0273-2289/05/121–124/529–540/$30.00

Lactic Acid Production from Cheese Whey by Immobilized Bacteria

Abolghasem Shahbazi,*[,1] Michele R. Mims,[1] Yebo Li,[1] Vestal Shirley,[1] Salam A. Ibrahim,[2] and Antrison Morris[2]

[1]Bioenvironmental Engineering Program, Department of Natural Resources and Environmental Design, and [2]Food and Nutritional Sciences Program, Department of Human Environment and Family Sciences, North Carolina A&T State University, 1601 East Market Street, Greensboro, NC 27411, E-mail: ash@ncat.edu

Abstract

The performance of immobilized *Bifidobacterium longum* in sodium alginate beads and on a spiral-sheet bioreactor for the production of lactic acid from cheese whey was evaluated. Lactose utilization and lactic acid yield of *B. longum* were compared with those of *Lactobacillus helveticus. B. longum* immobilized in sodium alginate beads showed better performance in lactose utilization and lactic acid yield than *L. helveticus*. In the spiral-sheet bioreactor, a lactose conversion ratio of 79% and lactic acid yield of 0.84 g of lactic acid/g of lactose utilized were obtained during the first run with the immobilized *L. helveticus*. A lactose conversion ratio of 69% and lactic acid yield of 0.51 g of lactic acid/g of lactose utilized were obtained during the first run with immobilized *B. longum* in the spiral-sheet bioreactor. In producing lactic acid *L. helveticus* performed better when using the Spiral Sheet Bioreactor and *B. longum* showed better performance with gel bead immobilization. Because *B. longum* is a very promising new bacterium for lactic acid production from cheese whey, its optimum fermentation conditions such as pH and metabolic pathway need to be studied further. The ultrafiltration tests have shown that 94% of the cell and cheese whey proteins were retained by membranes with a mol wt cutoff of 5 and 20 KDa.

Index Entries: Cheese whey; bifidobacteria; immobilized cell; lactose; lactic acid; membrane; fermentation.

Introduction

The manufacturing of cheese creates a vast quantity of whey as a byproduct, which is either discarded as waste or used in fertilizer or animal feed. The United States generates nearly 325 billion gallons of cheese whey annually. It is estimated that as much as 40–50% of the whey produced is disposed of as sewage. Because of its biochemical

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

potential for nutrient supplementation, this high level of waste disposal represents a substantial loss of a valuable resource. Cheese whey contains about 4.5–5% lactose, 0.6–0.8% soluble proteins, 0.4–0.5% (w/v) lipids, and varying concentrations of mineral salts *(1)*. These components can be used in the manufacturing of many food and other products.

There is interest in utilizing lactose from cheese whey to produce value-added products. Lactic acid is one such product. Lactic acid is a natural organic acid that has many applications in the pharmaceutical, food, and chemical industries. It is used as an acidulant and a preservative, as well as a substrate for the production of biodegradable plastics and some other organic acids *(2,3)*. Lactic acid can be produced by fermentation of a sugar-containing substrate such as cheese whey using *Lactobacillus helveticus (3,4)* which has been extensively evaluated in lactic acid research, and *Lactobacillus casei (5,6)*. *L. helveticus* is a thermophilic and acidophilic bacterium that can grow under conditions inhibitory for most contaminant microorganisms *(3)*. *Bifidobacterium longum* is a bacterium that can both convert lactose into lactic acid and produce an antibacterial compound, which can boost the immune system in its host. *Bifidobacterium* spp. produces a high yield of L (+) lactic acid compared with D (–) lactic acid *(7)*. By the *Bifidum* pathway, the fermentation of 2 mol of hexose results in 3 mol of acetate and 2 mol of lactate *(7)*. Most studies regarding *B. longum* have concentrated on increasing cell production by cell immobilization and optimized pH *(8,9)*. To date, there has been no report on using *B. longum* to produce lactic acid from cheese whey.

