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High-Yield Fermentation of Pentoses into Lactic Acid

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Abstract

Lactobacillus species capable of fermenting glucose are generally incapable of utilizing xylose for growth or fermentation. In this study, a novel aspect of a well-known *Lactobacillus* strain, *L. casei* subsp. *rhamnous* (ATCC 10863), was uncovered: it can ferment xylose as efficiently as glucose. This strain is a registered organism, extremely stable on long-term operation. Fermentation by this strain is characterized by an initial lag phase lasting 24–72 h before xylose consumption takes place. The yield (grams/gram) of lactic acid from xylose is in excess of 80% with initial volumetric productivity of 0.38 g/(L·h). Acetic acid is the primary byproduct formed at the level of about 10% of the lactic acid. In addition to xylose, it can ferment all other minor sugars in hemicellulose except arabinose. Subjected to mixed sugar fermentation, this strain consumes glucose first, then mannose, followed by almost simultaneous utilization of xylose and galactose. It shows high tolerance for lactic acid as well as extraneous toxins. It can ferment the mixed sugars present in acid-treated hydrolysate of softwood, giving yields similar to that of pure sugar but at a slower rate.

Index Entries: Lactic acid; xylose; *Lactobacillus*; fermentation.

Introduction

Lactic acid is a specialty chemical widely used in the food, chemical, and pharmaceutical industries *(1)*. It is produced by a synthetic or a fermentation route *(2)*. The fermentation route for lactic acid production has gained importance recently owing to a continued increase in demand for naturally produced lactic acid *(3)*. Lignocellulosic materials and starchy substances are potential feedstocks for production of fuels and chemicals including lactic acid by a fermentation route *(4)*. From that standpoint, there is a great deal of interest in utilizing all the carbohydrates in biomass. Considerable research has been directed to the search of organisms capable of

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high-performance xylose fermentation into lactic acid. This research has produced several alternatives: *Lactobacillus xylosus*, *L. pentosus*, *L. lactis* IO-1, *L. coprophilus*, and *L. hilgardii (5–8)*. In addition to these natural isolates, genetically engineered strains have been created for this purpose. A well-known example is the patented recombinant *Lactobacillus* $MONT_4$ culture that has been imbedded with external genes responsible for xylose fermentation into lactic acid *(9)*. The reported yield of lactic acid obtainable from this xylose-fermenting microorganism is in the range of 0.41–0.58 g/g. Acetic acid is usually produced as the major byproduct, and its proportion is about 60% of that of lactic acid *(5–8)*. The yield (grams/gram) of lactic acid from recombinant cells, although not available, may be higher, but the recombinant cells generally have a tendency of being genetically unstable on repeated use.

In our research, we made a startling experimental discovery: a registered *Lactobacillus* strain widely known as a lactic acid producer from glucose is also an efficient producer of lactic acid from xylose. The original name of this organism, at the time of our acquisition, was *Lactobacillus delbrueckii* (NRRL-B445). It is our understanding that it has since been renamed to *rhamnosus* and *Lactobacillus casei* subsp. *rhamnosus* (ATCC 10863) *(10)*. In this article, we report on various characteristics of this organism, focusing on its capability of fermenting xylose as well as mixed sugars.

Materials and Methods

Microorganism and Media

The microorganism employed in this work was *L. casei* subsp. *rhamnosus* (ATCC 10863). The culture was grown at 37°C for 48 h in agar slants made of 5% Elliker broth (Difco Lab., Detroit, MI) and 5% tomato juice agar (Difco), and was stored at 4°C. The fermentation medium contained 5 g/L of yeast extract (Sigma Chemical, St. Louis, MO), 0.2 g/L of K_2HPO_4, 0.2 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4{\cdot}7H_2O$, 0.03 g/L of $MnSO_4{\cdot}H_2O$, 0.03 g/L of $FeSO_4{\cdot}7H_2O$, and 2 g/L of succinic acid. The inoculum for fermentation was prepared by growing the culture anaerobically in a flask containing 5% Elliker broth at 37°C for 36 h. The average cell concentration in the inoculum was 0.7 g/L (dry cell mass).

