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Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars

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Recombinant *Escherichia coli* have been constructed for the conversion of glucose as well as pentose sugars into L-lactic acid. The strains carry the lactate dehydrogenase gene from *Streptococcus bovis* on a low copy number plasmid for production of L-lactate. Three *E. coli* strains were transformed with the plasmid for producing L-lactic acid. Strains FBR9 and FBR11 were serially transferred 10 times in anaerobic cultures in sugar-limited medium containing glucose or xylose without selective antibiotic. An average of 96% of both FBR9 and FBR11 cells maintained pVALDH1 in anaerobic cultures. The fermentation performances of FBR9, FBR10, and FBR11 were compared in pH-controlled batch fermentations with medium containing 10% w/v glucose. Fermentation results were superior for FBR11, an *E. coli* B strain, compared to those observed for FBR9 or FBR10. FBR11 exhausted the glucose within 30 h, and the maximum lactic acid concentration (7.32% w/v) was 93% of the theoretical maximum. The other side-products detected were cell mass and succinic acid (0.5 g/l). *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 259–264.

Keywords: lactic acid production; *Escherichia coli*; fermentation; pentoses

Introduction

Lactate production is approximately 130,000 metric tons per year globally [24], most of which is used by the food industry. Future demand for lactic acid is expected to expand with the introduction of polylactate into the market place. Polylactate is a high-quality plastic that has the advantages, compared to existing plastics, of being biodegradable and manufactured from renewable resources. In 1997, Cargill and Dow Chemical Company formed a new company (Cargill Dow Polymers) to develop and market polylactic acid polymers [10]. Optically pure lactic acid is preferred over racemic mixtures as a feedstock for producing polylactate.

Currently, lactate is produced by fermentation of glucose (for reviews, see Refs. [19,32]). Using lignocellulosic biomass might offer a less expensive feedstock for producing lactic acid. Sources of lignocellulosic biomass available for fermentation include agricultural residues, food-processing wastes, wood, municipal solid wastes, and wastes from the paper and pulp industry. Available biomass reserves in the U.S. are approximately 200 million tons [4]. Conversion of lignocellulosic biomass to lactic acid is problematic because these biomasses contain a mixture of sugars including (primarily) glucose and xylose. Xylose is a pentose that is not fermented by most lactic acid bacteria. Lactic acid-producing microorganisms that do ferment xylose, including the fungus *Rhizopus oryzae*, have low productivity and (usually) low yield and selectivity [16,17,23,37].

We are developing *E. coli* strains for selective production of L-lactic acid from mixed sugar streams. Wild-type *E. coli*, when growing fermentatively, produces a mixture of organic acids (acetic, lactic, formic, and succinic) and ethanol. *E. coli* engineered for production of lactic acid have several advantages compared to many other microorganisms available for lactic acid production. First, *E. coli* can be metabolically engineered to produce optically pure lactic acid with little in the way of other fermentation products [6]. Second, as is common for *E. coli*, the strains are capable of utilizing a wide variety of sugars, including xylose. *E. coli* strains have the added advantages of minimal nutritional requirements and being well characterized.

The lactic acid-producing strains described here were constructed by transforming *E. coli* strains that are unable to grow fermentatively on sugar(s) with a plasmid encoding the L-specific lactic acid dehydrogenase (LDH) gene from *Streptococcus bovis* [36]. Expression of the *ldh* gene compliments the mutations of the strains and allows them to grow fermentatively. In fact, these nonfermentative *E. coli* have frequently been used to screen genomic libraries for LDH activity by selecting for growth on anaerobic medium [3,11,25,36].

In this paper, three *E. coli* strains were screened for lactic acid production. Two are derived from K12 strains and the other from a B strain. All three strains were tested for lactic acid fermentation of glucose to lactate. One of the K12 strains and the B strain were further tested for plasmid maintenance in the absence of antibiotic selection. These two strains were also used to ferment xylose to lactic acid.

