0273-2289/95/5152—0527$06.00

Promising Ethanologens for Xylose Fermentation

Scientific Note

M. ZHANG,* M. A. FRANDEN, M. NEWMAN, J. MCMILLAN, M. FINKELSTEIN, AND S. PICATAGGIO

Applied Biological Sciences Branch, Alternative Fuels Division, National Renewable Energy Laboratory, 1617 Cole Blvd., Golden, CO 80401

Index Entries: Xylose fermentation; strain comparison; hydrolyzate tolerance; metabolic engineering.

INTRODUCTION

An economical biomass-to-ethanol process depends on the efficient conversion of both its cellulose and hemicellulose components. On a dry weight basis, the typical feedstock contains approx 25–50% (w/w) glucose, 10–30% (w/w) xylose, 15–30% (w/w) lignin, and 1–5% (w/w) of other minor pentose and hexose sugars (*1–3*). Although many microorganisms can ferment the glucose component in cellulose to ethanol, conversion of pentose sugars in the hemicellulose fraction, particularly xylose, has been hindered by the lack of a suitable biocatalyst. Despite the development of recombinant strains with improved fermentation performance (*4–8*), increased ethanol yields and concentrations and shorter fermentation times are key targets that have yet to be achieved from lignocellulosic hydrolyzates (*9*).

Our objective is to develop biocatalysts for the rapid and efficient conversion of xylose by engineering key metabolic pathways in selected organisms. To identify promising biocatalysts for these efforts, we have surveyed several industrial microorganisms according to several primary traits considered to be essential, as well as a number of secondary traits considered to be desirable, in a commercial biomass-to-ethanol process.

*Author to whom all correspondence and reprint requests should be addressed.

Table 1
Microorganisms Used in This Study

Zymomonas strain	*Lactobacillus* strain
Z. mobilis AG11[a]	*L. amylovorus* ATCC 33620
Z. mobilis CP3[a]	*L. delbrüeckii delbrüeckii* ATCC 9649
Z. mobilis CP4[a]	*L. delbrüeckii delbrüecki* BBL 445
Z. mobilis ATCC 10988	*L. delbrüeckii lactic* ATCC 21051
Z. mobilis ATCC 29191	*L. farciminus* ATCC 29644
Z. mobilis ATCC 29192	*Lactobacillus* MONT4[b]
Z. mobilis ATCC 31821	*L. salivarius* ATCC 11742
Z. mobilis ATCC 31822	*L. helveticus* ATCC 15009
Z. mobilis ATCC 31823	*L. sake* IFO 3541
Z. mobilis ATCC 39676	*L. sake* ATCC 15521
	L. plantarum ATCC 8014
	L. casei casei ATCC 39392
	L. casei rhamnosus ATCC 11443
	L. brevis ATCC 367
	L. brevis IFO 3960
	L. brevis DSM 20054
	L. pentosus ATCC 8041
	L. halotolerans ATCC 35410
	L. casei ATCC 49178

[a]See ref. (*10*).
[b]See ref. (*11*).

As a result of this survey, we have identified *Zymomonas* and *Lactobacillus* as promising microorganisms for metabolic engineering of ethanol production. Evaluation of the growth and fermentation performance of several *Zymomonas* and *Lactobacillus* strains on poplar wood hemicellulose hydrolyzate has allowed us to select the best strains for our metabolic engineering efforts.

MATERIALS AND METHODS

Microorganisms

Microorganisms used in this study are listed in Table 1.

Cultivation Media and Conditions

Zymomonas mobilis strains were routinely transferred every 2 wk on RM agar plates incubated anaerobically at 30°C. RM media contained 2% (w/v) glucose, 10 g/L yeast extract (Difco Laboratories, Detroit, MI), and 2 g/L KH_2PO_4 (pH 6.0). *Lactobacillus* strains were transferred on MRS agar

media (Difco Laboratories) and incubated anaerobically at either 30°C or 37°C.

