0273-2289/00/84–86/0455/$13.50

Production of Lactic Acid from Pulp Mill Solid Waste and Xylose Using *Lactobacillus delbrueckii* (NRRL B445)

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Abstract

Using the simultaneous saccharification and fermentation (SSF) technique, pulp mill solid waste cellulose was converted into glucose using cellulase enzyme and glucose into lactic acid using NRRL B445. SSF experiments were conducted at various pH levels, temperatures, and nutrient concentrations, and the lactic acid yield ranged from 86 to 97%. The depletion of xylose in SSF was further investigated by inoculating NRRL B445 into a xylose-only medium. On prolonged incubation, depletion of xylose with lactic acid production was observed. An experimental procedure with a nonglucose medium was developed to eliminate the lag phase. From xylose fermentation, *Lactobacillus delbrueckii* yielded 88–92% lactic acid and 2–12% acetic acid.

Index Entries: Xylose; pulp mill solid waste; lactic acid; *Lactobacillus*; fermentation.

Introduction

Lactic acid is an important chemical that has both food and industrial applications. The advantages of lactic acid polymers are their biodegradability, bioenvironmental compatibility, fabricability, thermoplasticity, and high strength *(1)*. The pulp and paper industry generates about 80 million of solid waste every year, and only about 42% of the waste is recycled. Therefore, it is environmentally and economically significant to consider the production of lactic acid using the solid waste from the pulp and paper industry.

The major components of lignocellulosic substrates are cellulose, hemicellulose, and lignin. Hemicellulose comprises 10–40% of the cellulosic materials. The predominant component of hemicellulose in hardwoods is xylan, and it can be converted into xylose using xylanase enzyme. During the course of simultaneous saccharification and fermentation (SSF) of pulp mill waste, it became evident that *Lactobacillus delbrueckii* (NRRL

B445) can convert xylose into lactic acid under substrate-limiting situations. This behavior was atypical of *L. delbrueckii*, which is known to convert only hexoses into lactic acid *(2,3)*. Now, *L. delbrueckii* (NRRL B445) is also being reported as *Lactobacillus rhamnosus (4,5)* and *Lactobacillus casei* subsp. *rhamnosus (6)*. This bacteria is known to convert galactose, glucose, mannose, ribose, rhamnose, and arabinose, but not xylose *(3)*. The three main organisms usually used for the fermentation of xylose into lactic acid in the Lactobacillaceae genera are *Lactobacillus pentosus*, *Lactobacillus plantarum*, and *Lactobacillus xylosus*. From xylose fermentation, *L. pentosus (7)* and *L. plantarum (2)* produce 60% lactic acid and 40% acetic acid. *L. xylosus* produces 41% lactic acid, 23% acetic acid, and .07% ethanol *(6)*. Another xylose-fermenting microorganism is *Lactococcus lactis*, which produces about 47% lactic acid *(8)*. Picataggio et al. *(9)* have patented a recombinant microorganism, *Lactobacillus* MONT4 (pLP3537-xyl), for producing a high yield of lactic acid (94%) from xylose fermentation. Herein, I report that *L. delbrueckii* (NRRL B445) can convert xylose into high amounts of lactic acid as the recombinant *Lactobacillus* MONT4 (pLP3537-xyl).

Materials and Methods

Raw Materials

Pulp mill solid waste was collected from Union Camp, and the composition was determined using the chemical analysis and standard procedures used by NREL Alternative Fuels Division *(10–14)*.

Enzymatic Hydrolysis

The enzymatic hydrolysis of the substrate was determined by using filter paper as standard because of its high digestibility. About 1 g of the solid waste and filter paper were weighed and placed in 100-mL bottles with an airtight caps containing 50 mL of 0.1 *M* sodium citrate buffer solution at pH 5.0. After the bottles were autoclaved and cooled, 60 international filter paper units (IFPU)/mL of cellulase enzyme were added to each bottle. The bottles were incubated in a 150 rpm shaker at 50°C. Samples were withdrawn occasionally up to 72 h, and stored in a refrigerator at 4°C after deactivating the enzyme. The glucose generated from the samples was analyzed using high-performance liquid chromatography (HPLC), and the digestibility of the samples was determined.

Microorganisms and Enzymes

L. delbrueckii (NRRL B445) was used for lactic acid fermentation. The enzyme cellulase cytolase CL (lot no. 17-92262-09, Environmental Biotechnologies, Menroe Park, CA) was used for saccharification. About 60 IFPU/mL of the enzyme was loaded into the fermentor. *L. pentosus* (NRRL B227) was used for comparative study.

Culture Medium

Elliker broth (Difco, Detroit, MI) was used as the culture medium for the fermentation.

SSF Medium

SSF medium consisted of solid waste (20–40 g/L), yeast extract (30 g/L), succinic acid (2 g/L), sodium hydroxide (1.25 g/L), $K_2H(PO)_4$ (0.2 g/L), $KH_2(PO)_4$ (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.6 g/L), $MnSO_4 \cdot H_2O$ (0.03 g/L), and $FeSO_4 \cdot 7H_2O$ (0.03 g/L).

Xylose Fermentation Mediums

Medium 1 contained xylose (15–30 g/L), yeast extract (30 g/L), succinic acid (2 g/L), NaOH (1 g/L), K_2HPO_4 (0.2 g/L), KH_2PO_4 (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.6 g/L), $MnSO_4 \cdot 7H_2O$ (0.03 g/L), and $FeSO_4 \cdot 7H_2O$ (0.03 g/L). Medium 2 contained xylose (15–30 g/L), tryptone (27.5 g/L), NaCl (4 g/L), sodium acetate (1.5 g/L), ascorbic acid (0.5 g/L), K_2HPO_4 (0.4 g/L), and $MgSO_4 \cdot 7H_2O$ (1 g/L).