Materials and methods

Bacterial strains, plasmid, and growth media

E. coli strains and plasmid used in this study are listed in Table 1. Nonfermenting *E. coli* strains FMJ39 and NZN111 were obtained from D.P. Clark (Southern Illinois University, Carbondale, IL). The plasmid used in this study, pVALDH1, was received from T.R.

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Table 1 List of bacterial strains and plasmid used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
ATCC11303	wild-type B strain	USDA Culture Collection
FMJ39	K12 *thr-1 leu 6 thi-1 lacY tonA22 rps*L *pfl-1 ldhA*::Kn	[22]
NZN111	K12 Δ*pfl*:: Cm *ldhA*::Kn	[5]
WA837	r_{B-} m_{B+} *gal met*	[35]
ND5	WA837 *ldhA*:: Kn, Δ*pfl*::Cm	This work
ND10	ATCC11303 Δ*pfl*:: Cam *ldhA*::Kn	This work
FBR9	FMJ39(pVALDH1)	[36]
FBR10	NZN111(pVALDH1)	
FBR11	ND10(pVALDH1)	This work
Plasmid		
pVALDH1	Em^r; *ldh* from *S. bovis*	[36]

Whitehead (National Center for Agricultural Utilization Research, USDA, Peoria, IL). The plasmid is an *E. coli*/streptococcal shuttle plasmid that encodes the *ldh* gene from *S. bovis* [36]. Strains were grown aerobically on LB broth (10 g tryptone, 5 g yeast extract, 5 g NACl/l and 15 g/l Bacto agar for a solid medium) supplemented with either 20 g/l glucose or xylose and, where indicated, either erythromycin (Em, 400 mg/l), chloramphenicol (Cm, 10 mg/l), or kanamycin (Kn, 50 mg/l). Antibiotics and sugars were added separately as sterilized solutions.

For anaerobic growth of *E. coli*, LB broth was prepared without NaCl and supplemented with 4 or 15 g/l xylose, 1 g/l acetate as an additional carbon source, 4 g/l sodium carbonate as a buffer, and 0.5 g/l cysteine HCl as a reducing agent. Where indicated, the redox indicator resazurin (1 mg/l) was added before autoclaving the medium. Anaerobic medium was flushed with a 20% CO_2/80% N_2 gas mixture and sealed with butyl rubber stoppers [8]. Sugars and acetate were prepared as separate stock solutions and autoclaved under 100% nitrogen atmosphere. The pH of the buffered medium was 7.

Growth and testing for plasmid maintenance

Unless stated otherwise, cultures were incubated at 37°C. Cultures were grown in either test tubes or serum bottles capped with butyl rubber caps and agitated gently. The continued presence of the plasmid and integrated mutations in anaerobic cultures was tested for as follows. Aliquots from liquid cultures were serially diluted and cultured aerobically on LB solid medium supplemented with 20 g/l of either glucose or xylose. Plates were incubated for 12–16 h. Fifty randomly selected colonies were transferred to a plate containing similar medium supplemented with the appropriate antibiotic(s). The selection plates were incubated for 18 h before checking them for growth.

Genetic procedures

Plasmid DNA was isolated from liquid cultures by standard procedures [31]. *E. coli* cells transformed with pVALDH1 were selected by plating on LB agar medium containing Em. The integrity of plasmid preparations was verified by agarose gel electrophoresis of undigested and restriction endonuclease (*Eco*RI)-digested plasmid.

A nonfermentative mutant (ND10) of *E. coli* B strain ATCC11303 was constructed by phage P1 transduction [21]: the *ldhA*::Kn and Δ*pfl*::Cm mutations were transduced from *E. coli* NZN111 into *E. coli* WA837, a B strain that is restriction-minus, modification-plus to create ND5. ND10 was constructed by transducing the mutations from ND5 into ATCC11303. Efforts to transduce the mutations directly from strain NZN111 to strain ATCC11303 failed, presumably because the latter's restriction system prevented the DNA from being transferred. ND10 was transformed with pVALDH1 to create strain FBR11.