Poplar wood (hybrid DN34) hemicellulose hydrolyzate (PWH) was prepared by dilute-acid treatment at 160°C for 10 min with approx 1% (w/w) sulfuric acid (*1,12,13*) and was stored at 4°C. The hydrolyzate was overlimed to decrease its toxicity (*14*), as follows: the pH was adjusted to 10–10.2 by addition of calcium hydroxide and held for 30 min before being adjusted to pH 7.0 with sulfuric acid. The overlimed hydrolyzate was further conditioned by stirring for 30 min at 50°C in a circulating water bath. Precipitates were removed by sterile filtration through a 0.2-μm filter. The conditioned hydrolyzates were stored at 4°C until use.

RM media with PWH (RM/PWH) was prepared by adding yeast extract and potassium phosphate (monobasic) to the appropriate concentration of PWH and sterile filtered. The RM/PWH media were supplemented with 5% (w/v) glucose. MRS (2% [w/v] glucose) media (Difco Laboratories), with PWH (MRS/PWH) was similarly prepared.

Z. mobilis was cultured anaerobically in RM media at 30°C until late-log phase. The cells were collected by centrifugation (3000*g*, 15 min) and used to inoculate 12 mL of RM/PWH (5% [w/v] glucose) media to an initial optical density at 600 nm (OD_{600}) of 0.5 in screw-cap test tubes (16 × 125 mm) containing six small glass beads to help disperse the CO_2 generated during fermentation. The cultures were incubated at 30°C with shaking at 150 rpm.

Lactobacillus strains were grown anaerobically in MRS medium either at 30 or 37°C. The twice-passaged cells were inoculated (5% [v/v] inoculum) into 12 mL of MRS/PWH (2% [w/v] glucose) media in screw-cap test tubes and were cultured at either 30 or 37°C without shaking.

Analysis

Growth of *Z. mobilis* was monitored by measuring OD_{600} without dilution (nonlinear OD) using a Spectronic 601 (Milton Roy, Rochester, NY) spectrophotometer. This approach was found to be suitable for monitoring the growth of *Z. mobilis* in the screening process. However, owing to higher final cell densities, *Lactobacillus* cultures were diluted into the linear range of the spectrophotometer before measuring OD_{600} (linear OD). Samples were taken at the beginning and the end of the fermentations for residual sugar and product concentration analyses. Glucose, xylose, ethanol, acetic acid, and lactic acid were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard (Wilmington, DE) 1090L HPLC equipped with an HP 1047A refractive index detector. HPLC analyses were carried using a Bio-Rad (Richmond, CA) HPX-87H organic acid analysis column operating at 65°C with a 0.01*N* sulfuric acid mobile phase flow rate of 0.6 mL/min. Calibration curves developed for each component were used to quantify solute concentrations.

RESULTS AND DISCUSSION

Fermentation Performance Criteria

A recent economic analysis of xylose fermentation (*15*) has identified higher ethanol yields and product concentrations as the most important factors influencing production costs, with increased volumetric productivity as an important secondary target. Accordingly, the microbial characteristics that appear to be indispensable in a commercial biomass-to-ethanol process include the ability to ferment sugars to ethanol at high yields and selectivity, and the ability to tolerate the high ethanol concentrations necessary for economical product recovery. An important prerequisite is that the biocatalyst must tolerate the inhibitory compounds typically present in hydrolyzates without sacrificing fermentation performance. These inhibitors include the acetic acid liberated by dilute-acid pretreatment of hemicellulose, furfurals, and the assorted phenolic compounds derived from lignin (*16*). The ability to ferment sugars at a low pH is another essential trait because it provides a measure of protection against contamination during prolonged, large-scale, continuous cultivation, and also avoids the cost associated with alkali addition. The biocatalyst should be a facultative anaerobe without an oxygen requirement, yet capable of tolerating any incidental introduction of oxygen during processing. Finally, the organism should possess a broad substrate utilization range and be capable of fermenting all of the sugars commonly present in cellulosic biomass. Compatibility with industrial health and safety regulations (i.e., general recognized as safe [GRAS] status) also was included as a key selection criterion. Additional desirable microbial characteristics that impact improved fermentation performance were also considered in the evaluation and are listed in Table 2.