The process of lactic acid production includes two key stages: fermentation and product recovery. Lactic acid fermentation is characterized by product inhibition, which affects cell growth and metabolism and, thus, limits the production of lactic acid *(10)*. Cell immobilization is one of the most attractive methods for maintaining a high cell concentration in bioreactors *(6,11,12)*. Entrapment in gel beads has been a popular technique for immobilization of lactic acid bacteria *(6,13,14)*. One potential limitation of these systems is disruption of the gel beads as a result of pressure buildup. Immobilization of cells attached to a solid matrix bioreactor has been an alternative means to maintain high cell concentrations *(10)*.

Usually, most separation protocols of microorganisms from fermentation broth are performed by small-scale centrifugation. Recently, cross-flow microfiltration has been used as a cell separator in continuous fermentation processes *(15)*. A successful lactic acid recovery approach has been that of continuous fermentation in a cell-recycle reactor in which the cells are separated by a filtration unit and returned to the fermentor while the product is removed in the permeate *(16,17)*. The long-term performance of membrane units at high cell densities is affected by fouling of the filtration membranes, requiring extensive cleaning protocols *(18)*.

The objectives of the present work were *(1)* to evaluate the performance of two bacteria, *B. longum* and *L. helveticus*, in converting cheese whey into

lactic acid; *(2)* to determine the performance of cells being immobilized on a spiral-sheet bioreactor and entrapped in gel beads; and *(3)* to evaluate the performance of lactic acid recovery by membrane filtration.

Materials and Methods

Raw Materials

Deproteinized cheese whey obtained from Davisco Foods International, (Eden Prairie, MN) was used in the preparation of the cheese whey medium. The composition of the deproteinized cheese whey powder was 6.8% crude protein, 0.8% crude fat, 78.6% lactose, 9.4% ash, and 4.4% moisture. Fifty-gram samples of the deproteinized cheese whey were dissolved in 1 L of deionized water and stirred for 5 min at ambient temperature. The whey medium was then autoclaved at 106°C for 10 min to prevent browning.

Microorganism and Culture Medium

B. longum was obtained from National Collection of Food Bacteria (NCFB 2259). Stock culture of this strain was maintained in 50% glycerol and Mann Rogosa Sharpe (MRS) broth medium at –80°C. Lypholized *L. helveticus* (ATCC 15009) was purchased from American Type Culture Collection (Rockville, MD). The stock cultures were maintained in autoclaved skim milk and stored at 4°C. Active cultures were propagated in 10 mL of MRS broth at a temperature of 37°C for 18–24 h under anaerobic conditions. This was used as a preculture to initiate cell production of higher volume with a 1% inoculation into 100 mL of fresh MRS broth, which was incubated at 37°C for 24 h.

Immobilization Techniques

Two different cell immobilization techniques were used: (1) immobilization in sodium alginate beads and (2) immobilization on spiral-sheet matrix. The two techniques were replicated for the two bacteria samples.

Cell Immobilization with Alginate Beads

A 1% sodium alginate gel was prepared by dissolving alginic acid in deionized H_2O that was stirred continuously at a temperature maintained above 50°C. Bacteria were grown in 1 L of MRS broth, and the harvested cell concentrations for *L. helveticus* and *B. longum* were about 2.7×10^9 and 8.0×10^9/mL, respectively. The cells were harvested by centrifuging at 25,000 rpm for 15 min. The centrifuged cells were then suspended in warm (45°C) sodium alginate. The homogeneous suspension was extruded drop by drop into a sterilized cold (4°C) calcium chloride solution (0.5 *M*) using laboratory-grade sterilized C-flex tubing (0.125 id and 0.188 od). Beads were allowed to harden for 30 min in the cold solution and then stored in a refrigerator overnight before aseptic transfer into a sterile column. The approximate diameter of the beads was 3 mm.