Experimental Setup

A bioreactor (BioFlow Model C30, New Brunswick Scientific, New Brunswick, NJ), pH controller (Chemtrix Type 45AR, New Brunswick Scientific, NJ), autoclave, incubator, and pump were used for the SSF.

Preparation of Inoculum

L. delbrueckii was incubated at 37°C for 1 d. The culture broth was scaled to a 200-mL flask and incubated again until it reached its exponential growth phase. About 10% of the culture inoculum was transferred into the fermentor, which contained 400 mL of the autoclaved SSF medium.

Experimental Procedure

The SSF medium was prepared, and then sterilized in an autoclave. The temperature was controlled using a sensor. Next, about 60 IFPU/mL of cellulase enzyme was added into the fermentor. The fluid in the fermentor was agitated at 150 rpm. The saccharification step alone was maintained for about 7.5–8 h. Then, approx 10% of the culture was inoculated into the fermentor. Ammonium hydroxide solution (6.0 *N*) was used to control the pH using a pH controller and a pump. The lactic acid concentration was checked every 12 h by taking samples from the fermentor.

Analytical Methods

The sugar contents of pulp mill solid waste were determined using Bio-Rad Aminex HPX-87H and Bio-Rad Aminex HPX-87P HPLC (Tustin, CA) columns. The former showed xylose, mannose, and galactose in one peak. The latter showed separate peaks for the same, and therefore was

used only for the determination of sugar contents. During the SSF experiment, samples were withdrawn from the fermentor every 12 h and analyzed using the Bio-Rad HPX-87H column for the composition of the reactants and the products.

Results

The pulp mill solid waste contained about 69.57% moisture. The chemical composition of the dried pulp mill solid waste showed 48.42% glucan, 9.55% xylan, 3% mannan, 1.39% arabinan, 7.22% ash, 20.55% Klason lignin, and 4.2% acid-soluble lignin. The digestibility in 72 h of the Union Camp solid waste was 88.9% and filter paper was 95.3%.

For SSF, the fermentable sugar in the yeast extract was determined by using about 30 g/L of yeast extract in the fermentor. It was found that about 1.1 g/L of lactic acid and 0.3 g/L of acetic acid were produced from the yeast extract fermentation; this was subtracted from all the results.

Table 1 gives the SSF of the Union Camp solid waste at pH 5.0 and 45°C. The lactic acid yield was 93% when all the hexose sugars were considered as the substrate. Interestingly, about 10% of acetic acid was formed as a byproduct when glucose was almost depleted. Xylose depletion began after the glucose was almost exhausted (*see* Table 1). The lactic acid yield with xylose as a possible substrate was 83%.

To find the variation of productivity with respect to different pH variations, the SSF experiment was conducted at 45°C and pH levels of 4.7, 5.0, and 5.5. Table 1 presents the results of solid waste SSF at 45°C and pH 4.7. The experimental yields of lactic acid and acetic acid production were 92 and 8%, respectively. Only hexoses were considered as the substrate utilized. This experiment did not show any depletion of xylose.

Figure 1 presents the profile of solid waste SSF into lactic acid at 45°C and pH 5.5. This experiment showed a simultaneous depletion of glucose and xylose. The lactic acid yield was more than 100% when hexoses were considered as the only substrate. This indicated that xylose was also consumed. With hexose and xylose as substrates, lactic acid and acetic acid yields were 93 and 7%, respectively.

Temperature was an important parameter in the production of lactic acid from solid waste using cellulase and *L. delbrueckii*. At pH 5.0, the temperature variations considered for the SSF were 40, 45, and 47°C.

The SSF experiment at pH 5.0 and 40°C is shown in Table 1. The lactic acid yield was calculated to be 97% and the acetic acid yield was 8%. Xylose depletion was not indicated for this experiment even at 139 h. The maximum yield was detected after 70 h.

Figure 2 depicts the SSF of solid waste pulp at 47°C and pH 5.0. The maximum lactic acid yield was detected after 43 h. Possible xylose depletion along with glucose was indicated at this pH level and temperature. The lactic acid yield was estimated to be 97%, and acetic acid yield was estimated to be 1%. Xylose was considered along with hexoses in calculating the yield. The overall fermentation rate was very fast. Lactic acid is