Batch fermentations of glucose and xylose

Bioreactors with automatic pH control were constructed and operated as described previously [2,8]. Cultures were grown at 35°C and pH 6.7, unless stated otherwise. The pH was controlled by addition of 2.5 N KOH+2.5 N NaOH. The cells were grown on 10 g/l tryptone and 5 g/l yeast extract supplemented with 8–10% w/v sugar(s) and antifoam 289 (0.1 ml/l) (Sigma, St. Louis, MO). Nitrogen was bubbled through the medium for 30 min followed by a 30-s burst of carbon dioxide to remove oxygen prior to inoculation and to supply carbon dioxide, which is required as a growth factor. The fermentation vessels were inoculated with a 5% v/v inoculum from a seed culture grown 18 h at 35°C, in pH-controlled bioreactors. The medium used for the seed culture was similar to that used for the fermentation except seed cultures were supplemented with 2% w/v glucose or xylose. Ethanol, sugars, organic acids, and OD_{550} nm were determined periodically with 1.5-ml samples of cultures. Each experiment was run in duplicate.

Analytical procedures

Optical densities (1 cm light path) for turbidity were monitored at 550 nm. Otherwise, optical densities were measured with a Spectronic 21 (Milton-Roy, UK) at 550 nm, or 660 nm for cultures containing resazurin. Concentrations of sugars and fermentation-generated organic acids (lactate and succinate) were determined by high-pressure liquid chromatography (HPLC) using an Aminex HPC-87H column (Bio-Rad, Richmond, CA) and refractive index detector. Samples were run at 65°C and eluted at 0.6 ml/min with 5 mM sulfuric acid.

Calculation of fermentation parameters

Two forms of lactic acid yield are reported: metabolic and production yields. Metabolic lactate yields were calculated as weight lactate produced per weight of sugar consumed and production yields based from weight lactate produced per weight of sugar(s) added to the fermentation.

Lactate production rates (V; g/l per h) were calculated over the time required for 95% of the maximum lactate concentration. The time required and maximum lactic acid concentration were determined by fitting a four-parameter sigmoid model to the lactic acid data. The model's parameters were calculated using a nonlinear curve fitting software package (Table Curve 2 D, version 4, SPSS, Chicago, IL). Each data set was fitted by the model with an adjusted correlation of 0.95 or greater. Both yields and V were corrected for the dilution of the culture from inoculation and for automatic base additions needed to maintain the set pH.

A carbon balance was carried out based on glucose or xylose consumed and products formed (biomass, lactic acid, and succinic

acid). Biomass production was determined from optical density measurements; 1.0 optical density at 550 nm was equal to a concentration of 0.30 mg/ml cell dry weight (data not shown). Dried *E. coli* biomass was assumed to be 45% w/w carbon [1].

Results and discussion

Construction of E. coli strains

E. coli strains FBR9, FBR10, and FBR11 were created by transforming nonfermentative strains FMJ39, NZN111, and ND10, respectively, with plasmid pVALDH1. This low copy number plasmid carries the *ldh* gene from *S. bovis* [36], which is specific for production of L-lactic acid. The growth of each transformant was compared to that of the original strain in anaerobic culture. Each strain was grown overnight on aerobic LB medium supplemented with glucose (4 g/l). A 1/100 (v/v) inoculum was transferred to a culture containing anaerobic LB supplemented with glucose and resazurin and optical densities were measured (Table 2). None of the untransformed strains showed significant growth in the 48-h period. In contrast, the transformants, which carry the *ldh* gene on pVALDH1, reached their maximum optical densities within 24 h.