Preparation of Acid Hydrolysates of Softwood

Softwood hydrolysates 98-05-14a and 98-05-14b obtained from the National Renewable Energy Laboratory under two different conditions were tested for fermentability/toxicity using the *Lactobacillus* species. The hydrolysate 98-05-14a was generated by pretreating softwood at 185°C for 10 min using 0.07 wt% sulfuric acid, then at 230°C with the same acid for 30 min; and the hydrolysate 98-05-14b was obtained by pretreating softwood at 175°C for 10 min using 0.07 wt% sulfuric acid, then at 238°C with the same acid for 35 min. The hydrolysates were held at 140°C for 3.5 h to

Table 1
Concentration of Acid Hydrolysates
of Softwood Obtained After Secondary Hydrolysis and Flashing

Effluent[a]	Glucose (g/L)	Xylose (g/L)	Mannose (g/L)	Galactose (g/L)	Arabinose (g/L)
98-05-14a[b]	14.41	2.95	3.79	1.61	1.20
98-05-14b[c]	16.47	4.95	4.68	2.14	1.45

[a]Both the effluents were held at 140°C for 3.5 h to break down oligomers and then flashed for concentrating.

[b]Effluent obtained from pretreating softwood at 185°C for 10 min using 0.07 wt% sulfuric acid, then at 230°C with the same acid for 30 min.

[c]Effluent obtained from pretreating softwood at 175°C for 10 min using 0.07 wt% sulfuric acid, then at 238°C with the same acid for 35 min.

break down oligomers and then were flashed for concentrating. This process may have removed volatile furfural generated during the acid hydrolysis. Table 1 gives the concentration obtained after flashing for each hydrolysate.

Batch Fermentation

Batch experiments were conducted at 45°C in a 250-mL glass bottle with a 100-mL working volume. Fermentation was initiated by adding a 10% (v/v) inoculum. The pH during the fermentation was controlled at 5.7 using calcium carbonate. Every 24 h, pH was monitored and adjusted to the operating pH using calcium carbonate and/or ammonium hydroxide. The bottles were purged with nitrogen when opened for sample collection or for pH adjustment. For fermentability/toxicity tests on actual hydrolysates, the hydrolysate loading was varied from 0 to 80% of culture volume, but the final concentrations of each sugar were made as follows: 15 g/L of glucose, 5 g/L of xylose, 5 g/L of mannose, 2.5 g/L of galactose, and 1.5 g/L of arabinose by external addition of sugars. In some runs, the hydrolysates were fermented as they were, at a loading of 80% of culture volume, without any external sugar supplementation but with yeast extract (Sigma) loading of 30 g/L. The pH adjustment and sample collection was done following the procedure described previously.

Analysis

The fermentation samples were taken every 24 h using aseptic techniques and were analyzed for sugar, lactic acid, and acetic acid by a high-performance liquid chromatograph (Water Associates) equipped with an RI detector. Bio-Rad's (Hercules, CA) HPX-87H column was used at 65°C with 0.005 *M* H_2SO_4 as the mobile phase. The flow rate was set at 0.6 mL/min. For substrate consumption profiles, Bio-Rad's HPX-87-P column was used at 85°C with deionized water as the mobile phase and the flow rate set at 0.55 mL/min.

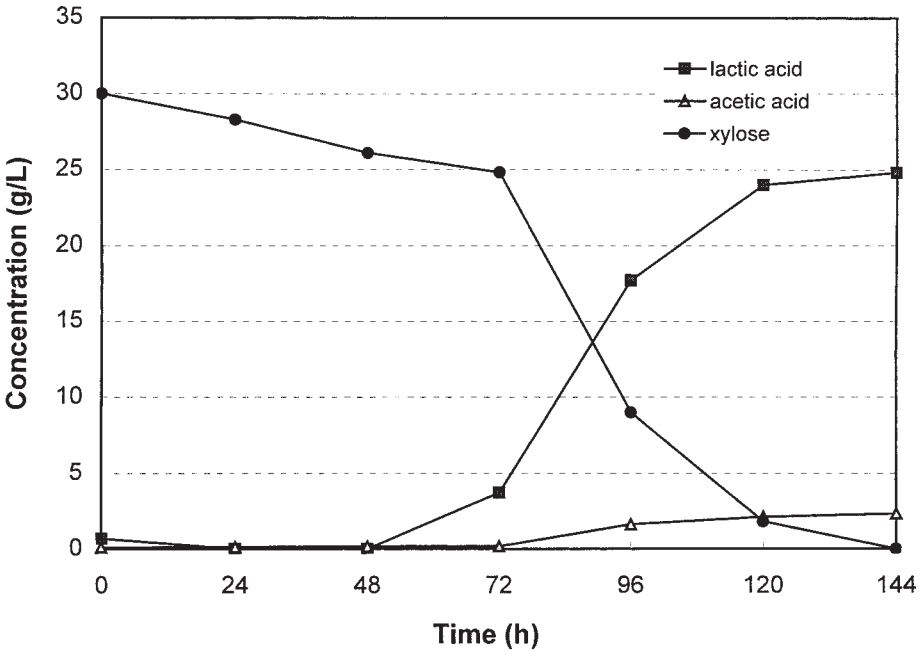


Fig. 1. Concentration profiles of lactic acid, acetic acid, and xylose in lactic acid fermentation from xylose. Fermentation conditions: 45°C, pH 5.7, 3% (w/v) xylose loading.