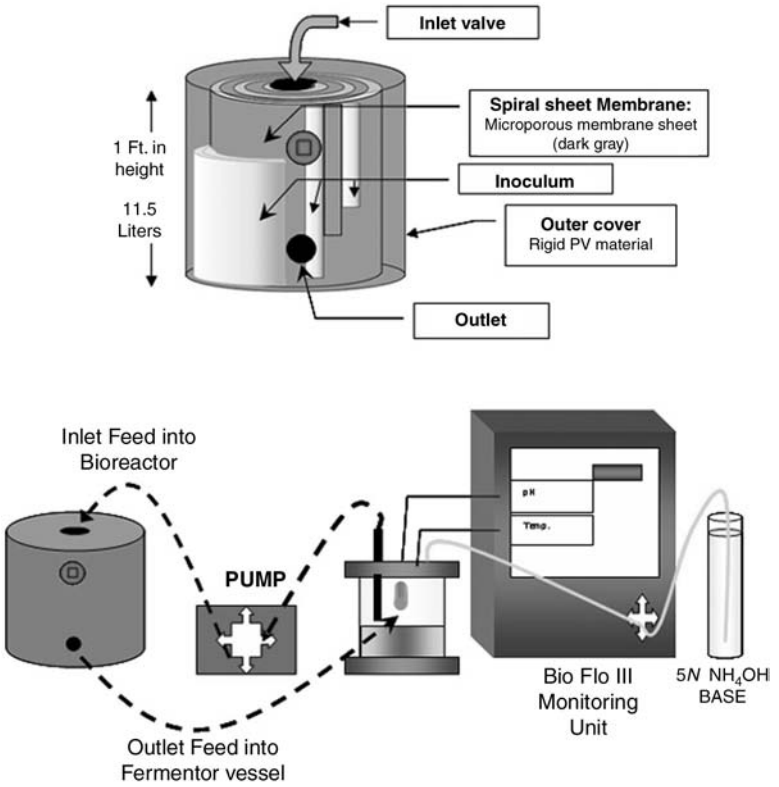

Fig. 1. Schematic diagram of spiral-sheet bioreactor and fermentation system.

Immobilization of Spiral-Sheet Matrix

The bioreactor was a spiral-sheet polymeric membrane cartridge that is used as a support matrix for cell immobilization (Fig. 1). The bioreactor was washed with a 1 *M* solution of NaOCl overnight and rinsed three times with sterile deionized H_2O. Nine liters of MRS inoculated with the designated bacteria was added to the reactor and allowed to stand overnight at room temperature. After the bacteria were immobilized, the MRS solution was drained off and fresh whey medium was added for fermentation.

Fermentation Process

Fermentation in Flasks with Immobilized Bacteria in Alginate Beads

Fermentation was performed in 500-mL flasks with a working volume of 250 mL. The initial cell concentrations of the medium were 5.4×10^9 and 1.6×10^{10}/mL for *L. helveticus* and *B. longum*, respectively. The fermentation was conducted for two runs using the same alginate beads. After 48 h, the fermentation broth was drained, and the cells were put into the MRS broth

Table 1
Conditions and Parameters of Fermentation Tests

Parameter	Immobilized cell in alginate beads	Spiral-sheet bioreactor
Bacteria	*L. helveticus* and *B. longum*	*L. helveticus* and *B. longum*
pH	Noncontrolled	6.5
Temperature	45°C for *L. helveticus*, 37°C for *B. longum*	37°C for both *L. helveticus* and *B. longum*
Agitation	No	150 rpm
Time	48 h	48 h
Nutrients	Nutrients added in second run	No nutrients added in second and third runs

at a temperature of 37°C for 12 h to revive the cells. After the MRS broth was drained, fresh cheese whey medium was added to start the second run of the fermentation. During each run, samples were withdrawn at 0, 4, 6, 24, and 48 h for analysis of lactose and lactic acid concentration.

