Journal of Microbiology and Biotechnology
Vol. 6. No. 2, 128-131, 1996

Growth of *Lactobacillus acidophilus* in Whey-based Medium and Preparation of Cell Concentrate for Production of Probiotics

HONG, SEOK-SAN*, WANG-JUNE KIM, SEONG-KWAN CHA, AND BYONG H. LEE[1]

Food Biotechnology Division, Korea Food Research Institute, San 46-1, Baekhyun-Dong, Bundang-Gu, Songnam, Kyonggi-Do 463-420, Korea, [1]Department of Food Science and Agricultural Chemistry, McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Lactobacillus acidophilus KFRI 233 (of human origin) exhibited a high tolerance to bile. The maximum cell yield was 6.6×10^9 CFU per gram of whey in a 5.0% whey medium. Cell growth was improved with the addition of 0.5% thiotone and 0.25% calcium carbonate. Cell growth reached a maximum level of 5.4×10^8 CFU/ml at 20 h. Eighty-nine percent of the viable cells in the centrifuged concentrate survived freezing at -70°C and this frozen concentrate showed no reduction in the viable cell count after 30 days at -70°C. Eight percent of the viable cells survived freeze-drying after the addition of 1 g/l sodium carbonate before harvesting by centrifuging and this freeze-dried concentrate showed only a slight reduction in the viable cell count after 30 days at 4°C.

The ingestion of lactic acid bacteria, which was initially proposed Metchnikoff as a means of reducing intestinal putrefaction and prolonging life (11), has been extensively investigated as a beneficial dietary adjunct for the enhancement of human health (1). Lilly and Stillwell (10) introduced the term 'probiotics' for growth promoting factors produced by microorganisms. Fuller (1) defined 'probiotic' as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'.

Lactobacillus acidophilus and *Bifidobacterium* species are frequently associated with health-promoting effects in the human intestinal tract (8). These probiotic effects are generally related to the inhibition of pathogenic species, reducing the risk of colon cancer (5), increasing immune responses (15), and decreasing serum cholesterol levels (3). The presence of *L. acidophilus* in milk is also beneficial to those individuals who cannot digest lactose efficiently (6). The bacterial cells serve as a source of an enzyme system for hydrolyzing lactose in the intestinal tract.

In a previous paper (7), *L. acidophilus* KFRI 233 was selected as a candidate for probiotics due to its excellent growth in MRS broth, antagonistic effect against *Clostridium perfringens*, and high β-galactosidase activity. In this article, we report several physiological characteristics and cell concentrate preparation from a whey-based medium of *L. acidophilus* KFRI 233.

*Corresponding author
Key words: *Lactobacillus acidophilus*, probiotics, cell concentrate, whey-based medium, bile tolerance

MATERIALS AND METHODS

Strains and Culture Conditions

Human-originated strains of *L. acidophilus*, designated as KFRI 150, 217, 233, were obtained from Korean Collection for Type Cultures, and the other *L. acidophilus* strains (KFRI 561, 572, 582), which were chosen from commercial probiotic products sold in Korea, were kindly donated by the College of Agriculture and Life Sciences, Seoul National University (Suwon, Korea). These strains were propagated in sterile 10% nonfat milk solids containing 0.5% yeast extract (Difco) by 1% inocula and incubation at 37°C for 20 h. The cultures were stored at 4°C between transfers.

Comparison of Bile Tolerance

Lactobacilli MRS broth (Difco) was prepared with and without 0.3% oxgall (Difco), dispensed in 10 ml volumes and sterilized by autoclaving at 121°C for 15 min. For each culture to be tested, one tube of each media was inoculated with 0.1 ml of a freshly prepared lactobacilli MRS broth culture. The inoculated media were incubated at 37°C in a water bath. Growth was monitored by the increase of A600nm with a spectrophotometer (Varian 634). Growth times required for turbidity to reach an optical density of 0.3 were determined.

Growth in Whey-based Media

Dried sweet whey (Dong Bo Industrial Co., Seoul)

was reconstituted in distilled water containing 0.1% Tween 80 (Sigma). Thiotone (BBL) and calcium carbonate were added to the reconstituted whey. After the added ingredients were dissolved, the medium was autoclaved for 15 min at 121°C. The medium was inoculated with 1% freshly prepared nonfat milk culture and incubated for 24 h at 37°C.