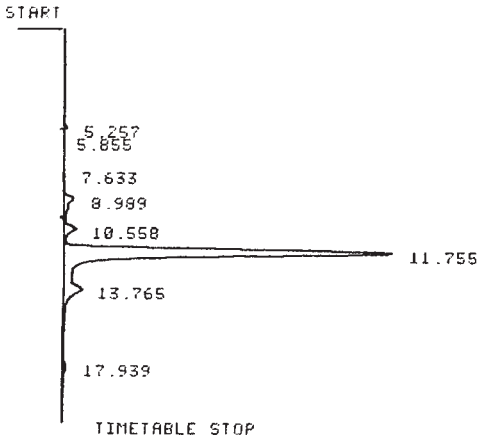
Fermentation Parameters

The reported lactic acid yield is defined as 1 g of lactic acid produced per gram of sugar consumed. In all cases, xylose and/or glucose were completely utilized. Therefore, the terminal yield calculated is also equivalent to 1 g of lactic acid produced per gram of sugar input. Initial lactic acid coming from the addition of 10% inoculum was subtracted to represent the true yield.

Results and Discussion

Profiles in Xylose Fermentation

Figure 1 presents the concentration profiles of lactic acid, acetic acid, and xylose in lactic acid fermentation from xylose by *L. casei* subsp. *rhamnosus* (ATCC 10863). The fermentation was carried out at 45°C, pH 5.7, and a xylose loading of 3% (w/v). For the initial 48 h, there was no sign of xylose consumption. After 72 h, both xylose consumption and lactic acid production began and proceeded in full swing until the fermentation was completed in 144 h. The yield of lactic acid is calculated to be 0.8 g/g, which is substantially higher than most reported yields of lactic acid from xylose *(5–8)*. Another important feature is that the amount of acetic acid (the primary byproduct) produced is much lower than those previously reported



Time (min)	Component
8.989	Xylose
10.558	Intermediate
11.755	Lactic acid
13.765	Acetic acid

Fig. 2. A typical HPLC chromatogram obtained close to the end of lactic acid fermentation from xylose. Column: Bio-Rad Aminex HPX-87H. Samples were diluted prior to injection.

with similar organisms. The amount of acetic acid was <10% that of lactic acid. Ethanol, another common byproduct, was detected only in a trace amount.

Conversion of pentoses by *Lactobacillus* has been projected to follow this overall stoichiometric equation *(11)*:

$$C_5H_{10}O_5 \rightarrow C_3H_6O_3 + CH_3COOH$$

This equation predicts the yield of acetic acid to be in excess of 65% of lactic acid. Two moles of adenosine triphosphate (ATP) are formed per mole of xylose consumed, the same amount of energy as produced per mole of glucose. Alternatively, ethanol may be produced in addition to acetate *(12–14)*, but 1 mol less of ATP per mole of xylose is formed utilizing this route. However, close examination of the high-performance liquid chromatography (HPLC) chromatogram (Fig. 2) reveals that production of lactic acid from xylose by this organism goes through an alternative pathway since neither ethanol nor acetate accumulates in a significant amount. However, an intermediate was detected that resembled the one observed during homofermentation of glucose by the same organism. One may speculate that a similar homofermentative pathway may apply to fermentation of xylose. Although the homofermentative characteristic is rare for pentose-utilizing organisms *(15)*, it is not totally unprecedented. Barre *(16)* reported that some thermophilic *Lactobacilli* can ferment pentoses (arabinose and ribose) homofermentatively. It has also been reported that strains related to *L. salivatrius (17)*, further described as *L. murinus (18)*, exhibited the same property.