Fermentation in Spiral-Sheet Bioreactor

The bioreactor containing immobilized cells was connected to a stirred 2.0-L benchtop fermentor to allow recirculation of the medium. The medium was continuously circulated between the bioreactor and the fermentor via a pump for temperature and pH control. The pH was maintained at 6.5 by neutralizing the acid with 5 *N* ammonium hydroxide. The agitation speed of the fermentor was maintained at 150 rpm and the temperature at 37°C. Samples were withdrawn at 6-h intervals and analyzed using high-performance liquid chromatography to determine the concentrations of lactose and lactic acid. MRS media was used to maintain stock cultures and for cell immobilization. The whey solution was used as the test media to determine lactosc conversion. In the bioreactor tests, after the bacteria were immobilized on the membrane, the MRS solution was drained off and fresh whey medium was added for fermentation. For the second and third runs, after the fermentation broth of the previous run was drained off, cheese whey medium of the same composition without nutrient supplementation was added. Table 1 summarizes the fermentation conditions.

Membrane System

The membrane filtration unit was an OPTISEP Filtration Module (North Carolina SRT, Cary, NC) equipped with a NADIR® membrane (flat module) with a mol wt cutoff of 5 and 20 kDa. The membrane unit was coupled to the fermentor. The medium was fed from the fermentor at constant flow rates via the recirculation pump. The concentrate was recycled to the fermentor while the permeate was collected in a container placed on an electronic balance. The balance interfaced via RS232 to a

computer that continually collected and recorded time and permeate weight. The transmembrane pressure and cross-flow velocity were adjusted with a manual valve and a pump controller. The pressure was measured by a standard pressure gage. The selected cross-flow velocities were 1 and 2 m/s. The time required to pass 1 L of liquid through the membrane unit was 16.6 and 8.3 s at a cross-flow velocity of 1 and 2 m/s, respectively.

Analysis

Lactose and lactic acid levels were determined with a high-performance liquid chromatograph (Waters, Milford, MA) with a KC-811 ion-exclusion column and a Waters 410 differential refractometer detector. The mobile phase was 0.1% H_3PO_4 solution at a flow rate of 1 mL/min. The temperature of the detector and column was maintained at 35 and 60°C, respectively.

Once lactic acid is produced, it is separated from residual cells, sugars, and proteins using membrane separation technology. To evaluate the protein retention from the treated whey total nitrogen was analyzed using the macro-Kjeldahl method. Samples were digested using a block digestion (FOSS Tecator, Sweden) and analyzed for nitrogen on a Tecator Kjeltec auto 2400 analyzer *(19)* as described in Foss Tecator Application Note, AN300 (Foss Tecator, 1999). When the protein nitrogen was determined, the samples were precipitated using a trichloroacetic acid solution before nitrogen analysis *(20)*. The digestion and analysis procedure for protein nitrogen was the same as that for total nitrogen analysis.

Lactic acid productivity was evaluated using the lactose conversion ratio and lactic acid yield. The conversion ratio was expressed as follows:

$$\text{Conversion ratio (\%)} = \frac{\text{intial lactose conc.} - \text{residual lactose conc.}}{\text{initial lactose conc.}} \times 100\%$$

Lactic acid yield was expressed as follows:

$$\text{Lactic acid yield (g / g)} = \frac{\text{lactic acid produced}}{\text{lactose utilized}}$$

Membrane separation of cheese whey was evaluated by two criteria: (1) permeate flux and (2) protein retention. The permeate flux was calculated by measuring the quantity of permeate collected during a certain time and dividing it by the effective membrane area for filtration:

$$\text{Permeate flux, } J = \frac{\text{permeate volume}}{\text{membrane area} \times \text{time}} (L / [m^2 \cdot h])$$

