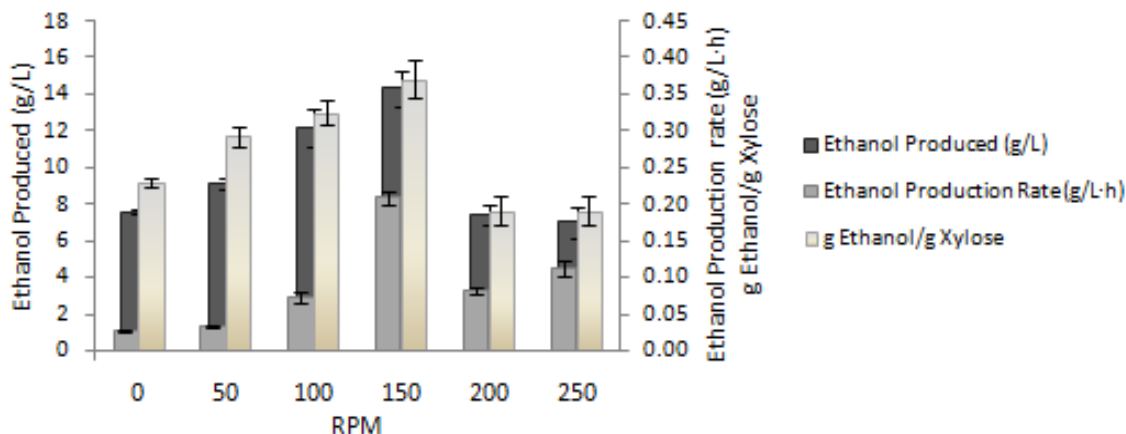


Fig. 2. Ethanol yield, conversion efficiency, and rate of production are represented for stationary, 50 rpm, 100 rpm, and 150 rpm treatments

Delgenes *et al.* (1986) reported higher ethanol productivity from xylose by *P. stipitis* NRRL Y-7124 microaerobically than anaerobically. In addition, Slininger *et al.* (1985) found the same microorganism yielded the highest ethanol concentration from xylose under aerobic conditions in comparison to microaerobic conditions. The results obtained in this study are consistent with these reports, except at excessive aeration rates, which resulted in substantially lower ethanol yields.

Hydrolysate Concentration

The wood hydrolysate contained a variety of carbohydrates, including glucose, xylose, mannose, arabinose, and cellobiose, all of which, with the exception of arabinose, *P. stipitis* is known to utilize. Cultures with 25% and 50% wood hydrolysate achieved maximum ethanol yields most rapidly (51.5 h), producing 6.23 ± 0.40 and $12.36 \pm 0.27 \text{ g/L}$, respectively (Fig. 3). Wood hydrolysate, at a concentration of 50%, was more favorable than other treatments, since the highest ethanol concentration was obtained in the shortest time and utilized the majority of sugars present. Concentrations of 25% hydrolysate had insufficient sugar concentrations ($15.0 \pm 0.53 \text{ g/L}$) to produce high ethanol concentrations. Inhibition of ethanol production occurred in the 75% and 100% treatments, evidenced by reduced rates of ethanol concentration and sugar consumption. This is likely due to inhibitory compounds typically present in lignocellulosic hydrolysates, such as acetic acid and furans (Nigam 2001b; Delgenes *et al.* 1996). Although inhibition occurred at higher concentrations of wood hydrolysate, the results demonstrate that the membrane filtration detoxification may be an alternative method to

other labor-intensive techniques such as overliming (Nigam 2001b).

Figure 4 indicates the rate, efficiency, and yield of ethanol production in all treatments. Wood hydrolysate at 25% resulted in high conversion efficiency of sugars to ethanol ($0.46 \pm 0.03 \text{ gg}^{-1}$), which was greater than 50% hydrolysate ($0.41 \pm 0.03 \text{ gg}^{-1}$), but 50% hydrolysate exhibited the highest rate of ethanol production ($0.24 \pm 0.01 \text{ g/L}\cdot\text{h}$). As anticipated, higher hydrolysate concentrations caused cellular inhibition, slowed the rate of production, and reduced conversion efficiency.

Concentrations of 25%, 50%, and 75% wood hydrolysate exhausted all xylose, suggesting that even when in a complex mixture of sugars, xylose is a preferred carbon source for *P. stipitis*. There occurred a minor lag phase in sugar consumption and ethanol production among all the wood hydrolysate concentrations. It is conceivable that since the inoculum did not contain the inhibitory compounds present in the wood hydrolysate, *P. stipitis* exhibited a brief lag phase, which was not as prominent in the shake flask experiment.