Selection of the Most Promising Microorganisms

We surveyed numerous industrial microorganisms by comparing their known metabolic characteristics to a weighted list of fermentation performance criteria. As shown in Table 3, *Z. mobilis* and *Lactobacillus* were identified as promising candidates for metabolic engineering.

Z. mobilis is used as a natural fermentative agent in the production of alcoholic beverages and demonstrates several potential advantages in industrial ethanol production (*17–20*). These include high ethanol yield and tolerance, high selectivity and specific productivity, the ability to ferment sugars at low pH, considerable tolerance to the inhibitors found in lignocellulosic feedstocks (*21*), and GRAS staus. Despite its advantages, fermentation processes based on the use of *Zymomonas* are still considered an immature technology compared to the practice of traditional yeast fermentation and have yet to be commercialized for fuel ethanol production from starch-based feedstocks. The major limitation of *Z. mobilis* is its nar-

Table 2
Fermentation Performance Criteria in a Biomass-to-Ethanol Process

Essential traits	Desirable traits
High conversion yield	High sugar consumption rate
High ethanol tolerance	High specific growth rate
Tolerance to hydrolyzates	High volumetric productivity
No oxygen requirement	High specific productivity
Low fermentation pH	C5/C6 co-fermentation
High fermentation selectivity	Minimal nutrient requirements
Broad substrate utilization range	High salt tolerance
GRAS status	Capable of Crabtree effect
	Entner-Douderoff pathway
	Facilitated sugar transport
	Nonsporeforming
	Nonconjugative
	Amenable to scale-up
	Availability of "industrial strains"
	Compatibility with SSF
	Cellulase producer
	Thermotolerance
	High shear tolerance
	Available gene-transfer system

Table 3
Ranking of Promising Biocatalysts

Ranking	Microorganism
1	*Zymomonas*
2	Recombinant *Saccharomyces*
3	Homofermentative *Lactobacillus*
4	Heterofermentative *Lactobacillus*
5	Recombinant *Escherichia coli*
6	Xylose-assimilating yeasts
7	*Clostridium*

row substrate utilization range, which is restricted to the fermentation of glucose, fructose, and sucrose (*17,19*). Metabolic engineering of pentose fermentation is required to make Z. *mobilis* an efficient biocatalyst for conversion of the xylose present in lignocellulosic feedstocks.

Lactobacillus is capable of fermenting many of the sugars commonly found in biomass and offers potential advantages in biomass fermentations, including high ethanol tolerance (*22*), resistance to the inhibitors present in hydrolyzates (*21*), fermentation at low pH, thermotolerance, and high lactate yield (wild-type obligate homofermentative *Lactobacilli*