Effect of Initial Xylose Loading

The effect of initial xylose loading on lactic acid fermentation was studied at 45°C, pH 5.7. Figure 3 summarizes the results. An increase in

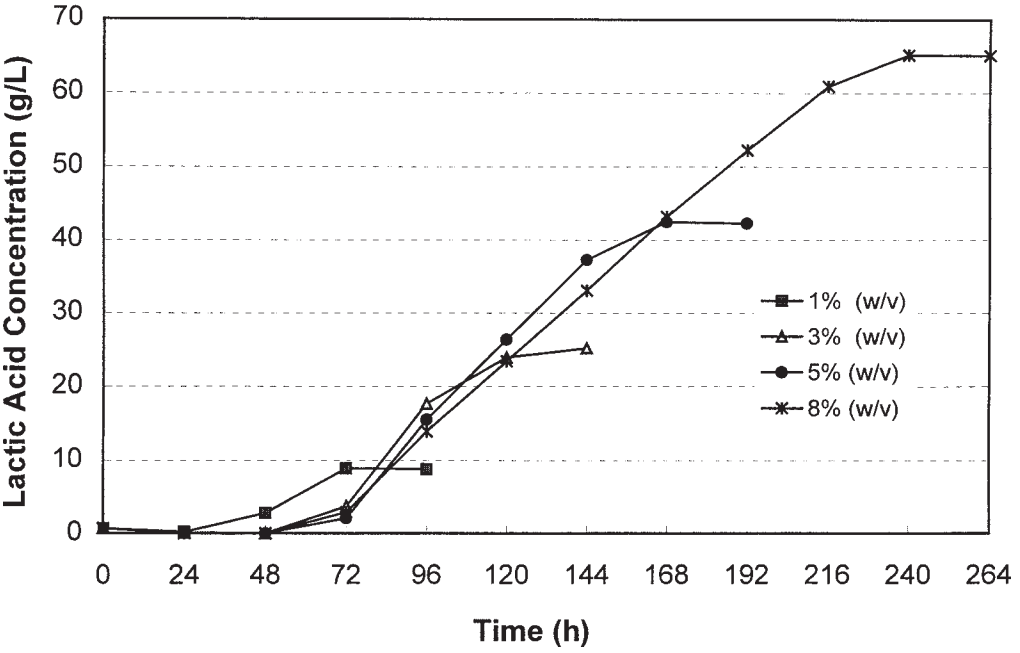


Fig. 3. Time course of lactic acid production at different initial xylose loading. Fermentation conditions: 45°C, pH 5.7.

xylose loading induced an increase in lag time. The fermentation nevertheless went to completion in all cases. The fermentation was complete at 72 h for a 1% (w/v) xylose loading, and 240 h for 8% (w/v). However, no clear trend on the lactic acid yield was noticed. The calculated yields were 0.81, 0.81, 0.83, and 0.80 because the xylose loading was increased from 1 to 8% (w/v). The relatively long initial lag phase perhaps can explain why the xylose utilization behavior of this organism has gone unnoticed.

Effect of pH

The effect of pH was studied over the range 4.0–7.5 with xylose loading of 3% (w/v) (Fig. 4). Fermentation runs made at pH 5.2 and 6.0 were complete, whereas the runs made at pH 4.0 and 7.5 were incomplete, even after 168 h. The fermentation rate was much higher at pH 6.0 than at pH 5.2. From this observation, the best estimate of the optimum pH for xylose fermentation is 6.0.

Fermentation of Minor Sugars and Mixed-Sugar Substrates

The capability of a microorganism to ferment xylose was the focal point of our study. We also were interested in determining how this organism reacts to mixed-sugar substrates of glucose and xylose because they are the two major carbohydrates in biomass feedstock. A series of fermentation tests were therefore made to address this issue using an initial sugar loading of 2% (w/v) glucose + 2% (w/v) xylose, at 45°C and pH 5.7. Figure 5