The nontransformed strains were unable to grow fermentatively, as previously reported for strains FMJ39 and NZN111 [8,13]. The *pfl*- and *ldhA*- mutations carried by these strains prevent them from recycling the NADH generated from oxidation of the sugars being metabolized into pyruvate. Complementation of the *ldhA*- mutation with the cloned lactate dehydrogenase gene allows for pyruvate conversion to L-lactate, which supplies the needed electron sink and allows the transformed strains to grow under fermentative conditions. Strain FMJ39 has been used to screen genomic libraries for LDH activity by selecting for growth on anaerobic medium [3,11,25,36]. Similar results as for the LDH transformants were obtained when the same parental strains were transformed with pLO1297, which provides an electron sink *via* conversion of pyruvate into ethanol (Refs. [8,13] and unpublished results).

Genetic stability of FBR9 and FBR11 in repeated batch cultures

Plasmid-carrying microorganisms might be undesirable for industrial production of lactic acid because without addition of antibiotics, the production strain might lose the plasmid after the large number of transfers required to grow enough inoculum to seed an industrial sized bioreactor. However, because *E. coli* require LDH for fermentative growth, the plasmid encoding the *ldh* gene might be positively selected for as long as the medium was kept anaerobic.

Plasmid stabilities for two of the transformed strains (FBR9 and FBR11) were tested using serial batch cultures. Cultures were grown anaerobically in media supplemented with glucose or xylose and cultures were transferred twice per day using a 2% v/v inoculum.

Once per day, a sample was removed, diluted, and plated aerobically on LB agar plates supplemented with the same sugar used for the liquid medium. Colonies were transferred onto LB agar plates supplemented with Em for detection of pVALDH1. The ratio of cells carrying and not carrying pVALDH1 (Em^r) was determined from the number of transferred colonies that grew after being incubated for approximately 18 h. When strains FBR9 and FBR11 were transferred on glucose, the plasmid was identified for both strains in an average of 96±5% of the cells during the 10 transfers with no decrease over time. Plasmid maintenance was also similar for FBR11 transferred on xylose (94±4%). As expected, pVALDH1 was maintained by FBR9 and FBR11 cells in the absence of antibiotics. That 100% of the colonies did not grow on the Em^r selection plates does not imply that a subpopulation of cells can grow in the absence of LDH activity. The cells sampled from the anaerobic culture were initially grown aerobically on nonselective plates. Some of the sampled cells that appear to have lost the plasmid might have lost the plasmid only after being transferred to the solid nonselective medium.

Table 2 Growth in anaerobic medium

Strain	Turbidity[a]	
	24 h	48 h
Untransformed strains		
FMJ39	0.01	0.05
NZN111	0.02	0.05
ND10	0.03	0.02
Transformed strains		
FBR9	0.60	0.62
FBR10	0.51	0.54
FBR11	0.80	0.75

[a]Optical density measured at 660 nm.

Table 3 Effect of pH and temperature on lactic acid production for strain FBR9

pH	Temperature (°C)	Maximum lactate concentration (g/l)
5.5	30	11.4±4.2
5.5	35	23.7±0.3
6.5	30	53.3±1.2
6.5	35	62.7±6.1
7.5	30	64.0±4.8
7.5	35	64.6±4.8

Specific growth rates on glucose and xylose

The specific growth rates of FBR9 and FBR11 were measured in anaerobic test tube cultures supplemented with glucose or xylose. The cultures were inoculated from seed cultures grown on the same sugar and incubated at 35°C. Growth rates were determined by measuring changes in optical density during the exponential growth phase. Both strains grew faster on glucose (FBR9 0.76±0.00 h^{-1}; FBR11 0.94±0.00 h^{-1}) compared to xylose (FBR9 0.47±0.01 h^{-1}; FBR11 0.62±0.01 h^{-1}) as expected from previous results reported for *E. coli* [8,13]. Strain FBR11 grew faster on both sugars compared to FBR9.