The protein retention ratio was defined as follows:

$$\text{Rention ratio (\%), } R = \left(1 - \frac{C_P}{C_F}\right) \cdot 100$$

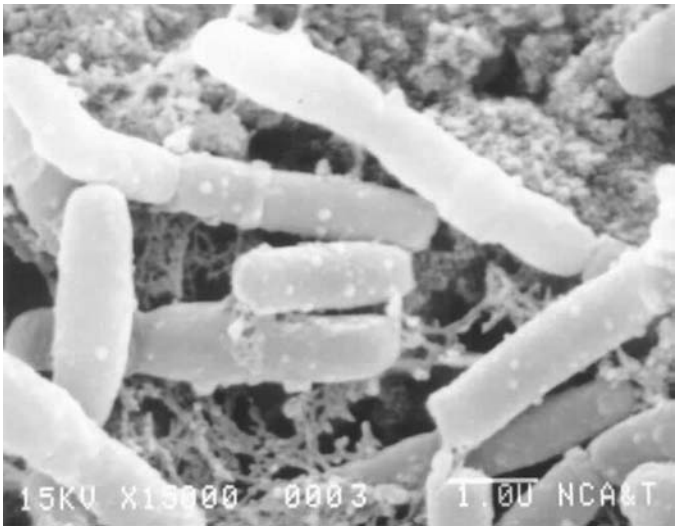

Fig. 2. Scanning electron microscopy showing *L. helveticus* immobilized on spiral-sheet polymeric matrix. Magnification: 1:15,000.

in which C_F is the concentration of protein in the feed stream, and C_P is the concentration of protein in the permeate.

Results and Discussion

Cell Growth

Samples of the spiral-sheet polymeric matrix immobilized by *L. helveticus* were prepared for microscopic examination of cell attachments. Electron micrographs (Fig. 2) show that *L. helveticus* cells were successfully attached onto and spread over the surface of the spiral-sheet polymeric matrix. Some cells also penetrated into the open pores of the matrix. When the medium was circulated at flow rates <1 L/min, these attached cells were not detached or washed out with the medium.

Batch Fermentation with Cells Immobilized in Alginate Beads

For the batch fermentation with immobilized cells in alginate beads without pH control, the fermentation temperature was 45°C for *L. helveticus* and 37°C for *B. longum* (Tables 2 and 3). Lactose conversion ratios of 31.2 and 53.6% were obtained with *B. longum* for the first and second run, respectively, and 20.4 and 38.0% were obtained with *L. helveticus.* It can be seen that *B. longum* shows significant improvement in lactose utilization over *L. helveticus* ($p < 0.05$ for the first run and $p < 0.001$ for the second run, respectively). Both *B. longum* and *L. helveticus* had a lactic acid yield of 0.60 *g* of lactic acid/g of lactose utilized during the first run of fermentation. The statistical analysis showed there was no significant difference between the lactic acid yield of *B. longum* and *L. helveticus* during the first run ($p > 0.5$).

Table 2
Lactose Conversion Ratio and Lactic Acid Yield Using Immobilized *L. helveticus* in Gel Beads with Nutrient Supplement in Second Run

Time (h)	Lactose concentration (g/L)	Lactic acid concentration (g/L)	Lactose utilized (g/L)	Conversion ratio (%)	Yield (glactic acid/ glactose)
First run					
0	39.0	0	—	—	—
4	35.7	0	3.3	8.5	0.00
6	34.9	1.2	4.1	10.4	0.30
24	32.2	3.7	6.8	17.3	0.55
48	31.1	4.8	7.9	20.4	0.60
Second run					
0	39.0	1.5	—	—	—
4	28.9	5.4	10.1	25.9	0.39
6	29.0	6.4	11.0	28.2	0.45
24	24.8	8.5	14.2	36.5	0.49
48	24.2	8.5	14.8	38.0	0.47

Table 3
Lactose Conversion Ratio and Lactic Acid Yield Using Immobilized *B. longum* in Gel Beads with Nutrient Supplement in Second Run