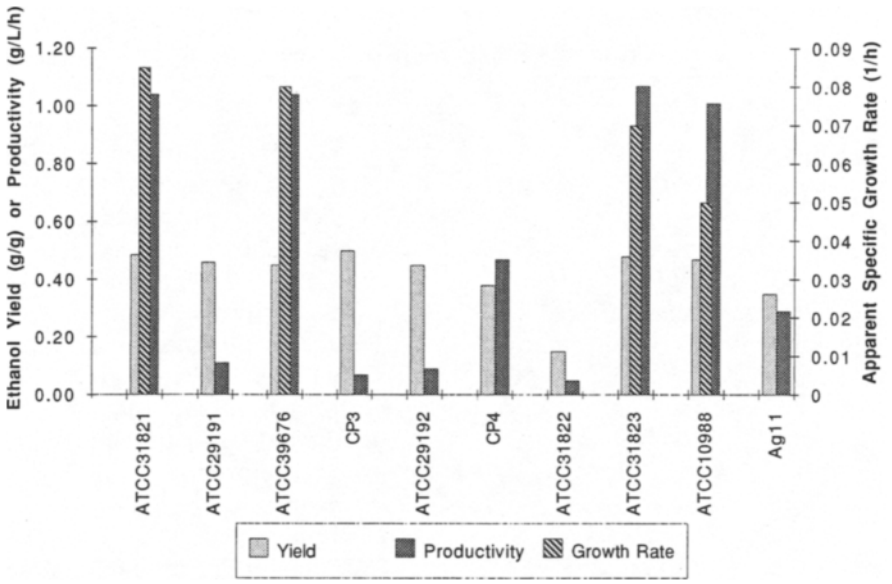

Fig. 1. Fermentation performance of Z. *mobilis* strains in 40% (v/v) PWH at 24 h. For the strains that grew, cell growth is expressed as the apparent specific growth rate calculted from the change in nonlinear optical density. Ethanol yield was calculated as gram of ethanol produced per gram of glucose consumed. Average volumetric productivity was calculated from the final product concentration divided by the fermentation time (same as in Fig. 2).

do not produce ethanol). *Lactobacillus* is used commercially in the preparation of a variety of food and feed products. Being thermotolerant, *Lactobacillus* could be compatible with simultaneous saccharification and fermentation (SSF) processes using commercially available *Trichoderma* cellulase preparations with optimal enzyme activities about 50°C. Metabolic engineering of *Lactobacillus* for ethanol production would require the introduction of genes encoding enzymes for both pentose metabolism and ethanol production.

Strain Comparison of Fermentation Performance in PWH

Clearly, Z. *mobilis* and *Lactobacillus* offer several potential advantages for xylose fermentation. Since tolerance to hydrolyzates is a key requirement for a commerical process, several strains were screened for their resistance prior to selecting the best strains for our metabolic engineering efforts. Ten *Zymomonas* and 19 *Lactobacillus* strains were compared for growth, product yield, and productivity in PWH.

As shown in Fig. 1, four of the Z. *mobilis* strains demonstrated superior growth and fermentation performance in 40% (v/v) PWH. These strains

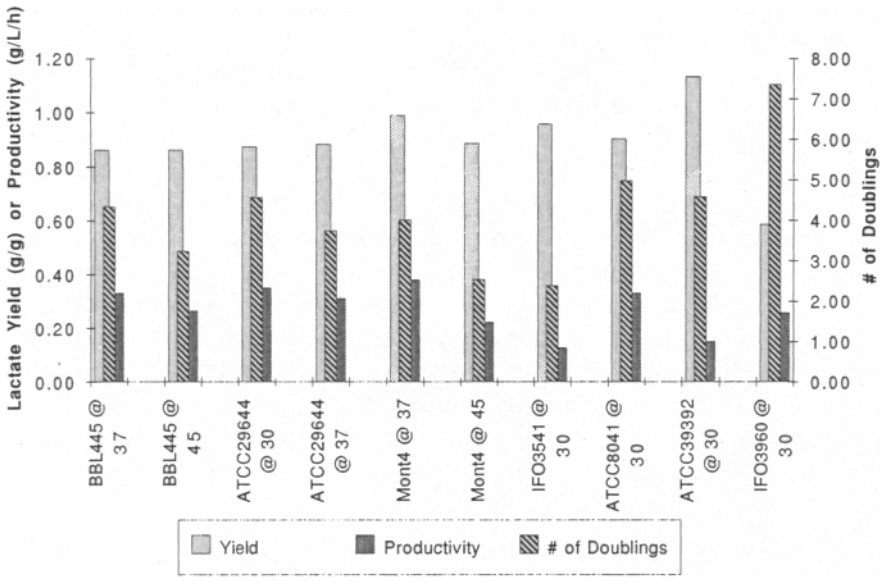

Fig. 2. Fermentation performance of *Lactobacillus* strains in 80% (v/v) PWH 48 h. Cell growth is expressed as the number of the cell doublings calculated from initial and final linear optical density. Lactate yield was calculated as gram of lactate produced per gram of glucose consumed (except in the case of *L. brevis* IFO 3960, where the yield was calculated from both consumed xylose and glucose).

were ATCC 31821, ATCC 39676, ATCC 31823, and ATCC 10988. Among the four strains, ATCC 31821 grew the best followed by ATCC 39676, ATCC 31823, and ATCC 10988. In all cases, glucose (at 50 g/L) was completely converted to ethanol within 24 h. As expected in these wild-type strains, xylose was not consumed. Ethanol yields ranged from 0.45 to 0.49 g/g consumed glucose, with ATCC 31821 demonstrating the highest yield. All four strains achieved similar productivities (1.01–1.07 g/L/h in 24 h).