Table 1
SSF of Pulp Mill Solid Waste

Time	Lactic acid	Glucose	xmg[a]	Cellobiose	Acetic acid
pH = 5.0, Temperature = 40°C					
7.5	0.3635	4.04	1.115	4.2643	0.2
20.08	3.058	4.72	1.216	3.9192	0.37
32.33	12.26	0.161	1.23	0.9173	0.534
44.08	13.64	0.156	1.36	0.922	0.528
55.33	13.67	0.173	1.368	0.6055	0.751
67.16	14.57	0.217	1.438	0.6098	0.987
79.25	14.99	0.194	1.431	0.57	1.23
90.66	15	0.188	1.42	0.5178	1.36
102.83	15.04	0.195	1.393	0.4551	1.52
115.83	15.12	0.177	1.372	0.3461	1.75
127.66	15.21	0.219	1.48	0.2679	1.94
139.25	15.4	0.127	1.316	0	2.15
pH = 5.0, Temperature = 42°C					
7.583	0.1447	3.5756	1.0849	3.7424	0
19.62	2.96	3.927	1.2486	3.1444	0.33
31.78	10.83	0.1962	1.1994	0	0.53
44.25	12.39	0	1.2873	0	0.66
55.5	12.7	0	1.3463	0	0.791
68.92	12.86	0	1.3541	0	0.932
79.17	12.12	0	1.354	0	1.09
91.75	12.14	0	1.4058	0	1.23
103.25	12.84	0	1.514	0	1.26
115.42	13.07	0	1.326	0	1.39
127.37	12.13	0	1.4484	0	1.5
140.17	12.15	0	1.4187	0	1.62
pH = 5.0, Temperature = 45°C					
0	0.7621	0.0962	0	0.6428	0.0859
6.833	0.7967	4.53	1.224	3.782	0.1074
19.25	2.9641	4.842	1.371	2.821	0.161
31.25	6.0633	3.2115	1.335	1.707	0.344
43.17	10.68	0.3423	1.306	0.3036	0.483
54.42	11.6305	0.3077	1.143	0.1429	0.7516
67.25	12.2514	0.2308	0.7551	0.1071	0.934
79.17	13.2664	0.1923	0.3265	0.1071	0.9448
90.83	13.7546	0.1538	0.0898	0.1071	1.396
102.33	13.788	0.1154	0.0816	0.1071	1.5036
118.08	14.2276	0.1538	0.0816	0.1071	1.7716
pH = 4.7, Temperature = 45°C					
8.3	0.9925	3.7721	1.2271	3.881	0
18.47	1.6555	4.8591	1.2924	3.6127	0.2549
30.63	3.9044	4.2625	1.3722	2.6905	0.4508
42.46	8.5978	1.4622	1.3346	1.4465	0.4234
54.13	11.7821	0	1.2833	0	0.563
66.21	12.5036	0	1.404	0	0.8379
77.96	12.597	0	1.3636	0	1.1257
92.21	12.9265	0	1.5122	0	1.5736
101.46	12.5235	0	1.4839	0	1.7597
114.29	12.4851	0	1.4935	0	1.8816
125.38	12.5498	0	1.5015	0	2.1993
137.38	12.3455	0	1.5225	0	2.6276

[a]Xylose, mannose, and galactose.

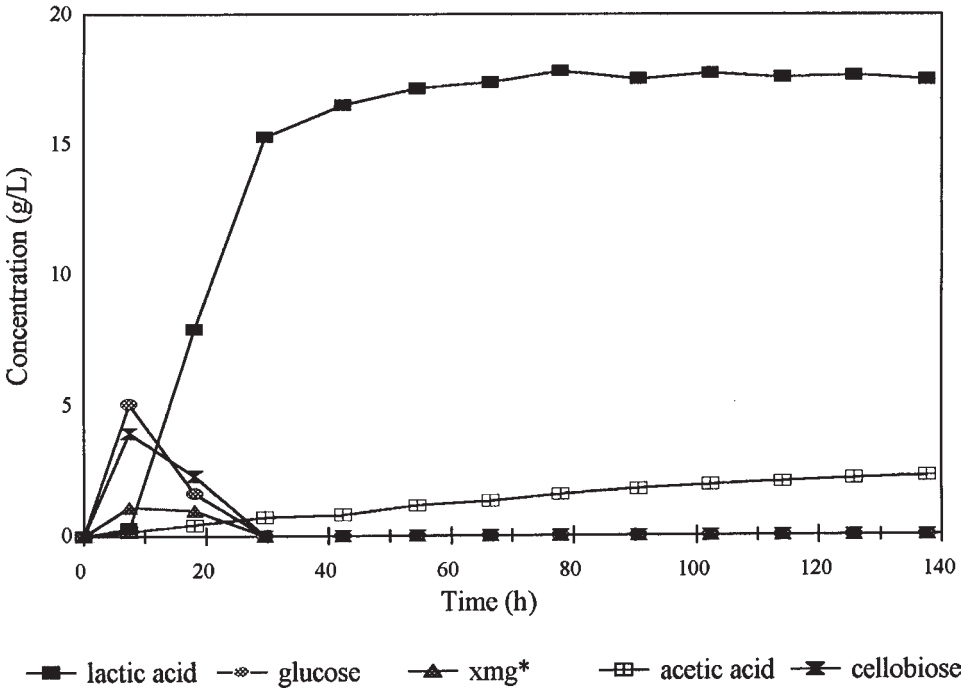

Fig. 1. SSF of pulp mill solid waste at pH 5.5 and 45°C. *Xylose, mannose, and galactose.

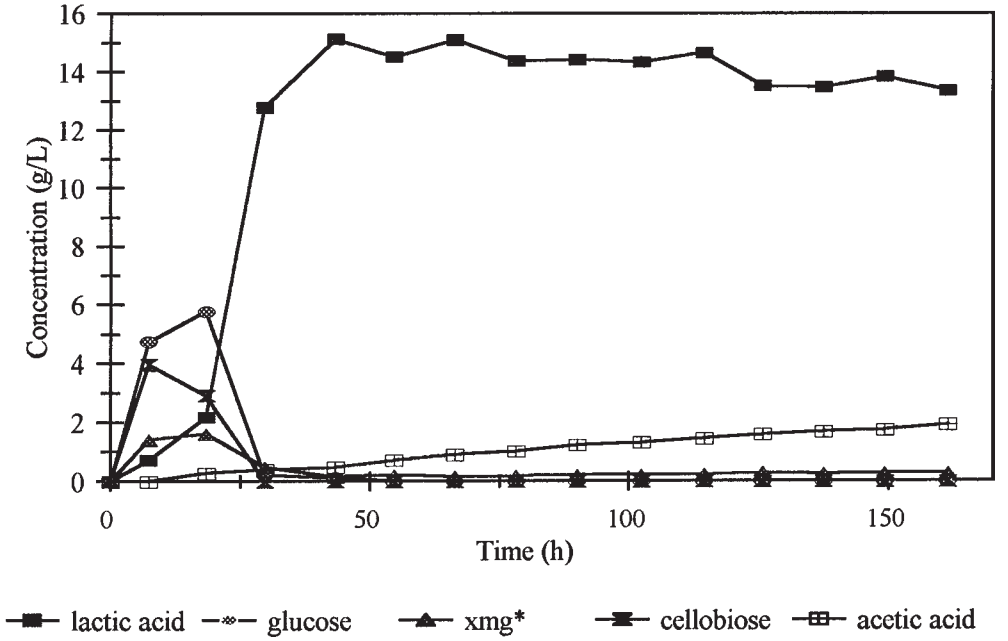

Fig. 2. SSF of pulp mill solid waste at pH 5.0 and 47°C. *Xylose, mannose, and galactose.