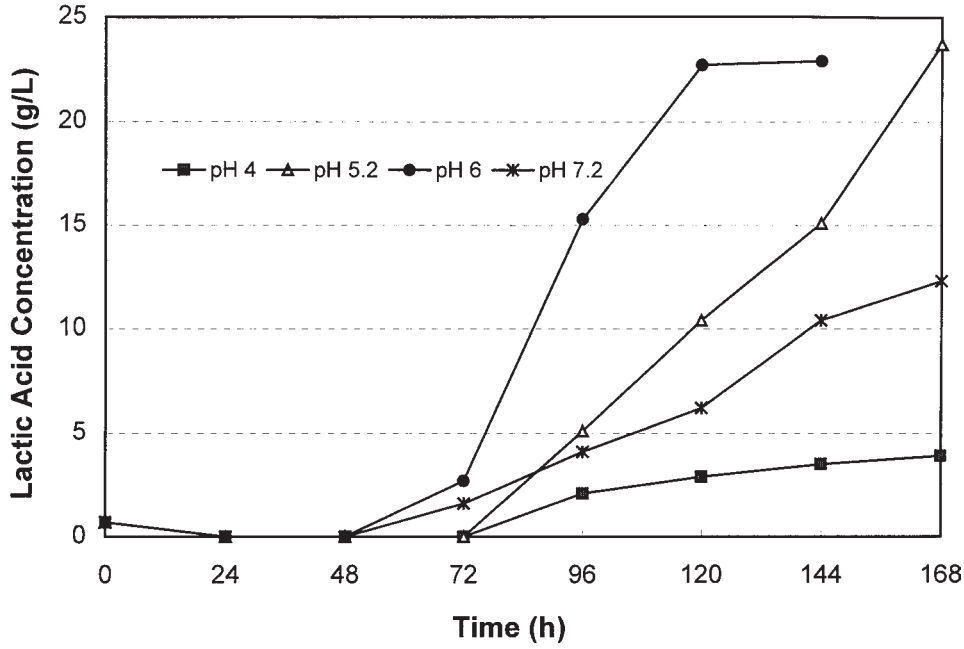


Fig. 4. Effect of pH on lactic acid production from xylose. Fermentation conditions: 45°C, 3% (w/v) initial xylose loading.

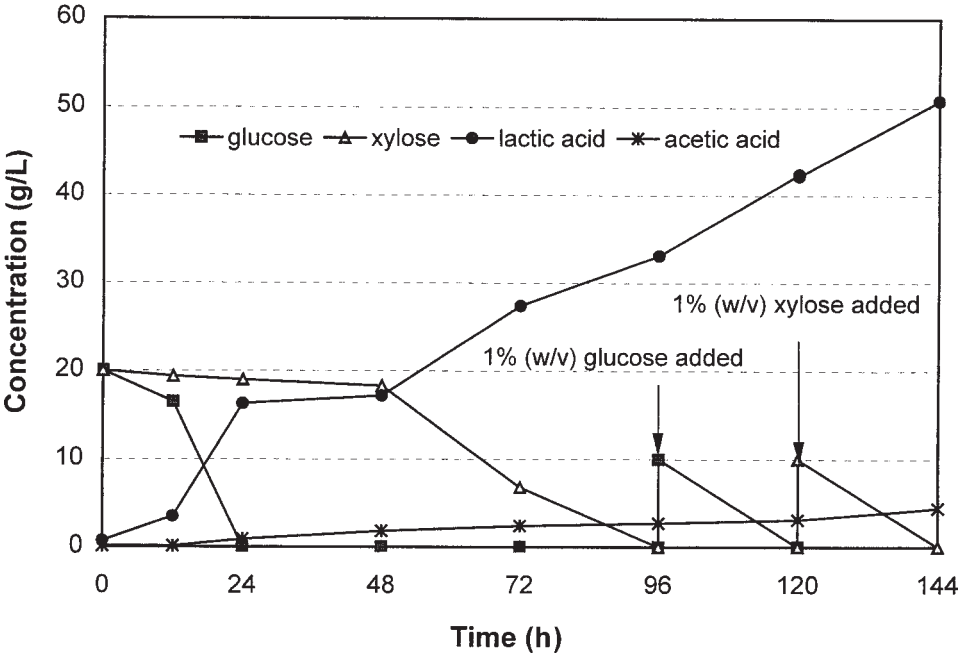


Fig. 5. Lactic acid production from a mixed sugar fermentation of glucose and xylose. Fermentation conditions: 45°C, pH 5.7, 2% (w/v) glucose + 2% (w/v) xylose initial sugar loading.