Preparation of Cell Concentrate

Cells were harvested when the culture of *L. acidophilus* reached the late logarithmic and early stationary phase (15-20 h). Twenty percent solution of sodium carbonate was added to the culture and the cells were harvested by centrifuging for 10 min at 4,000×g and 4°C. To prepare frozen cell concentrate, the centrifuged concentrate was dispensed aseptically in 1 g quantities into sterile 2 ml polyethylene screw cap freezing vials, frozen and stored at -70°C. To prepare freeze-dried cell concentrate, 1 g aliquots of the centrifuged concentrate were placed in 10 ml butyl rubber-stoppered serum bottles and frozen at -70°C. The stoppers were loosened, and the contents were freeze-dried with Maxi-Dry FD-4.5-60 (FTS Systems Inc., Stone Ridge, NY). Bottles were sealed under vacuum. An aluminum cap was then crimped over the rubber stoppers to hold them secure. The bottles were stored at 4°C.

Enumeration Procedures

Serial dilutions were prepared with dilution blanks composed of 0.1% peptone (Difco) and 0.001% Antifoam A Emulsion (Sigma) in distilled water. Viable cells were counted using spread plates with MRSO agar (9). The MRSO agar was prepared by dissolving 0.1% oxgall and 1.5% Bacto Agar (Difco) in lactobacilli MRS broth prior to sterilizing (15 min at 121°C). The plates were incubated in an anaerobic jar (BBL) at 37°C for 48 h. Bile-containing medium in anaerobic condition was used to measure the ability to survive and grow in the intestinal tract. To evaluate cell concentrates, vials of frozen concentrate were thawed by submerging them in tap water at 30°C for 3 min.

RESULTS AND DISCUSSION

Bile Tolerence of *L. acidophilus* KFRI 233

It is important to select a probiotic strain having a high degree of bile resistance because the more bile-tolerant strain developed better in the upper small intestine than did the lesser bile tolerant strain (4). Comparison of the ability of the six strains of human-originated *L. acidophilus* to grow in the MRS broth with and without 0.3% oxgall revealed considerable variation among strains (Table 1). When compared with the control broth, 0.3% oxgall exerted inhibitory effect on all strains. Strains KFRI 217, 233, and 493 were the most resistant strains. Strains 150 and 491 had not reached an absorbance of 0.3 at 8 h. Among the six strains of lactobacilli, KFRI 233 showed the highest growth both with and without 0.3% oxgall.

Optimization of Whey-based Medium Composition for the Growth of *L. acidophilus* KFRI 233

Table 2 shows the growth of *L. acidophilus* KFRI 233 at 2.5, 5.0 and 7.5% whey solids. The highest cell growth attained, based on counts on MRSO agar in anaerobic condition, was 6.1×10^8 CFU/ml in the 7.5% whey medium. But the maximum cell yield was 1.2×10^{10} CFU per gram of whey in the 5.0% whey medium.

Table 3 shows the effect of thiotone concentration on cell growth. At 0.5% thiotone, cell growth increased 6.2 times as compared to that without thiotone. At 1.0 and 1.5% thiotone, cell growth increased little when compared with the growth at 0.5% thiotone. Cell growth did not increase with the addition of yeast extract or the hydrolysis of whey protein with pepsin.

L. acidophilus is sensitive to acidity (12). During cell growth, lactobacilli produce lactic acid and lower the pH of the media. Table 4 shows the effect of $CaCO_3$ addition on cell growth. By the addition of 2.5 g/l $CaCO_3$, cell

Table 1. Comparison of bile tolerance of strains of human-originated *L. acidophilus*.

Strain	Hours to reach $A_{660\,nm}=0.3$	
	MRS broth	MRS broth+0.3% oxgall
KFRI 150	4.25	> 8
217	2.74	3.17
233	2.46	3.05
491	4.03	> 8
493	3.17	3.50
582	3.98	4.33

Table 2. Effect of whey concentration on the growth of *L. acidophilus* KFRI 233 and cell yield in a medium containing 1.0% thiotone and 0.5% calcium carbonate.