These four strains were further tested for their ability to adapt to higher concentrations of hydrolyzates. One of them, ATCC 31821, showed superior performance in 50% (v/v) hydrolyzate following one passage of growth in 30% (v/v) hydrolyzate. This result suggests that tolerance to higher concentrations of hydrolyzate may be achieved through progressive adaptation.

The *Lactobacillus* strains were much more tolerant to the hydrolyzates. Seven strains, selected for their carbohydrate utilization patterns and ethanol tolerance (data not shown), grew well in up to 80% (v/v) hydrolyzate (Fig. 2). These include the obligate homofermentative strains *L. delbrüeckii delbrüeckii* BBL 445, *L. farciminus* ATCC 29644, *Lactobacillus* MONT4, the facultative homofermentative strains *L. sake* IFO 3541, *L. pentosus* ATCC 8041, *L. casei casei* ATCC 39392, and the obligate homofermentative strain *L. brevis* IFO 3960. The three obligate homofermentative

strains consumed about 80% of the initial glucose (20 g/L) in 48 h at 37°C and achieved lactate yields ranging from 0.86 to 0.99 g/g consumed glucose. *L. delbrüeckii delbrüeckii* BBL 445 and *Lactobacillus* MONT4 performed relatively well even at 45°C, achieving yields of 0.86 and 0.89 g/g, respectively. However,the productivities of L. *delbrüeckii delbrüeckii* BBL 445 and *Lactobacillus* MONT4 were reduced by about 20 and 40%, respectively, in comparision with those at 37°C. The three facultative homofermentative strains achieved relatively high lactate yields from glucose, but were incapable of fermenting xylose in the presence of glucose. The obligate heterofermentative stain *L. brevis* IFO 3960 fermented glucose and xylose simultaneously, but demonstrated low lactate yields because of mixed product formation (ethanol and acetate) (data not shown). All strains showed relatively low productivities (0.13–0.38 g/L/h in 48 h). The major drawbacks for the *Lactobacillus* strains appear to be their relatively lower sugar consumption rates compared with Z. *mobilis*.

Approaches for Metabolic Engineering

Comparative performance trials suggest that *Zymomonas* may become an important industrial ethanol-producing microorganism because of its 5–10% higher yield and up to fivefold higher productivity compared to traditional yeast fermentations (*23*). Recent studies, however, indicate that this organism lacks the key pentose phosphate pathway enzymes (*24*). Thus, both the xylose assimilation genes and key pentose phosphate pathway genes will probably be required to enable Z. *mobilis* to ferment xylose to ethanol. Metabolic engineering efforts to introduce such genes into Z. *mobilis* are now under way.

Its superior tolerance hydrolyzate makes *Lactobacillus* an attractive candidate for metabolic engineering of xylose fermentation for ethanol production. Xylose assimilation genes will be required for metabolic engineering of the obligate homofermentative *Lactobacillus* strrains since they do not utilize xylose. Additional genes will be required to redirect the carbon flow from pyruvate to ethanol. Furthermore, inactivation of lactate dehydrogenases may be required to achieve high ethanol yields. Deregulation of catabolite repression may be necessary to allow simultaneous fermentation of both glucose and xylose.

CONCLUSIONS

Several essential and desirable traits have been established for selection of promising ethanologens for xylose fermentation. As a result of our detailed survey, we have identified Z. *mobilis* and *Lactobacillus* as promising microorganisms for metabolic engineering of ethanol production. Strain evaluation studies identified four Z. *mobilis* strains that exhibit superior

performance in 40% (v/v) PWH in terms of growth, ethanol yield, and productivity. Several species of *Lactobacillus* demonstrate excellent fermentation performance and tolerance in up to 80% (v/v) PWH. The key biochemical pathways for metabolic engineering of ethanol production from pentose sugars have been identified, and the metabolic engineering efforts in Z. *mobilis* have been initiated.