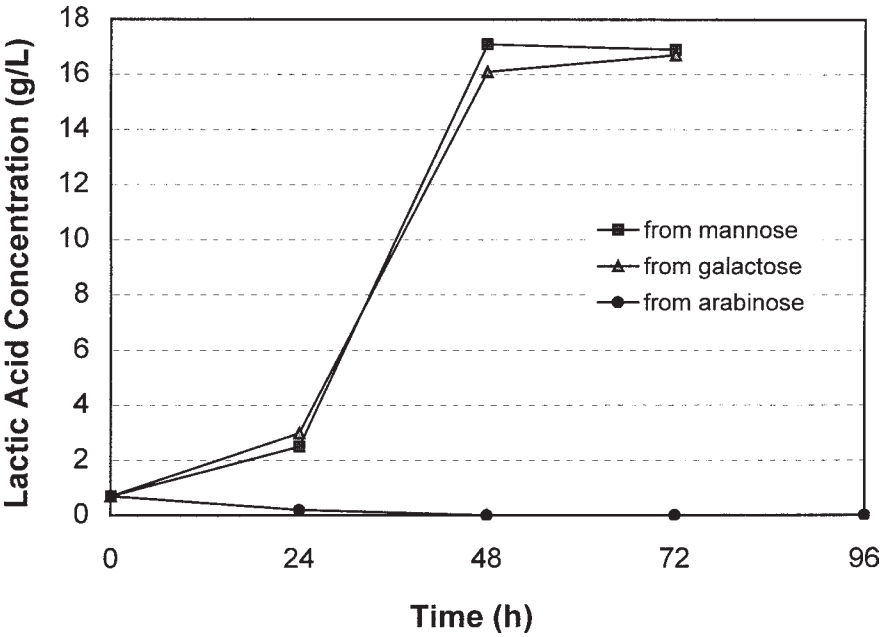


Fig. 6. Lactic acid production from mannose, galactose, and arabinose fermentation. Fermentation conditions: 45°C, pH 5.7, 2% (w/v) initial sugar loading.

presents the concentration profiles of glucose, xylose, lactic acid, and acetic acid. It is quite evident that glucose is utilized preferentially. Only after glucose is completely depleted does xylose uptake begin. It occurred after another lag period. When both xylose and glucose were utilized completely, an additional 1% (w/v) glucose was put into the fermentor. It was consumed without any lag time. At the 120-h point, 1% (w/v) xylose was then added. The additional portion of the xylose was also consumed without any lag period. The lag period for xylose utilization was observed only at the start of the first xylose utilization step; it was not seen in the subsequent switching of substrates between glucose and xylose.

Lignocellulosic biomass contains a variety of minor sugars in addition to xylose and glucose. From an economic standpoint, it is highly beneficial if all the sugar substrates are utilized. A series of additional tests were made with this strain to verify its ability to ferment minor sugars. The fermentation was conducted at 45°C and pH 5.7 using individual sugars of mannose, galactose, and arabinose at a 2% (w/v) level. Figure 6 presents the lactic acid profiles for each sugar substrate. It is clearly seen that mannose and galactose were fermented rather efficiently whereas arabinose was not. The yields of lactic acid were 0.82 and 0.80 g/g for mannose and galactose, respectively. The organism was then subjected to a mixed-sugar substrate containing all five sugars. The consumption profiles of Fig. 7 indicate that this microorganism utilizes glucose first, then mannose, followed by almost simultaneous utilization of xylose and galactose. Arabinose was again left unutilized, confirming our previous finding.

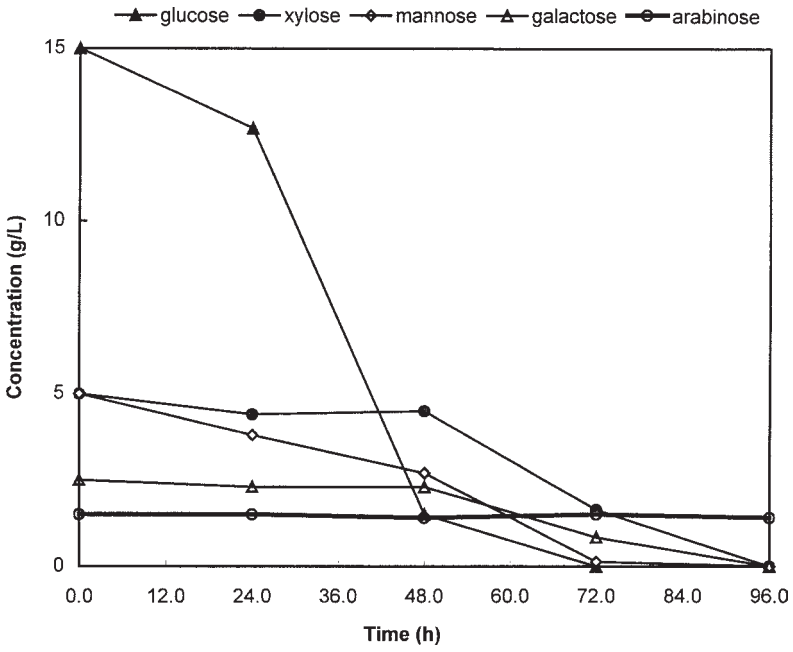


Fig. 7. Substrate consumption profiles obtained in mixed sugar fermentation. Fermentation conditions: 45°C, pH 5.7, 15 g/L glucose, 5 g/L xylose, 5 g/L mannose, 2.5 g/L galactose, and 1.5 g/L arabinose.