Time (h)	Lactose concentration (g/L)	Lactic acid concentration (g/L)	Lactose utilized (g/L)	Conversion ratio (%)	Yield (glactic acid/ glactose)
First run					
0	39.0	0.0	—	—	—
4	33.6	1.4	5.4	13.7	0.26
6	32.5	2.2	6.5	16.6	0.34
48	26.8	0.7	12.2	31.2	0.60
Second run					
0	39.0	2.9	0.0	0.0	0.0
4	27.4	6.8	11.6	29.7	0.33
6	26.1	7.8	12.9	33.1	0.38
24	20.5	13.1	18.5	47.4	0.55
48	18.1	15.7	20.9	53.6	0.62

However, *B. longum* showed significant improvement in lactic acid yield (0.62 g of lactic acid/g of lactose utilized) over *L. helveticus* (0.47 g of lactic acid/g of lactose utilized) during the second run ($p < 0.01$).

The alginate beads recovered from the first run were reused with nutrient supplement in the second run. For *L. helveticus*, lactose conversion ratio increased from 20.4 to 38% for the first and second run, respec-

tively. The conversion ratio for *B. longum* increased from 31.2 to 53.6% for the first and second run, respectively. It can be seen that significant improvement in lactose conversion was obtained for both of the two bacteria ($p < 0.002$) during the second run. No significant decrease was observed for lactic acid yield in the second fermentation run with immobilized *B. longum* ($p > 0.5$). The lactic acid yield of the second run (0.47 g of lactic acid/g of lactose utilized) was significantly lower than that of the first run (0.60 g of lactic acid/g of lactose utilized) with immobilized *L. helveticus* ($p < 0.04$).

B. longum showed a better performance than *L. helveticus,* with a maximum lactose conversion ratio of 53.6% and maximum lactic acid yield of 0.62 g of lactic acid/g of lactose utilized for the second run. Nutrient supplementation significantly improved the lactose conversion ratio for both bacteria, and no significant decrease in lactic acid yield was observed during the second runs for *B. longum.* In batch fermentations, the lactose conversion was inhibited with the accumulation of lactic acid because the pH was not controlled. To obtain a higher lactose conversion ratio and higher lactic acid yield, optimum fermentation conditions such as pH control are required.

Immobilized Cell Fermentation in Spiral-Sheet Bioreactor

Table 4 shows the lactose conversion ratio and lactic acid yield when immobilized *L. helveticus* was used to ferment cheese whey in a spiral-sheet bioreactor at 37°C. It can be seen that when *L. helveticus* was immobilized on a spiral-sheet matrix, a lactose conversion ratio of 79% and a lactic acid yield of 0.84 g of lactic acid/g of lactose utilized were obtained during the first run. When the immobilized beads were reused for the second and third runs without any nutrient supplementation, no significant decrease in lactose conversion ratio was observed ($p > 0.20$); however, lactic acid yield decreased to 0.72 and 0.57 g of lactic acid/g of lactose utilized for the second and third run, respectively. The decrease in lactic acid yield was probably caused by utilization of lactose in a different pathway owing to a lack of nutrients in the successive runs.

Compared to the batch fermentation with immobilized *L. helveticus* cells in alginate beads, the lactose conversion ratios were increased from 22.4 and 38.0% to 79 and 76% for the first and second run, respectively. The lactic acid yields were increased from 0.60 and 0.57 g of lactic acid/g of lactose utilized to 0.84 and 0.72 g of lactic acid/g of lactose utilized for the first and second run, respectively.

A lactose conversion ratio of 68.5% and a lactic acid yield of 0.51 g of lactic acid/g of lactose utilized were obtained with the *B. longum* immobilized on the spiral-sheet bioreactor (Table 5). Compared to the batch fermentation with immobilized cells in alginate beads, the lactose conversion ratio was increased from 32 to 68.5%, but there was no significant improve-

Table 4
Lactose Conversion Ratio and Lactic Acid Yield Using Immobilized *L. helveticus* in Bioreactor Without Nutrient Supplementation in Second and Third Runs