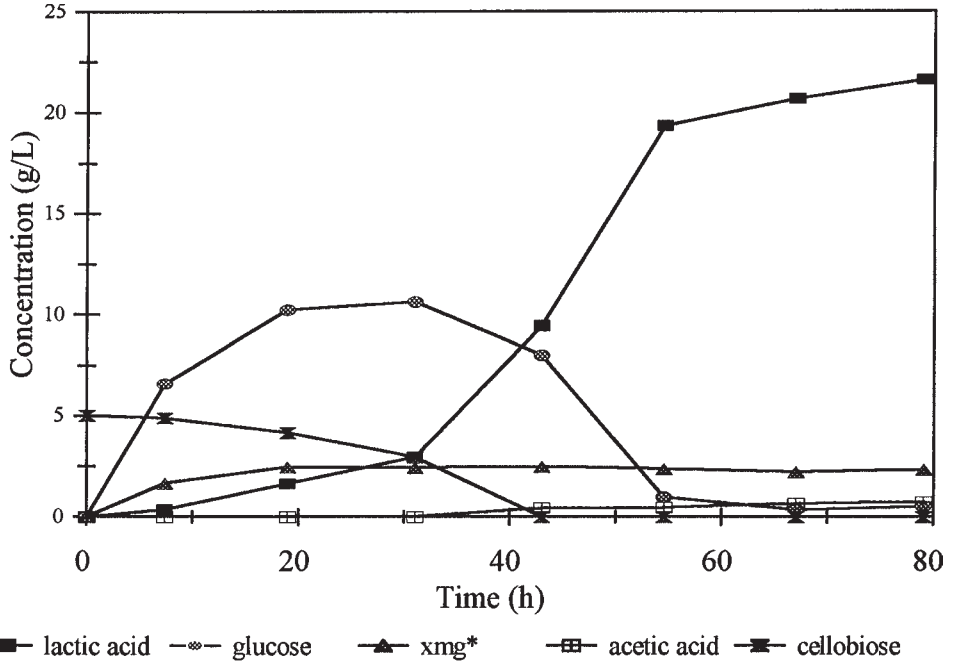

Fig. 3. SSF of pulp mill solid waste (double loading) at pH 5.0 and 45°C. *Xylose, mannose, and galactose.

unstable at this temperature. Once all the carbon sources were depleted, acetic acid was produced from lactic acid.

Finally, an experiment was conducted at pH 5.0 and 45°C with double the normal loading of the substrate (30 g/L) (*see* Fig. 3). The lactic acid yield was 88%, and the acetic acid yield was 6%. Xylose was not depleted, and the acetic acid formation was low. Therefore, it can be concluded that the acetic acid formation is substantially lower at higher substrate loading.

At 45°C and pH 5.5, the reaction was faster and the productivity of lactic acid was very high. Lactic acid yield was 93% and acetic acid yield was 7%. Xylose was fermented simultaneously with glucose under these conditions. Also, it was found that a higher loading of the substrate will decrease the formation of acetic acid. Another favorable experiment with faster and higher production was conducted at 47°C and pH 5.0. The yield of lactic acid was 97% and acetic acid was 1%. The only problem was the instability of lactic acid at operating temperature once the sugars were depleted. This problem can be avoided by adding sugars as a fed-batch process. The upper limit of the temperature that is tolerated by the microorganism is 48°C, and, therefore, caution should be exercised or a loss of culture could result.

Xylose depletion, which was observed during the SSF experiment, was further investigated by placing 14 g/L of glucose and 7.5 g/L of xylose medium 1 in the fermentor at 45°C and pH 5.5 (*see* Fig. 4). After approx 50 h, glucose was almost fully utilized. Then, xylose utilization began, with its

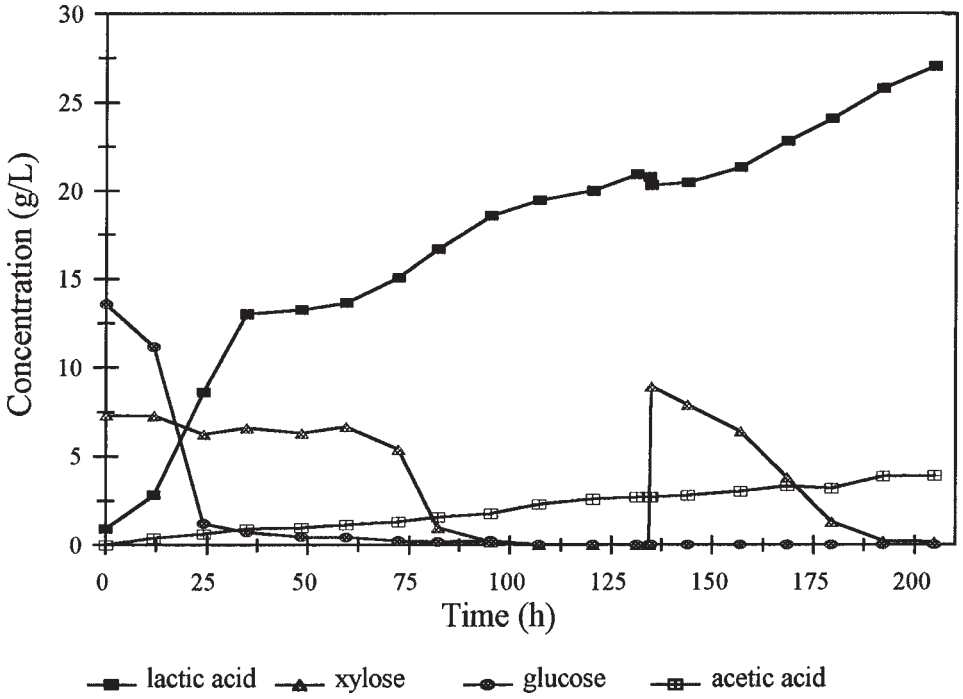

Fig. 4. Glucose and xylose fermentation at pH 5.5 and 45°C in medium 1.