Product Inhibition

Lactic acid is generally known to be a strong inhibitor for fermentation even with pH control *(19)*. The extent of inhibition by lactic acid in xylose fermentation was investigated. For this purpose, a fed-batch fermentation was carried out with 2% (w/v) initial xylose, and subsequent additions of xylose at 96-, 144-, 216-, and 288-h points. It was run at 45°C and pH 5.7. Figure 8 shows the concentration profiles of lactic acid, acetic acid, and xylose. This operation scheme minimizes the substrate inhibition effect, if any, so that any decrease in fermentation rate can be attributed solely to product inhibition. The profiles in Fig. 8 indicate that there is a gradual buildup of inhibition on the microbial activity. The extent of inhibition is such that the volumetric productivity declines to 0.13 g/(L·h) (a 66% reduction from the initial productivity) at a point where the lactic acid concentration reaches 70 g/L.

Fermentability/Toxicity Tests on Acid Hydrolysate of Softwood

Acid-treated softwood hydrolysates generated under two different conditions were put through fermentability/toxicity tests for this organism at different dilutions. Table 2 gives the fermentation data for the first condition. The concentration of lactic acid generally increased with fermentation time. All of the sugar substrates were completely consumed

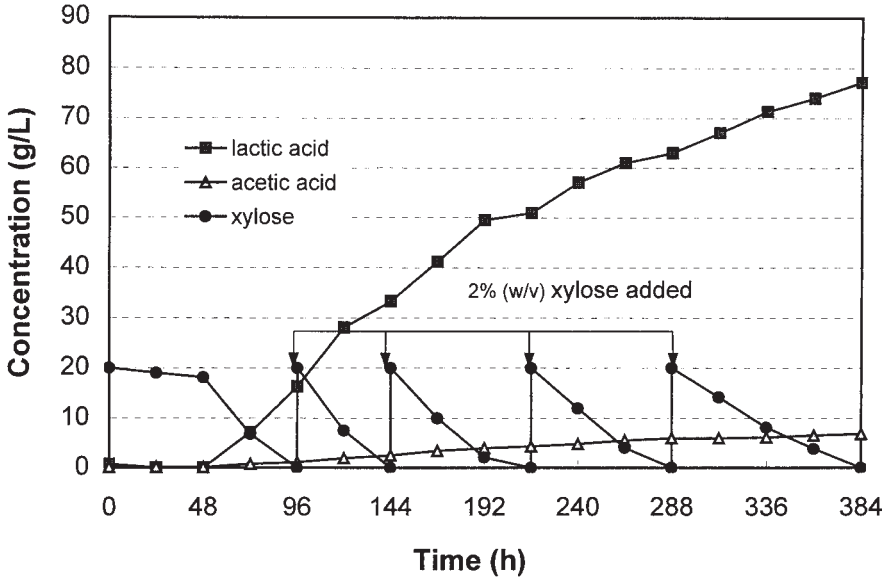


Fig. 8. Lactic acid production from xylose employing fed-batch addition of xylose. Fermentation conditions: 45°C, pH 5.7, 2% (w/v) initial xylose loading.

except arabinose. The time required for complete fermentation (or complete substrate utilization) generally increased with an increase in hydrolysate loading. This is a clear indication that certain components in the acid hydrolysate are inhibitory to the fermentation. The potential inhibitors/toxins are dissolved lignin, its derivatives, hydroxymethyl furfural, and acetic acid. Similar fermentation profiles were obtained for softwood hydrolysate generated under the second pretreatment condition. Table 3 gives the yield (grams/gram) and volumetric productivity for condition no. 2. With pure sugars (0% hydrolysate loading, control) the yield was 0.83 g/g. With hydrolysate loading of 40 and 60%, the yields were only slightly lower, at 80% on average. Even with 80% loading, the yield reached a respectable level of 74 to 75%. Noted that when the substrate with 80% loading of hydrolysate was supplemented with additional yeast extract, the yield improved to about the same level of the control. However, the volumetric productivity decreased significantly with hydrolysate loading of 60% and above.