Effect of pH and temperature on lactic acid production

Using strain FBR9, fermentations were run to determine the effect of pH and temperature on production of lactate. Fermentations were carried out with 8% w/v glucose at pH 5.5–7.5 and at 30°C and 35°C; cultures were inoculated at pH 7 and the pH was allowed to drift to the set-point to avoid shocking the cells. Maximum lactate concentrations at 120 h were similar for fermentations controlled at

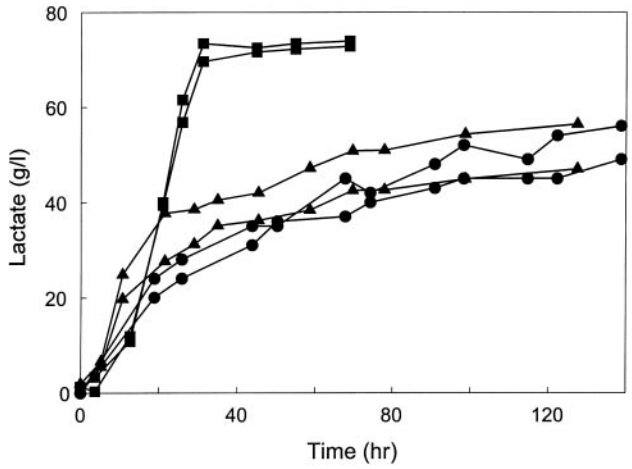

Figure 1 Fermentation of glucose (10% w/v) by lactic acid strains FBR9 (●), FBR10 (▲), and FBR11 (■). Data from two experiments shown.

pH 6.5 and 7.5 and temperatures of 30°C and 35°C (Table 3). The lower lactate production at pH 5.5 compared to the other fermentations can be accounted for by residual glucose. The poorer results observed at pH 5.5 can be accounted for in part because pH 5.5 is below the optimal growth conditions for *E. coli* (pH 6–8; [14]) and because lactic acid becomes more inhibitory with decreasing pH.

Lactic acid inhibition of bacterial growth has been attributed primarily to two mechanisms: uncoupling of the proton gradient across the cellular membrane and intracellular accumulation of the anion. The first step in both mechanisms is the diffusion of the undissociated lactic acid molecule across the cellular membrane. Once acid enters the cell, it dissociates because of the intracellular pH (approximately 7.8, [9,29]). The cell maintains the intracellular pH by pumping H^+ out *via* cation-dependent proton pumps, which is an energy-intensive process [26]. Meanwhile, the anion concentration increases because the charge on the anion prevents it from transversing the membrane (unless through a transport protein), which leads to an osmotic imbalance [28,29]. Therefore, increasing the amount of undissociated lactic acid, as occurs when lactic acid concentration is increased or the pH is lowered, leads to greater inhibition of cell growth because of pH and osmotic imbalances. For a given lactic acid concentration, decreasing the fermentation pH from 7.5 to 5.5 increases the undissociated lactic acid present in the medium 100-fold, from 0.02% of the lactic acid to 2%.

Comparison of strains FBR9, FBR10, and FBR11 for fermentation of glucose and xylose

Strains FBR9, FBR10, and FBR11 were used to ferment 100 g/l glucose in bioreactors equipped with automatic pH control. Strain FBR11 exhausted the glucose in 30 h, while fermentations run using strains FBR9 and FBR10 had 2.1–2.3% w/v residual glucose even after 120 h (Figure 1). The average lactic acid productivity of FBR11 (2.2 g/l per h) was three- to fivefold greater than for FBR9 and FBR10 (Table 4). FBR11 also had a very high lactic acid yield, 93% of theoretical. The theoretical lactic acid yield for conversion of sugars is 1.0 g acid produced per gram of sugar consumed. As a direct consequence of the large amounts of residual glucose, both FBR9 and FBR10 had low yields, only 61–63% of the theoretical maximum yield. The only side-products detected were a small amount of succinic acid (0.9–2.2 g/l) and cell biomass.