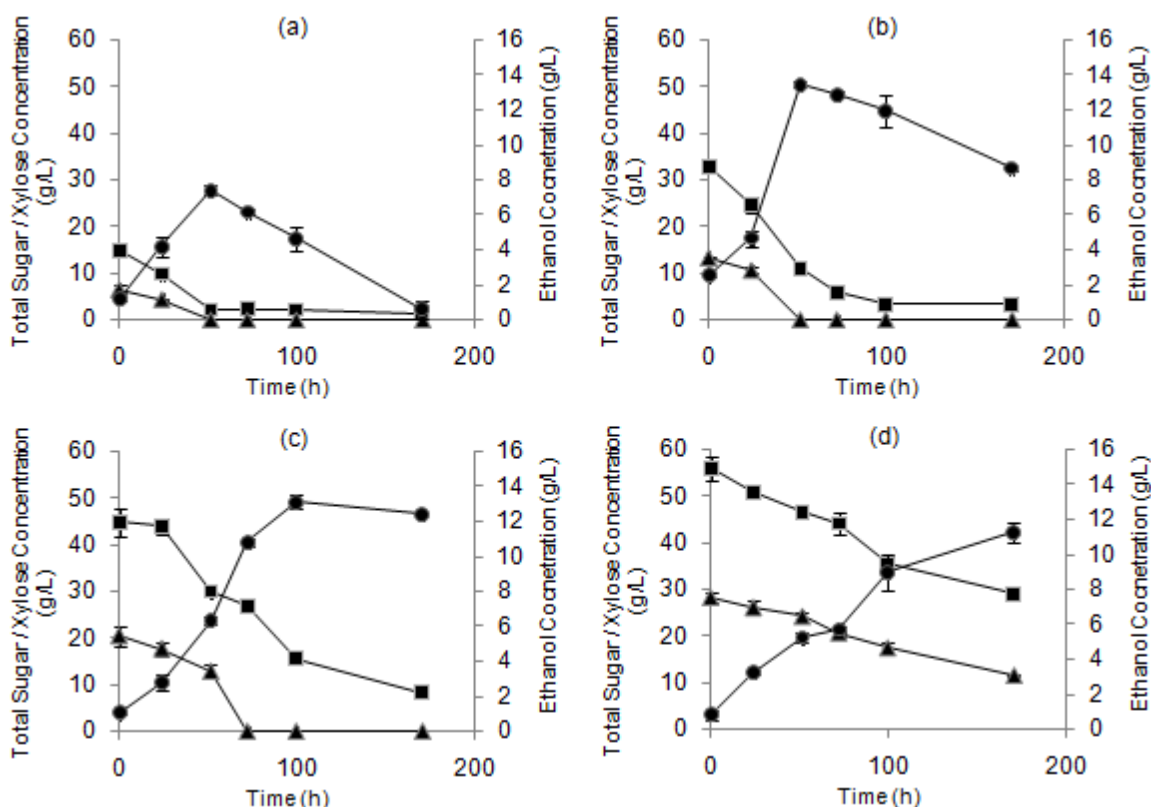


Fig. 3. Wood hydrolysate cultures with *P. stipitis* at concentrations of (a) 25%, (b) 50%, (c) 75%, and (d) 100%. For each, ethanol concentration (—●—), D-xylose concentration (—▲—), and total sugar concentration (—■—) are represented for this 170.5 h experiment. Where standard error bars cannot be seen, the error is smaller than the data point symbols.

Nigam (2001a) achieved an ethanol yield by an adapted *P. stipitis* of 14.5 g/L with a red oak acid prehydrolysate in approximately 100 h, a result comparable to that of this study. However, a hydrolysate-adapted strain of *P. stipitis* achieved 20.2 g/L of ethanol from spent sulfite liquor derived from red oak in approximately 20 h, suggesting that

hydrolysate preparation and strain adaptation are crucial to ethanol yields (Nigam 2001b). Adaptation of *P. stipitis* for tolerance to inhibitory compounds including ethanol, acetic acid, furfural, and phenolics may further improve yield from that achieved in this study. Recombinant microorganisms have achieved 16.9 to 30 g/L of ethanol from wood hydrolysates, suggesting that yield could be greatly increased by genetic manipulation (Lawford and Rousseau 1991; Katahira *et al.* 2006).