Conclusion

An unknown property of a well-known strain of *Lactobacillus*, *L. casei* subsp. *rhamnous* (ATCC 10863), was uncovered: it utilizes xylose to produce lactic acid in high yield. Exposed to a xylose medium, this strain underwent an initial lag phase of 24–72 h before xylose consumption took place. It had a pH optimum of 6.0. The lag time increased as the initial xylose loading was increased. The yield (grams/gram) of lactic acid from

Table 2
Fermentation Data of Acid Hydrolysate of Softwood at Different Dilutions (condition no. 1)

Effluent loading (% of culture volume)[a]	Fermentation time (h)	Glucose (g/L)	Xylose + mannose + galactose (g/L)	Arabinose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0 (control)	0	15[b]	12.5 (5 + 5 + 2.5)[b]	1.5[b]	0.71	0.1
	24	12.7	10.1	1.52	4.72	0.23
	48	1.5	9.2	1.43	6.41	0.38
	72	0	2.6	1.54	19.62	0.78
	96	0	0	1.42	23.51	0.96
40	0	15[b]	12.5 (5 + 5 + 2.5)[b]	1.5[b]	0.75	0.21
	24	13.6	12.8	1.56	1.26	0.35
	48	10.1	11.1	1.43	4.12	0.46
	72	0.8	8.3	1.43	13.63	0.71
	96	0	2.2	1.45	20.54	0.94
	108	0	0	1.39	23.75	1.09
60	0	15[b]	12.5 (5 + 5 + 2.5)[b]	1.5[b]	0.65	0.32
	24	13.78	12.7	1.42	1.11	0.23
	48	12.3	11.3	1.41	1.34	0.32
	72	7.75	10.1	1.39	5.76	0.46
	96	0	7.1	1.36	13.9	0.93
	120	0	3.2	1.28	20.9	0.91
	132	0	0	1.23	22.56	1.24
80	0	15[b]	12.5 (5 + 5 + 2.5)[b]	1.5[b]	0.76	0.45
	24	14.5	12.4	1.56	1.07	0.73
	48	13.8	11.8	1.43	1.1	0.46
	72	12.9	11.3	1.47	1.14	0.56
	96	8.5	10.9	1.39	6.73	0.78
	120	2.3	8.9	1.35	12.46	0.85
	144	0	2.7	1.31	19.13	1.07
	156	0	0	1.28	21.1	1.23

[a]Effluent was obtained from softwood pretreated at 185°C for 10 min using 0.07 wt% sulfuric acid, then at 230°C with the same acid for 30 min. Effluent was held at 140°C for 3.5 h to break down oligomers and then flashed for concentrating.

[b]External glucose, xylose, mannose, galactose, and arabinose were added to adjust to the same initial concentration as shown at 0 h for all effluent loading levels.

Table 3
Fermentation Data of Acid Hydrolysates of Softwood (condition no. 2)

Effluent	Effluent loading (% of culture volume)	Yield (g/g)	Volumetric productivity (g/[L·h])
98-05-14a[a]	0 (control)	0.83	0.23
	40	0.83	0.21
	60	0.79	0.16
	80	0.74	0.13
	80[b]	0.82	0.15
98-05-14b[a]	0 (control)	0.83	0.23
	40	0.80	0.20
	60	0.78	0.16
	80	0.75	0.13
	80[b]	0.80	0.16

[a]*See* Table 1 footnotes.
[b]Effluent fermented as is, without external sugar addition but with yeast extract loading of 30 g/L. For all cases, inoculum volume = 10% (v/v), and inoculum cell concentration = 0.7 g/L was used.

xylose was in excess of 80%, and acetic acid was the main byproduct, at a level of about 10% that of lactic acid. When subjected to mixed-sugar substrates, this strain utilized glucose first, then mannose, followed by simultaneous utilization of xylose and galactose. This strain, however, could not catabolize arabinose. Fermentation was inhibited by lactic acid even with pH control. A strong inhibition was prevalent above the lactic acid concentration of 70 g/L. The extent of inhibition was such that the volumetric productivity declined from the initial level of 0.37 to only 0.13 g/(L·h). Finally, this organism exhibited a high level of tolerance to toxins. When applied to acid hydrolysates of softwood containing mixed sugars, it produced lactic acid with yields approaching that of pure sugars but at a slower rate.

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