ACKNOWLEDGMENTS

We would like to thank Dr. D. Eveleigh of Rutgers University for providing us with Z. *mobilis* strains CP3, CP4, Ag11, and ATCC 29192. This work was funded by the Ethanol from Biomass Program of the US Department of Energy Biofuels Systems Division.

REFERENCES

1. Torget, R., Werdene, P., Himmel, M., and Grohmann, K. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 115–126.
2. Torget, R., Walter, P., Himmel, M., and Grohmann, K. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 75–86.
3. Torget, R., Himmel, M., and Grohmann, K. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 115–123.
4. Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W., and Preston, J. F. (1987), *Appl. Environ. Microbiol.* **53,** 2420–2425.
5. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 893–900.
6. Kötter, P., Amore, R., Hollenberg, C. P., and Ciriacy, M. (1990), *Curr. Genet.* **18,** 493–500.
7. Hallborn, J., Walfridsson, M., Airaksinen, U., Ojamo, H., Hahn-Hägerdal, B., Penttila, M., and Keranen, S. (1991), *Bio/Technology* **9,** 1090–1095.
8. Tantirungkij, M., Nakashima, N., Seki, T., and Yoshida, T. (1993), *J. Ferment. Bioeng.* **75,** 83–88.
9. Picataggio, S. K., Zhang, M., and Finkelstein, M. (1994), in *Enzymatic Conversion of Biomass for Fuels Production,* Himmel, M. E.. Baker, J. O., and Overend, R. P., eds., American Chemical Society, Washington, DC, chapter 17, pp. 342–362.
10. Eveleigh, D. E., Stokes, H. W., and Dally, E. L. (1983), in *Organic Chemicals from Biomass,* Wise, D., ed., Benjamin-Cummings, Menlo Park, CA, chapter 2, pp. 69–91.
11. Barre, P. (1978), *J. Appl. Bacteriol.* **44,** 125–129.
12. Grohmann, K., Torget, R., and Himmel, M. (1985), *Biotech. Bioeng. Symp.* **No. 15,** 59–80.
13. McMillan, J. (1994), in *Enzymatic Conversion of Biomass for Fuels Production,* Himmel, M. E., Baker, J. O., and Overend, R. P., eds., American Chemical Society, Washington, DC, chapter 15, pp. 292–324.

14. Lawford, H. G. and Rousseau, J. D. (1993), in *Energy from Biomass and Wastes XVI,* Klass, D. L. ed., Institute of Gas Technology, Chicago, pp. 559–597.
15. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
16. McMillan, J. (1994), in *Enzymatic Conversion of Biomass for Fuels Production,* Himmel, M. E., Baker, J. O., and Overend, R. P., eds., American Chemical Society, Washington, DC, chapter 21, pp. 411–437.
17. Swings, J. and De Ley, J. (1977), *Bacteriol. Rev.* **41,** 1–46.
18. Rogers, P. L., Lee, K. J., and Tribe, D. E. (1979), *Biotechnol. Lett.* **1,** 165–170.
19. Montenecourt, B. S. (1985), in *Biology of Industrial Microorganisms,* Demain, A. L. and Solomon, N., eds., Benjamin-Cummings, Menlo Park, CA, chapter 9, pp. 261–289.
20. Doelle, H. W., Kirk, L., Crittenden, R., Toh, H., and Doelle, M. (1993), *Crit. Rev. Biotechnol.* **13,** 57–98.
21. Olsson, L., Linden, T., and Hahn-Hägerdal, B. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 359–368.
22. Gold, R. S., Meagher, M. M., Hutkins, R., and Conway, T. (1992), *J. Indust. Microbiol.* **10,** 45–54.
23. Beavan, M., Zawadzki, B., Droniuk, R., Lawford, H., and Fein, J. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326.
24. Feldman, S. D., Sahm, H., and Sprenger, G. A. (1992), *Appl. Microbiol. Biotechnol.* **38,** 354–361.