Time (h)	Lactose concentration (g/L)	Lactic acid concentration (g/L)	Lactose utilized (g/L)	Conversion ratio (%)	Yield (g (glactic acid/ glactose)
First run					
0	44.1	9.5	—	—	—
6	41.5	11.0	2.6	5.9	0.58
12	36.5	16.3	7.6	17.2	0.90
18	29.0	21.7	15.1	34.2	0.81
24	23.4	25.5	20.6	46.8	0.78
30	18.8	28.9	25.3	57.4	0.77
42	12.3	36.4	31.8	72.2	0.84
48	9.3	38.8	34.8	78.9	0.84
Second run					
0	34.2	15.2	—	—	—
6	29.0	18.0	5.3	15.4	0.54
12	23.2	20.4	11.1	32.3	0.48
18	18.6	23.3	15.6	45.6	0.52
24	15.3	25.7	18.9	55.3	0.56
30	12.6	28.1	21.6	63.2	0.60
36	10.7	31.2	23.5	68.8	0.68
42	9.3	32.8	24.9	72.8	0.71
48	8.4	33.7	25.8	75.5	0.72
Third run					
0	42.3	11.6	—	—	—
6	33.9	14.3	8.4	19.7	0.33
12	25.5	17.7	16.8	39.6	0.36
18	19.4	19.1	22.9	54.2	0.33
24	15.7	23.1	26.6	63.0	0.43
30	11.1	25.9	31.2	73.8	0.46
36	9.0	29.3	33.3	78.8	0.53
42	7.6	31.9	34.7	82.0	0.59
48	0.0	35.8	42.3	100.0	0.57

ment in the lactic acid yield. The lactose conversion ratio and lactic acid yield of immobilized *B. longum* in the bioreactor were lower than those of *L. helveticus*.

Membrane Separation

With the ultrafiltration membrane system, lactic acid and some lactose were removed from the growth medium while cells and some lactose were kept in the bioreactor. As shown in Table 6, the protein retention ratios were about 94% for membranes with a mol wt cutoff of 5 and 20 kDa. The specific fluxes were 31.2 and 46.6 L/($m^2 \cdot h$) for membranes with a mol wt

Table 5
Lactose Conversion Ratio and Lactic Acid Yield
Using Immobilized *B. longum* in Bioreactor

Time (h)	Lactose concentration (g/L)	Lactic acid concentration (g/L)	Lactose utilized (g/L)	Conversion ratio (%)	Yield (glactic acid/ glactose)
0	52.6	0.6	—	—	—
12	43.1	7.0	9.5	18.1	0.67
18	37.3	9.6	15.3	29.1	0.59
24	32.7	11.8	19.9	37.9	0.56
36	23.8	15.9	28.9	54.9	0.53
42	20.2	18.1	32.5	61.7	0.54
48	16.6	19.2	36.1	68.5	0.51

Table 6
Retention Ratio and Specific Flux of Membrane Separation System

Mol wt cutoff (Daltons)	Raw protein retention ratio (%)	Protein retention ratio (%)	Specific flux (L/[$m^2 \cdot h$])
5	71.97	93.99	31.2
20	53.93	94.12	46.6

cutoff of 5 and 20 kDa, respectively. As expected, a higher membrane molecular weight cutoff caused a higher specific flux and a lower retention ratio (raw protein). The raw protein was calculated according to the total nitrogen measured in the samples. For the protein retention ratios that were calculated according to the protein nitrogen, both of the membrane cutoffs had a high retention ratio (94%). It can be concluded that most of the proteins could be separated as concentrate using a membrane with a mol wt cutoff <20 kDa. If nanofiltration is incorporated to further separate the lactic acid from lactose, the bioreactor combined with the membrane separation system might be used for continuous production and separation of lactic acid.