It was not expected that FBR11 would perform twice as well (based upon final lactic acid concentration) as either FBR9 or FBR10. The major difference among the strains is that FBR11 was constructed from an *E. coli* B strain and both FBR9 and FBR10 were constructed from *E. coli* K12 strains. The specific *E. coli* B strain chosen was based upon an earlier screening of *E. coli* strains genetically engineered for ethanol production [15]. While few studies exist comparing growth for different *E. coli* strains, when cultured aerobically *E. coli* B has significantly higher cell yields and specific growth rates compared to *E. coli* K12 [20]. FBR11 also grew faster anaerobically than either FBR9 or FBR10 on both glucose and xylose. Some acid-resistant *E. coli* have the ability to lower their internal pH, which gives them a significant growth advantage at lower external pHs [9]. Decreasing the difference between the internal and external pHs mitigates the inhibitory effects of weak organic acids such as lactic acid. However, it was reported that *E. coli* B shows the same proportional decrease in growth rate as *E. coli* K12 for equal concentrations of acetate [20]. Therefore, the increase in lactic acid production by strain FBR11 compared to the other strains is most likely related to its superior growth properties and not to increased tolerance to lactic acid.

Wild-type *E. coli* produce a mixture of organic acids, including D-lactate and ethanol when grown fermentatively [7]. By introducing the lactate dehydrogenase gene from *S. bovis* into a *pfl*-, *ldhA*- background, the transformed strains have been engineered to selectively produce the more economically desirable L-lactate isomer in high yields. The isomer form of the product was confirmed enzymatically (data not shown). Another difference between the *S. bovis* and *E. coli* LDHs is that 1,6 diphosphofructose (FDP) is an allosteric activator of the *S. bovis* LDH [12][34]. Dependence of L-LDH activity on FDP is thought to regulate

Table 4 Fermentations of 100 g/l glucose or xylose[a]

Strain	Carbon source	Maximum lactic acid (g/l)	Residual sugar (g/l)	Base addition (mol/l)	Succinic acid (g/l)	Y_p[b] (g/g)	Y_m[c] (g/g)	V[d] (g/l/h)	Carbon recovery (%)
FBR9	Glucose	53.0±3.0	21±1	0.54±0.01	0.09±0.00	0.63±0.03	0.84±0.03	0.48±0.02	92±3
FBR10	Glucose	51.7±4.7	23±6	0.52±0.01	0.10±0.01	0.61±0.01	0.84±0.01	0.70±0.08	101±12
FBR11	Glucose	73.2±0.6	0±0	0.86±0.02	0.22±0.02	0.93±0.01	0.93±0.01	2.33±0.11	101±1
FBR9	Xylose	56.0±6.0	17±8	0.58±0.08	0.10±0.02	0.67±0.00	0.84±0.00	0.47±0.05	94±0
FBR11	Xylose	63.3±1.8	10±3	0.70±0.02	0.17±0.00	0.78±0.01	0.89±0.01	0.73±0.10	94±1

[a]Duplicate fermentations were performed for each trial at pH 6.7 and 35°C.
[b]Lactic acid production yield.
[c]Lactic acid metabolic yield.
[d]Average lactic acid productivity.

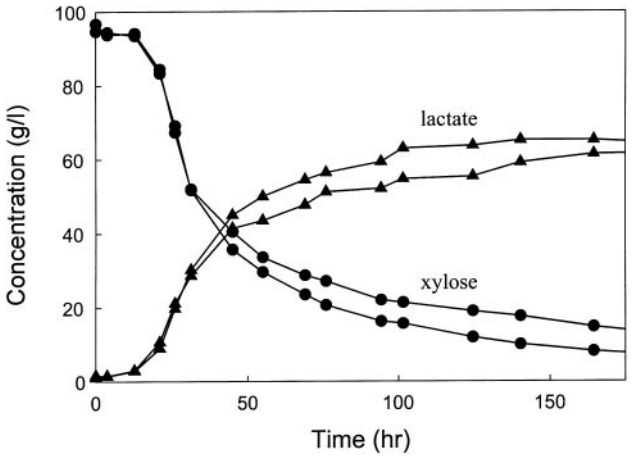

Figure 2 Fermentation by xylose (100 g/l) by strain FBR11; lactic acid (▲), xylose (●). Results of two experiments shown.

lactate production by *S. bovis*, in a fermentation rate-dependent manner [30]. Presumably, the same regulatory control of *S. bovis* LDH activity can be expected to operate in the *E. coli* strains because FDP is an intermediate of glycolysis.