completion in 100 h. The yield of lactic acid was 86% and the yield of acetic acid was 13%. About 9 g/L of xylose was added to the fermentor at about h 135, and it was completely utilized. The depletion of xylose was investigated by using xylose (13 g/L) as the only substrate (medium 1) at pH 5.0 and 45°C. After 60 h, xylose depletion with a spike in lactic acid production was noted (*see* Fig. 5). Fermentation was completed at approx 130 h, with 88% lactic acid yield and 12% acetic acid yield. Again, to prove these unusual results by NRRL B445, the experiment was repeated with an initial xylose loading of 27 g/L. Xylose was fermented (*see* Fig. 6), with 93% lactic acid yield and 6% acetic acid yield. About 4.5 g/L of xylose was added at about h 200 and h 250, and xylose was completely converted into lactic acid.

To alleviate the problem of the lag phase formed by the *L. delbrueckii*, the xylose adapted *L. delbrueckii* from the fermentor was cultured in modified Elliker broth *(7)*. The microorganism was transferred to the fermentor containing medium 2. Figure 7 shows the production of lactic acid by *L. delbrueckii*. Maximum production of lactic acid was reached within 24 h. The lactic acid yield was 92% and the acetic acid yield was 2%. The initial presence of acetic acid (medium 2) in the fermentor caused a feedback inhibition for the decreased formation of acetic acid. The xylose fermentation experiment using medium 2 was repeated with double the xylose loading (25 g/L). Figure 8 shows the utilization of xylose by *L. delbrueckii* with

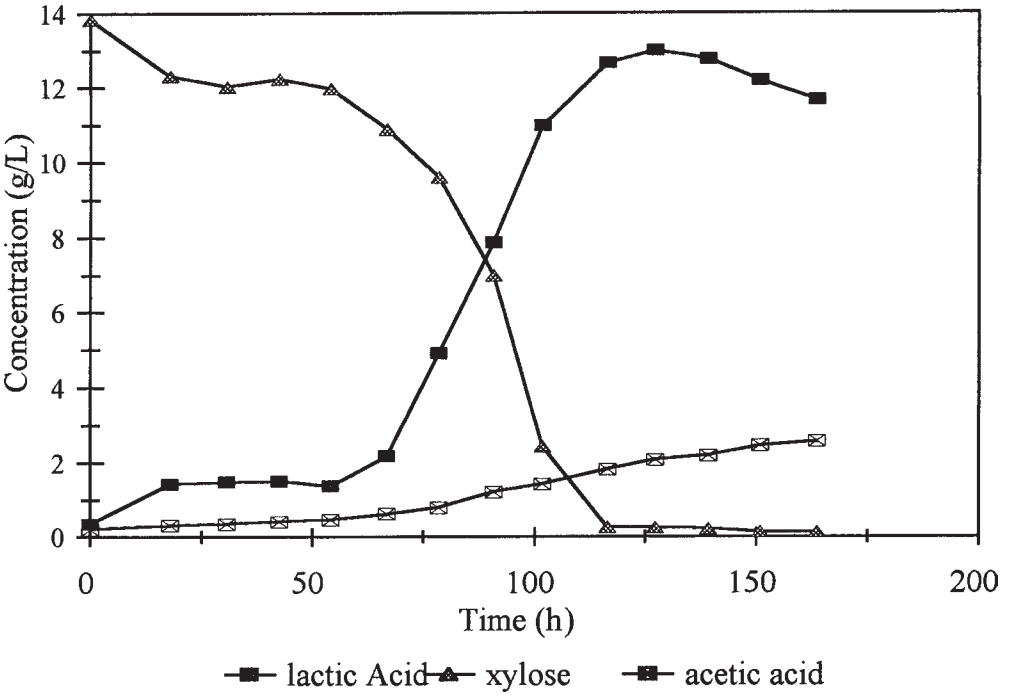

Fig. 5. Xylose fermentation at 13 g/L loading, pH 5.0, and 45°C in medium 1.