Conclusion

A bioreactor with a spiral-sheet matrix could successfully immobilize bacteria on its matrix for the continuous production of lactic acid from cheese whey. The immobilized cells in the spiral-sheet bioreactor showed better performance than bacteria immobilized in alginate beads. Fermentation with immobilized *L. helveticus* in the bioreactor reached a lactose conversion ratio of 79% and lactic acid yield of 0.84 g of lactic acid/g of lactose utilized. To reuse the immobilized cells, nutrient supplementation in successive runs is required to prolong fermentation without a sig-

nificant drop in performance. *B. longum* was demonstrated to be promising bacteria for lactic acid production from cheese whey. During the batch fermentation with cells immobilized in alginate beads, *B. longum* performed better than *L. helveticus*. Further experiments to develop optimal fermentation conditions need to be conducted with *B. longum* to determine how to improve its ability to produce a high yield of lactic acid. The metabolic pathway for *B. longum* in converting lactose needs to be investigated further.

Acknowledgments

This research was supported in part by a research grant from the USDA-CSREES Evans-Allen Program and the Agricultural Research Program at North Carolina A&T State University.

References

1. Siso, M. I. G. (1996), *Bioresour. Technol.* **57,** 1–11.
2. Shahbazi, A., Salameh, M., and Ibrahim, A., (2005), Milchwissenschaft. **60**, in press.
3. Tango, M. S. A. and Ghaly, A. E. (2002), *Appl. Microbiol. Biotechnol.* **58,** 712–720.
4. Roy, D., Goulet, J., and LeDuy, A. (1986), *Appl. Microbiol. Biotechnol*. **24,** 206–213.
5. Bruno-Barcena, J. M., Ragout, A. L., Cordoba, P. R., and Sineriz, F. (1999), *Appl. Microbiol. Biotechnol.* **51,** 316–324.
6. Roukas, T. and Kotzekidou, P. (1998), *Enzyme Microbiol. Technol*. **22,** 199–204.
7. Gomes, A. M. P. and Malcata, F. X. (1999), *Trends Food Sci. Technol*. **10,** 139–157.
8. Doleyres, Y., Paquin, C., LeRoy, M., and Lacroix, C. (2002), *Appl. Microbiol. Biotechnol*. **60,** 168–173.
9. Song, S. H., Kim, T. B., Oh, H. I., and Oh, D. K. (2003), *World J. Microbiol. Biotechnol.* **19,** 721–731.
10. Senthuran, A., Senthuran, V., Mattiasson, B., and Kaul, R. (1996), *Biotechnol. Bioeng.* **55(6),** 841–853.
11. Mostafa, N. A. (1995), *Energy Convers. Mgmt*. **37(3),** 253–260.
12. Senthuran, A., Senthuran, V., Hatti-Kaul, R., and Mattiasson, B. (1999), *J. Biotechnol.* **73,** 61–70.
13. Guoqiang, D., Kaul, R., and Mattiasson, B. (1992), *Appl. Microbiol. Biotechnol*. **36,** 309–314.
14. Corton, E., Piuri, M., Battaglini, F and Ruzal, S. M. (2000), *Biotechnol. Prog*. **16(1),** 59–63.
15. Persson, A., Jonsson, A., and Zacchi, G. (2001), *Biotechnol. Bioeng*. **72(3),** 269–277.
16. Mehaia, M. A. and Cheryan, M. (1986), *Enzyme Microbiol. Technol*. **8,** 289–292.
17. Jeantet, R., Maubois, J. L., and Boyaval, P. (1996), *Enzyme Microbiol. Technol.* **19,** 614–619.
18. Hjorleifsdottir, S., Holst, O., and Mattiasson, B. (1991), *Bioprocess. Eng*. **6,** 29–34.
19 Foss Tecator. (1999). Application Note. The Determination of Nitrogen According to Kjeldahl using Block Digestion and Steam Distillation. 2400/24600 Kjctcc® Auto Sampler System User Manual 10009165. Revl.l.
20 AOAC. (1995), Official Methods of Analysis of AOAC International, 17th ed. S587.A3: AOAC Official Method 991. 22. Arlington, VA: AOAC International.