Whey concentration (%)	Cell growth (CFU/ml)	Cell yield (CFU/g whey)
2.5	2.5×10^8	9.9×10^9
5.0	5.8×10^8	1.2×10^{10}
7.5	6.1×10^8	8.1×10^9

Table 3. Effect of thiotone concentration on the growth of *L. acidophilus* KFRI 233 in a medium containing 5.0% whey solids.

Thiotone concentration (%)	Cell growth (CFU/ml)
0	7.7×10^7
0.5	4.8×10^8
1	4.9×10^8
1.5	4.8×10^8

growth showed a 19% increase and the pH after cell growth increased to 5.0 from 4.2 as compared to the control. At 5.0 and 7.5 g/l $CaCO_3$, there was no increase in cell growth or pH as compared with that at 2.5 g/l $CaCO_3$. This result suggests that cell growth was inhibited by low pH and $CaCO_3$ addition increased the cell growth as a result of pH increase.

Fig. 1 shows the growth curve and pH decrease during cell growth. Cell growth reached maximum of 6.0×10^8 CFU/ml at 20 h and then remained constant with no reduction in numbers for the remainder of the incubation time. However, the pH decreased to 4.9 continuously throughtout incubation. Generally probiotic cell cultures are used in the ultrafrozen or freeze-dried state. The physiological condition of the culture plays an important part in the survival of the bacterium after liquid-nitrogen freezing and freeze-drying. The bacterial culture shows the maximum stability and resistance to liquid-nitrogen freezing and freeze-drying in the late logarithmic and early stationary phase, respectively (2). It is therefore best to harvest the culture of *L. acidophilus* KFRI 233 at 15-20 h to produce frozen or freeze-dried cell concentrate.

Preparation of Cell Concentrate of *L. acidophilus* Grown in Whey-based Medium

In consideration of the above results, *L. acidophilus* KFRI 233 was cultivated in a medium containing 5.0% whey solids, 0.5% thiotone, and 0.25% calcium carbonate for 15-20 h for the preparation of cell concentrate.

Sodium carbonate is added to milk cultures of lactic acid bacteria to increase pH before cell concentration (13). In this study, viable cell recovery of frozen cell concentrate of *L. acidophilus* grown in whey-based medium was not improved by adding sodium carbonate to the culture before centrifugation. Eighty-nine percent of the viable cells in the cell concentrate centrifuged without sodium carbonate survived during freezing at -70°C (Table 5). After 30 days of storage at -70°C, this frozen concentrate showed no reduction in viable cell counts.

Table 5 shows the effect of the addition of sodium carbonate before centrifugation of the culture on the viable cell recovery of freeze-dried cell concentrate of *L. acidophilus* grown in whey-based medium. There was little difference between the dry weights or the viable cell counts of the freeze-dried or frozen concentrate with or without sodium carbonate but the addition of sodium carbonate increased the viable cell counts of freeze-dried concentrate when compared to the control. Eight percent of the viable cells in the frozen cell concentrate survived during freeze-drying by the addition of 1 g/l sodium car-

Table 4. Effect of $CaCO_3$ addition on the growth of *L. acidophilus* KFRI 233 and pH change in a medium containing 5.0% whey solids and 0.5% thiotone.

Amount of $CaCO_3$ added (g/l)	Cell growth (CFU/ml)	pH
0	4.8×10^8	4.2
2.5	5.9×10^8	5.0
5.0	5.9×10^8	5.1
7.5	5.8×10^8	5.1

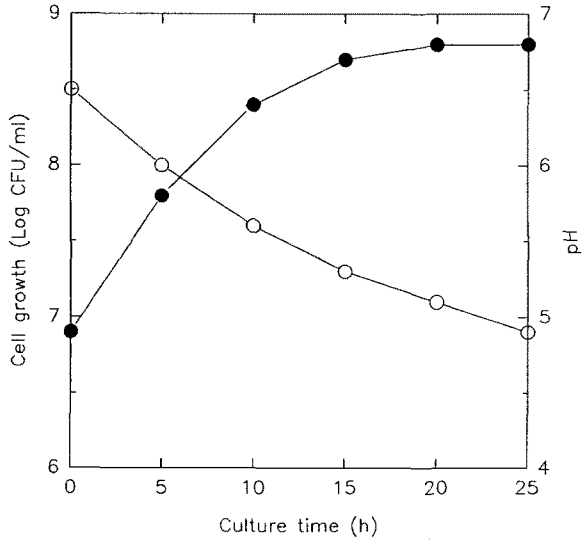

Fig. 1. Growth of *L. acidophilus* KFRI 233 and pH in a medium containing 5.0% whey solids, 0.5% thiotone, and 0.25% calcium carbonate.
●, cell growth; ○, pH.