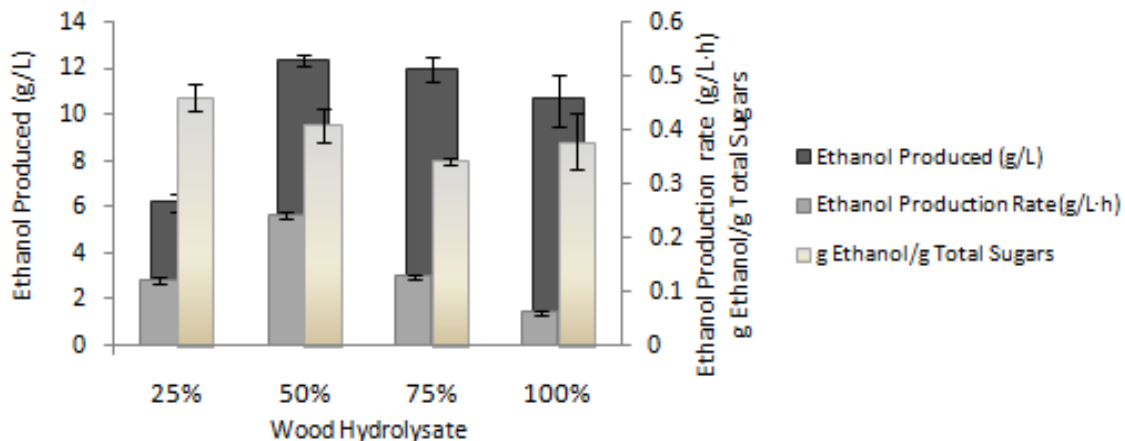


Fig. 4. Yield, rate, and efficiency of ethanol production for 25%, 50%, 75%, and 100% wood hydrolysate

CONCLUSIONS

1. Sugar maple hydrolysate detoxified and concentrated by membrane filtration has potential for use as a carbon source for ethanol production.
2. *P. stipitis* is capable of fermenting a hemicellulosic hydrolysate of sugar maple at a concentration of 50% to produce 12.4 g/L ethanol with 0.41 g ethanol produced per gram of sugar.
3. At 75% and 100% hydrolysate, although carbohydrate concentrations were greater, inhibitory compounds reduced ethanol yield by *P. stipitis*.
4. The shake flasks experiment exhibited the greatest ethanol yield with agitation of 150 rpm.
5. Careful monitoring of dissolved oxygen concentrations will be needed in future fermentor experiments.
6. Xylitol concentration was reduced with increasing aeration which enabled *P. stipitis* to divert additional carbon to ethanol formation (up to 150 rpm),.

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REFERENCES CITED

- Agbogbo, F. K., and Wenger, K. S. (2006). "Effect of pretreatment chemicals on xylose fermentation by *Pichia stipitis*," *Biotechnol. Let.* 28(24), 2065-2069.
- Amidon, T. E., Wood, C. D., Shupe, A. M., Wang, Y., Graves, M., and Liu, S. (2008). "Biorefinery: Conversion of woody biomass to chemicals, energy, and materials," *J. Biobased Materials Bioenergy* 2(2), 100-120.
- Bell, D. S., Ingram, L. O., Ben-Bassat, A., Doran, J. B., Fowler, D. E., Hall, R. G., and Wood, B. E. (1992). "Conversion of hydrolysates from corn cobs and hulls into ethanol by recombinant *Escherichia coli* B containing integrated genes for ethanol production," *Biotechnol. Let.* 14(8), 857-862.
- Cheng, K., Cai, B., Zhang, J., Ling, H., Zhou, Y., Ge, J., and Xu, J. (2008). "Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process," *Biochem. Eng. J.* 38(1), 105-109.
- Delgenes, J. P., Moletta, R., and Navarro, J. M. (1986). "The effect of aeration on D-xylose fermentation by *Pachysolen tannophilis*, *Pichia stipitis*, *Kluyveromyces marxianus* and *Candida shehatae*," *Biotechnol. Let.* 8(12), 897-900.
- Delgenes, J. P., Moletta, R., and Navarro, J. M. (1996). "Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*," *Enzy. Microb. Technol.* 19(3), 220-225.
- Dominguez, J. M., Cao, N., Gong, C. S., and Tsao, G. T. (2000). "Ethanol production from xylose with the yeast *Pichia stipitis* and simultaneous product recovery by gas stripping using a gas-lift loop fermentor with attached side-arm (GLSA)," *Biotechnol. Bioeng.* 67(4), 336-343.
- Eken-Saraçoğlu, N. and Arslan, Y. (2000). "Comparison of different pretreatments in ethanol fermentation using corn cob hemicellulosic hydrolysate with *Pichia stipitis* and *Candida shehatae*," *Biotechnol. Let.* 22(10), 855-858.
- Ferrari, M. D., Neirotti, E., Alborno, C., and Saucedo, E. (1992). "Ethanol production from eucalyptus wood hemicellulose hydrolysate by *Pichia stipitis*," *Biotechnol. Bioeng.* 40(7), 753-759.
- Hammerschlag, R. (2006). "Ethanol's energy return on investment: a survey of the literature 1990-present," *Environ. Sci. Technol.* 40(6), 1744-1750.
- Jönsson, L. J., Palmqvist, E., Nilvebrant, N. O., and Hahn-Hägerdal, B. (1998). "Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*," *Appl. Microbiol. Biotechnol.* 49(6), 691-697.
- Katahira, S., Mizuike, A., Fukuda, H., and Kondo, A. (2006). "Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cello oligosaccharide-