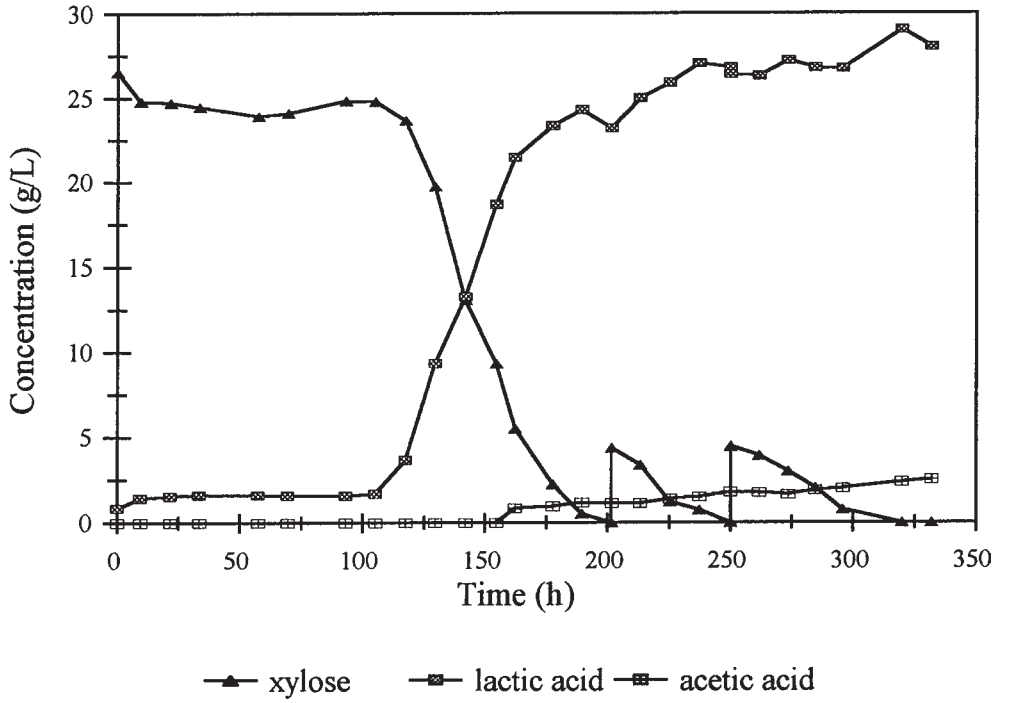

Fig. 6. Xylose fermentation at 27 g/L loading, pH 5.0, and 45°C in medium 1.

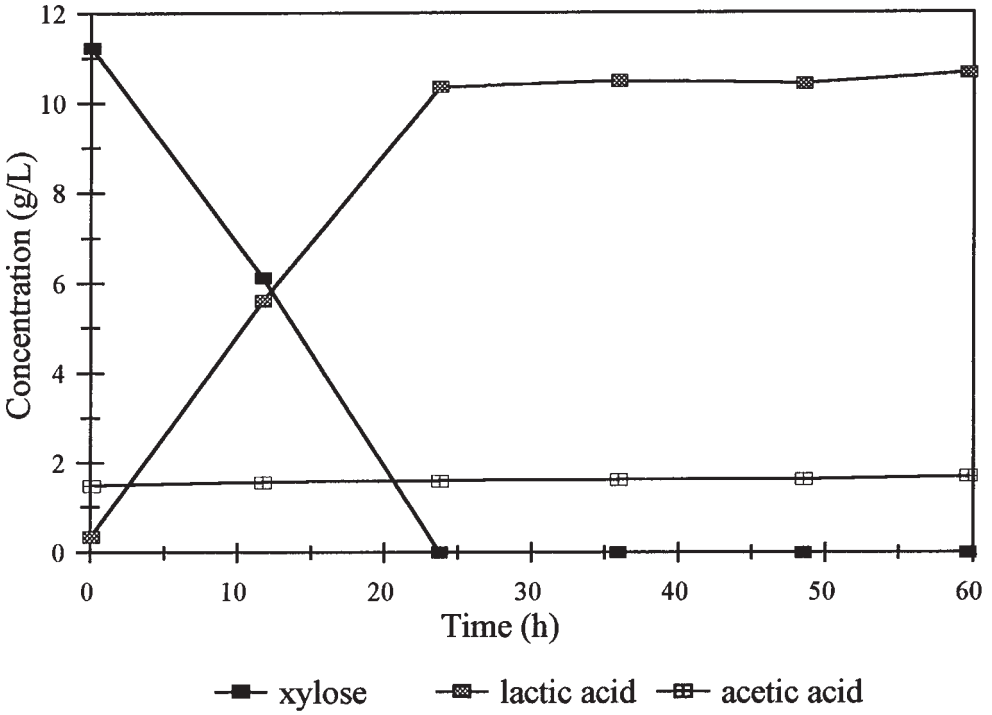

Fig. 7. Xylose fermentation at pH 5.0 and 45°C in medium 2.

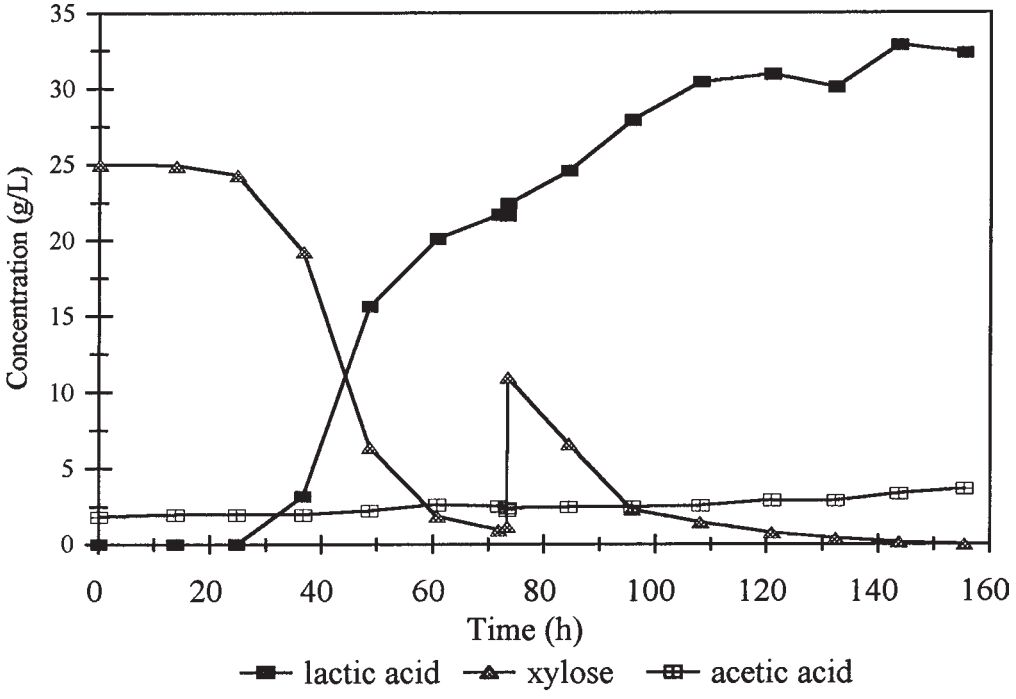

Fig. 8. Xylose fermentation at 25 g/L loading, pH 5.0, and 45°C in medium 2.