The lactate yield and productivity observed for strain FBR11 compare favorably to another *E. coli* strain recently engineered for L-lactic acid fermentation of glucose [6]. That strain (JP204) carries a plasmid encoding the L-lactate specific *ldh* from *Lactobacillus casei* and contained a genomic *pta-1* (phosphotransacetylase) mutation, which prevents the strain from producing acetate fermentatively. The resulting strain produced only 45 g of L-lactic acid in 67 h with a metabolic yield that was 69% of theoretical. When a *ppc*- (phosphoenolpyruvate carboxylase) mutation was introduced, which eliminated succinic acid production, the strain produced only 0.43% w/v of lactic acid in 33 h. A D-lactic acid production strain was also described in the same report. This strain carried a *pta*$^-$, *ppc*$^-$ background and relied on native D-LDH activity. While the product yield was similar (0.9 g/g), the final concentration (62 g/l) and productivity (1 g/l per h) were still lower than that observed for FBR11 (Table 4).

Strains FBR9 and FBR11 were used to ferment the pentose xylose (10% w/v), using a similar set-up as that used for the glucose fermentations. The strains fermented xylose more slowly than glucose although, once again, strain FBR11 gave the highest yield (Figure 2, Table 4). Strain FBR11 obtained its maximum lactic acid concentration (63.3 g/l) within 100 h. FBR9 also obtained its maximum lactic acid concentration in 100 h, but reached only 56 g/l. The lactic acid production yield for strain FBR9, based upon added xylose, was 78% of the maximum possible. It is also significant that the lower yield compared to that for the glucose fermentation can be attributed to residual carbohydrate. Therefore, even though the yield was diminished, selectivity for lactic acid production remained very high in fermentation of xylose.

The yield and selectivity of strain FBR11 fermenting xylose are comparable to results reported for other microorganisms. With very few exceptions, lactic acid bacteria that ferment pentose sugars use the heterofermentative lactic acid pathway that yields only 60% lactic acid, the remainder of products being a mixture of ethanol and acetate [18]. One *Lactobacillus* strain was reported to convert arabinose and ribose to lactic acid with a 90% yield [18]. Picataggio *et al* [27] metabolically engineered this strain to also ferment xylose by expressing the xylose operon from *Lactobacillus pentosus*. The resulting strain produced 15 g/l lactic acid in 5 days [27]. More recently, there has been reported a *Lactobacillus casei* subsp. *rhamnosus* (ATCC 10863) strain that fermented xylose to L- and D-lactic acid with a yield greater than 80% [17,33]. However, average lactic acid productivity was low because of a 1- to 2-day lag phase [17,33]. The fungus *R. oryzae*, which is also used industrially to convert glucose to lactic acid, can also ferment xylose. But again productivity was low and only 2.3% w/v lactic acid was produced in a fermentation that lasted 144 h [37].

While the results for strain FBR11 fermenting xylose compare favorably with reported values for other microorganisms, the yield and productivity are still lower than was desired. We are currently developing a new series of lactic acid-producing *E. coli* using strains with improved characteristics for pentose utilization. The parent strains carry a glucose phosphotransferase (*pts*-) mutation in addition to the *pfl* and *ldh*A mutations, which are required for plasmid maintenance. The phenotypic effect of the *pts*- mutation is to suppress catabolite repression, which allows for the co-utilization of pentose sugars in media containing glucose.

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