Table 5. Effect of Na_2CO_3 addition before centrifugation of the culture on the dry weight (DW) of freeze-dried cell concentrate and the viable cell counts of cell concentrates frozen and freeze-dried.

	Concentration of Na_2CO_3 (g/l)			
	0	0.5	1.0	1.5
DW of freeze-dried concentrate from 1 liter culture (g)	7.9	7.8	7.9	7.7
Viable cell counts of frozen concentrate[a] (VCF, CFU/g DW)	6.8×10^{10}	6.9×10^{10}	6.8×10^{10}	6.9×10^{10}
Viable cell counts of freeze-dried concentrate[b] (VCFD, CFU/g DW)	1.9×10^9	4.8×10^9	5.4×10^9	5.6×10^9
Survival ratio during freeze-drying (VCFD/VCF)	0.028	0.069	0.080	0.081

[a]The frozen concentrate was prepared by freezing the centrifuged concentrate of *L. acidophilus* for 10 h at -70°C. [b]The freeze-dried concentrate was prepared by drying the frozen concentrate with a vaccum below 30 μmHg for 24 h.

bonate before harvesting by centrifuging. During 30 days of storage at 4°C, this freeze-dried concentrate showed only a slight reduction in viable cell counts.

Further research is needed on the use of cryogenic compounds to increase the cell viability of frozen and freeze-dried cell concentrates of *L. acidophilus* (14).

REFERENCES

1. Fuller, R. 1989. Probiotics in man and animals. *J. Appl. Bacteriol.* **66**: 365-378.
2. Gherna, R. L. 1994. Culture preservation, p. 278-292. *In* P. Gerhardt (ed.-in-chief), *Methods for General and Molecular Bacteriology*, ASM, Washington.
3. Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **49**: 377-381.
4. Gilliland, S. E., T. E. Staley, and L. J. Bush. 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *J. Dairy Sci.* **67**: 3045-3051.
5. Havenaar, R. and J. H. J. Huis in't Veld. 1992. Probiotics: a general view, p. 151-170. *In* B. J. B. Wood (ed.), *The Lactic Acid Bacteria*, vol. **1**. Elsevier Applied Science, London.
6. Kim, H. S. and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* **66**: 959-966.
7. Kim, W. J., S. S. Hong, and S. K. Cha. 1994. Selection of human-originated *Lactobacillus acidophilus* for production of probiotics. *J. Microbiol. Biotechnol.* **4**: 151-154.
8. Klaver, F. A. M. and R. van der Meer. 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. *Appl. Environ. Microbiol.* **59**: 1120-1124.
9. Koch, A. R. 1994. Growth measurement, p. 248-277. *In* P. Gerhardt (ed.-in-chief), *Methods for General and Molecular Bacteriology*, ASM, Washington.
10. Lilly, D. M. and R. H. Stillwell. 1965. Probiotics: growth promoting factors produced by microorganisms. *Science* **147**: 747-748.
11. Metchnikoff, E. 1907. *The Prolongation of Life*, G. P. Putman's Sons, New York.
12. Robinson, R. K. and A. Y. Tamime. 1990. Microbiology of fermented milks, p. 291-343. *In* R. K. Robinson (ed.), *Dairy Microbiology*, vol. **2**. Elsevier Applied Science, London.
13. Speckman, C. A., W. E. Sandine, and P. R. Elliker. 1974. Lyophilized lactic acid starter culture concentrates: preparation and use in inoculation of vat milk for Cheddar and cottage cheese. *J. Dairy Sci.* **57**: 165-173.
14. Tamime, A. Y. 1990. Microbiology of starter cultures, p. 131-201. *In* R. K. Robinson (ed.), *Dairy Microbiology*, vol. **2**. Elsevier Applied Science, London.
15. Yasui, H., A. Mike, and M. Ohwaki. 1989. Immunogenicity of *Bifidobacterium breve* and change in the antibody production in Peyer's patches. *J. Dairy Sci.* **72**: 30-35.

(Received December 22, 1995)