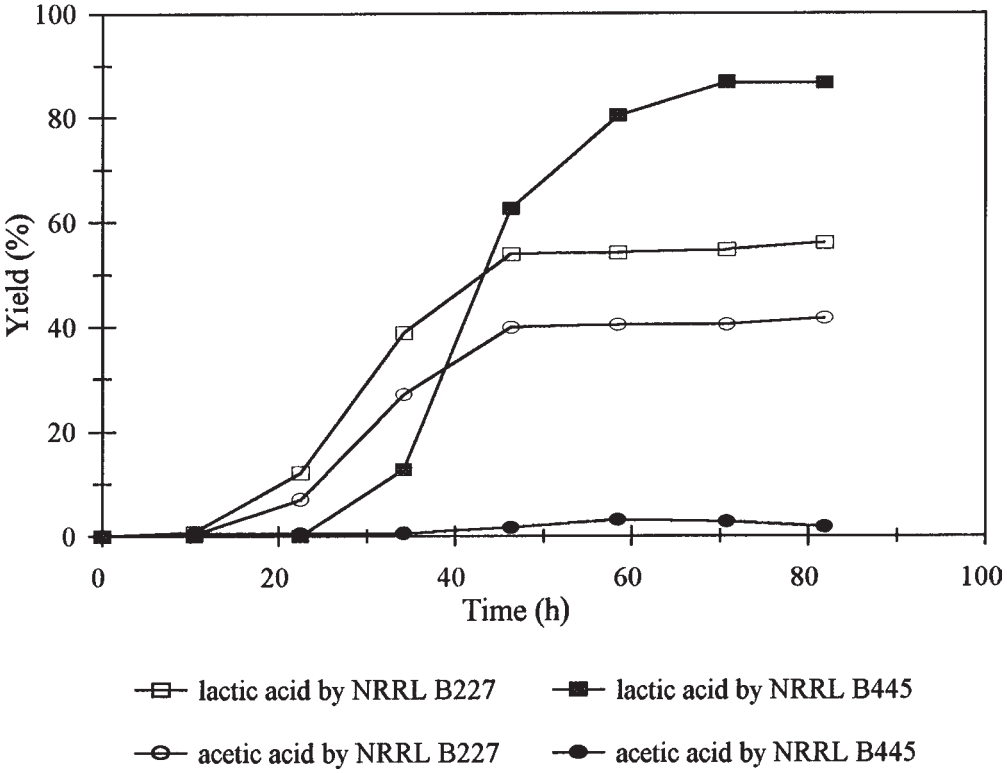

Fig. 9. Comparative study of lactic acid production by NRRL B445 and NRRL B227.

the production of lactic acid. The lactic acid yield was estimated to be 90% and the acetic acid yield was estimated to be 2%. Once the xylose was almost depleted after 80 h, about 11 g/L of xylose was again added to the fermentor. Fermentation was completed after 155 h, and the lactic acid yield was 86%; the acetic acid yield was only 5%.

For comparison of the performance of *L. delbrueckii* culture in terms of lactic acid production, another experiment was conducted with initial xylose loading of 15.5 g/L using *L. pentosus* at optimal pH 6.5 and 33°C *(7)*. After 60 h, xylose fermentation was completed with a production of 60% lactic acid and 40% acetic acid. The conversion was almost 100%. Xylose fermentation using *L. pentosus* was repeated with a higher xylose loading (23 g/L) at the same conditions. About 56% of lactic acid and 40% of acetic acid were formed. Figure 9 presents a comparison of the lactic acid and acetic acid yield from xylose fermentation by *L. pentosus* and *L. delbrueckii*. Note that *L. delbrueckii* produces more lactic acid and less acetic acid than *L. pentosus*.

Again, the xylose fermentation by *L. delbrueckii* with an initial loading of 34 g/L of xylose and high cell loading to the fermentor was investigated (*see* Fig. 10). The lactic acid yield was 78.5% and the acetic acid yield was 5%.