- assimilating yeast strain,” *Appl. Microbiol. Biotechnol.* 72(6), 1136-1143.
- Lawford, H. G. and Rousseau, J. D. (1991). “Fuel ethanol from hardwood hemicellulose hydrolysate by genetically engineered *Escherichia coli* B carrying genes from *Zymomonas mobilis*,” *Biotechnol. Lett.* 13(3), 191-196.
- Liu, S., Amidon, T. E., and Wood, C. D. (2008). “Membrane filtration: concentration and purification of hydrolysates from biomass,” *J. Biobased Materials Bioenergy* 2(2), 121-134.
- Moniruzzaman, M., Dien, B. S., Skory, C. D., Chen, Z. D., Hespell, R. B., Ho, N. W. Y., Dale, B. E., and Bothast, R. J. (1997). “Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain,” *World J. Microbiol. Biotechnol.* 13(3), 341-346.
- Moniruzzaman, M., and Ingram, L. O. (1998). “Ethanol production from dilute acid hydrolysate of rice hulls using genetically engineered *Escherichia coli*,” *Biotechnol. Lett.* 20(10), 943-947.
- Nigam, J. N. (2001a). “Development of xylose-fermenting yeast *Pichia stipitis* for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate,” *J. Appl. Microbiol.* 90(2), 208-215.
- Nigam, J. N. (2001b). “Ethanol production from hardwood spent sulfite liquor using an adapted stain of *Pichia stipitis*,” *J. Indust. Microbiol. Biotechnol.* 26(3), 145-150.
- Nigam, J. N. (2001c). “Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*,” *J. Biotechnol.* 87(1), 17-27.
- Parekh, S. R. (1986). “Adaptation of *Candida shehatae* and *Pichia stipitis* to wood hydrolysates for increased ethanol production,” *Appl. Microbiol. Biotechnol.* 25(3), 300-304.
- Saez-Miranda, J. C., Saliceti-Piazza, L., and McMillan, J. D. (2006). “Measurement and analysis of intracellular ATP levels in metabolically engineered *Zymomonas mobilis* fermenting glucose and xylose mixtures,” *Biotechnol. Prog.* 22(2), 359-368.
- Saha, B. C. and Cotta, M. A. (2006). “Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw,” *Biotechnol. Prog.* 22(2), 449-453.
- Slininger, P. J., Bothast, R. J., Okos, M. R., and Ladisch, M. R. (1985). “Comparative evaluation of ethanol production by xylose-fermenting yeasts presented high xylose concentrations,” *Biotechnol. Lett.* 7(6), 431-436.
- Slininger, P. J., Bothast, R. J., Van Cauwenberge, J. E., and Krutzman, C. P. (1982). “Conversion of D-xylose to ethanol by the yeast *Pachysolen tannophilus*,” *Biotechnol. Bioeng.* 24(2), 371-384.
- Slininger, P. J., Dien, B. S., Gorsich, S. W., and Liu, Z. L. (2006). “Nitrogen source and mineral optimization enhance D-xylose conversion to ethanol by *Pichia stipitis* NRRL Y-7124,” *Appl. Microbiol. Biotechnol.* 72(6), 1285-1296.
- Telli-Okur, M. and Eken-Saraçoğlu, N. (2006). “Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* under uncontrolled pH conditions in a bioreactor,” *Turkish J. Eng. Env. Sci.* 30(5) 317-322.

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