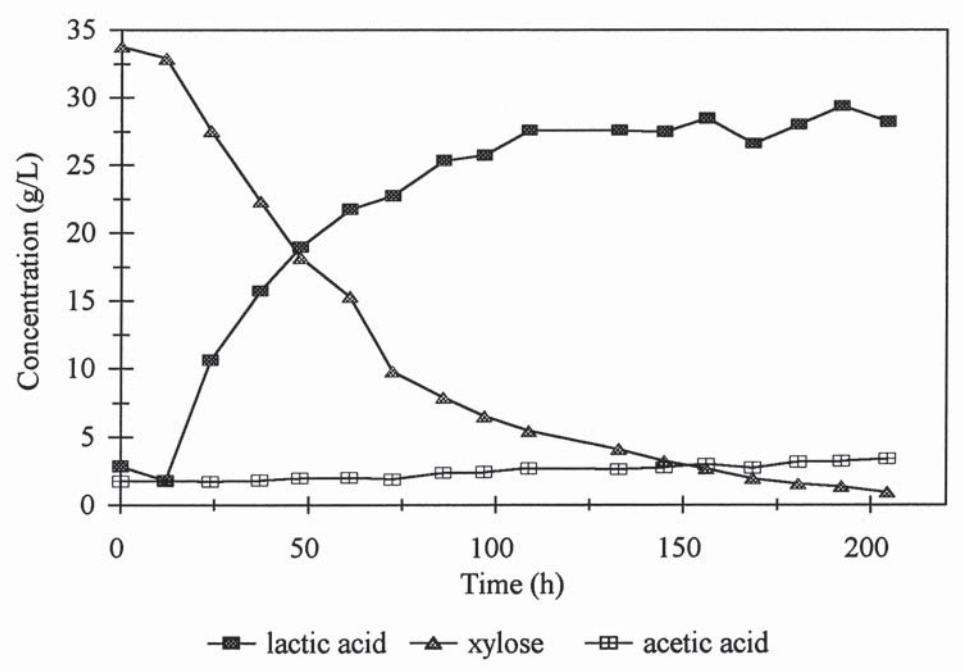

Fig. 10. Xylose fermentation at 34 g/L loading, pH 5.0, and 45°C in medium 2.

Discussion

This study proves that under substrate-limiting conditions, a homofermentative organism such as *L. delbrueckii* will behave in a heterofermentative manner. For example, acetic acid is formed and other sugars such as xylose are fermented. Therefore, it was proven that *L. delbrueckii* (NRRL B445) can ferment xylose into lactic acid and acetic acid, contrary to previous knowledge *(2,3,9)*. Initially, the experimental run with both glucose and xylose as substrates indicated that xylose started to ferment 60 h after the glucose was completely fermented. This type of behavior was not typical of xylose-fermenting microorganisms such as *L. xylosus*, *L. lactis*, and *L. pentosus*, which are not known to exhibit a long lag phase when both glucose and xylose are present *(6–8)*.

Nevertheless, the yield of lactic acid was less and of acetic acid was higher for these microorganisms when compared with *L. delbrueckii*. For example, Borch et al. *(15)* conducted a study on the heterofermentative behavior of homofermentative *Lactobacillus* sp. 93 SMRICC 235 under glucose-limiting conditions in an anaerobic continuous culture with complete recycle. They found that under glucose-limiting situations, high levels of acetate, formate, and ethanol were produced along with the complete utilization of amino acids such as methionine, phenyl alanine, and threonine and 50% utilization of aspartic acid, glutamic acid, glycine, isoleucine, leucine, lysine, proline, serine, and valine. Under glucose-limiting situations, De Bruyn et al. *(16)* found that *Lactobacillus divergens* utilized other carbon

sources available to produce lactic acid, formic acid, and acetic acid. Also, De Vries et al. *(17)* observed that *L. lactis*, a homofermentative organism, adopts heterofermentative metabolic pathways producing formic acid, acetic acid, and ethanol in anaerobic conditions under glucose-limiting situations. Thomas et al. *(18)* reported the switch from homofermentative to heterofermentative behavior by *S. lactis* by forming formate, ethanol, and acetate. These studies indicate that under glucose-limiting conditions, microorganisms such as *L. delbrueckii* can produce acetic acid from lactic acid or utilize xylose to produce lactic acid when glucose is limited to maintain the metabolizing of its bacterial cells.

A homofermentative lactic acid bacteria follows the Embden-Meyerhof-Parnas or glycolytic pathway to convert 1 mol of glucose into 2 mol of pyruvate. First, glucose is converted into fructose 1,6 biphosphate, which is converted into pyruvate. Pyruvate is converted into lactic acid by lactate dehydrogenase. Factors that influence the switch of a homofermentative organism to a heterofermentative organism under glucose-limited situations are lactate dehydrogenase levels, pH levels, and oxygen *(19)*. Fructose 1,6 biphosphate is converted into lactate, and its presence activates lactate dehydrogenase. When glucose is limited, fructose 1,6 biphosphate is also low, and the reversible reaction of lactate into pyruvate will occur. Then, pyruvate is diverted into different end products such as acetic acid. Lactate dehydrogenase synthesis, which controls the pyruvate metabolism, is lower at higher pH levels. Therefore, alkaline conditions can alter the homofermentative behavior of *Lactobacilli*. The xylose fermentation was noticed only at pH higher than 5.0 and temperatures higher than 45°C. Since *Lactobacilli* are aerotolerant, the presence of oxygen, an electron acceptor, induces pyruvate oxidase, which dissimilates pyruvate into acetic acid.

Conclusion

The lactic acid yield from the pulp mill solid waste SSF was more than 100% when only hexoses were considered as the substrate for *L. delbrueckii*. Hence, fermentation of xylose into lactic acid was evident.

On the basis of glucose and xylose consumed, the overall lactic acid yield in SSF was 86–97% and the acetic acid yield was 6–13%. The formation of acetic acid was observed under substrate-limiting situations. When the initial loading of solid waste pulp (30 g/L) was increased, it was found that less acetic acid was formed.

The utilization of xylose to produce lactic acid by *L. delbrueckii* during SSF was further investigated. The fermentation capability of this microorganism was investigated using xylose as the sole carbon source. Sixty hours after culture inoculation, conversion of xylose into lactic acid became evident. The overall lactic acid yield was 86–92% and acetic acid yield was 2–12% from xylose fermentation. In a separate test using glucose and xylose, glucose seemed to be consumed first. Xylose was fermented only in the complete absence of glucose. In the mixed sugars fermentation, the overall

yield of lactic acid was 83% and acetic acid was 13%. The lag phase in xylose fermentation by NRRL B445 was eliminated by using a xylose-adapted culture and a medium free of glucose.

Acknowledgments

I would like to acknowledge Dr. Y. Y. Lee for being the advisor. I am also grateful to Dr. McCaskey for help in the fermentation techniques. Financial support was provided by the Pulp and Paper Research Education Center.

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