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High Cell Density Cultivation of *Bifidobacterium longum* Using a Calcium Carbonate-Alginate Beads System

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Abstract A $CaCO_3$-alginate beads system was developed for high cell density cultivation of *Bifidobacterium longum* and the cost-effective media were also screened. In batch process with $CaCO_3$ beads, two strains of *B. longum* showed both the highest viable cells and optical density in TPY medium, resulting in maximum optical density and viable cell counts of 12.40, 2.22×10^{10} cfu/ml for *B. longum* ATCC 15707 and 13.71, 3.93×10^{10} cfu/ml for *B. longum* HLC 3742. Released size distribution, according to $CaCO_3$-alginate bead size preparation, was smaller than others. These results were also examined by observing their morphology. The skim milk-based medium was most adequate to cultivate *B. longum* as the cheapest medium, and 10% skim milk supplemented with 2% glucose and 1% yeast extract was a suitable medium, supporting the growth to 5.57×10^{10} cfu/ml for ATCC 15707 and 6.82×10^{9} cfu/ml for HLC 3742. During the long-term storage at 4°C and −20°C, *B. longum* cultivated with $CaCO_3$ beads had the highest stability. Consequently, $CaCO_3$-alginate beads buffer was found to be useful not only to cultivate *B. longum* but also to preserve cultures.

Key words: *Bifidobacterium longum*, $CaCO_3$ beads, high cell density cultivation, skim milk-based medium, stability

Bifidobacterium species are known for their health promoting effects in both human and animal intestinal tracts. Their probiotic effects are generally related to inhibition of pathogenic species, reducing the risk of colon cancer, increasing the immune response, and decreasing serum cholesterol level [1, 3, 5, 6, 8, 15]. Reuter [13] surveyed commercial fermented milk products containing bifidobacteria in several countries and found that *Bifidobacterium longum* is widely used in Germany.

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However, strict anaerobes such as bifidobacteria are difficult to cultivate, since they produce organic acids in the cultivation process. Generally, high cell density cultivation of lactic acid bacteria have been controlled with solutions such as NaOH, KOH, NH_4OH, and Na_2CO_3 [4, 14]. Peebles *et al.* [12] harvested the maximum concentration of *Streptococcus cremoris* by controlling pH with ammonium hydroxide as a neutralizer. Recently, the characteristics of the reaction of calcium carbonate immobilized with alginate as the buffer system for the cultivation of bifidobacteria in fermenter have been described by the mathematical model [9]. Lloyd and Pont [11] reported that the biomass and viability of lactic acid bacteria were affected by specific buffer and culture conditions during cultivation.

Moreover, it is generally accepted that bifidobacteria have nutritional stringentness, such that complex and expensive media are frequently used [4]. In order to lower the production cost in the commercial scale, development of cheap media should be established.

In this study, it was investigated whether a $CaCO_3$-alginate beads buffer system was useful to obtain high cell density of *B. longum* in a skim milk-based medium.

Materials and Methods

Bacterial Strains and Maintenance

B. longum ATCC 15707 was purchased in a lyophilized form from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.), and *B. longum* HLC 3742 was isolated from feces of a healthy Korean [10]. These two strains were routinely prepared as an inoculum by incubation in an anaerobic system (Forma Scientific Inc., U.S.A.) filled with mixed gases of N_2 (75%), H_2 (10%), and CO_2 (5%) for 20 h at 37°C. They were transferred twice to trypticase-proteose peptone-yeast extract (TPY) broth, containing 0.5% glucose as the single carbohydrate source, before cultivation.

Preparation of $CaCO_3$ Beads

Sodium alginate (medium viscosity, Sigma Chemical Co., U.S.A.) was dissolved at 2% (w/v) in distilled water by using impeller, and then 20% (w/v) $CaCO_3$ was homogenized with the above alginate solution. This mixture was dropped into sterile 100 mM $CaCl_2$ solution through a 24G blunt-ended needle, using compressed air (2.3 kg/cm^2) filtered through a sequence of 5 μm, 1 μm, and 0.5 μm air filters set in an air compressor. The beads were gently stirred with a magnetic stirrer, hardened for 1 h in this solution, and recovered using a mesh sieve.

High Cell Density Cultivation

The high cell density cultivation of *B. longum* was conducted under anaerobic conditions with shaking at 100 rpm for 20 h at 37°C in a 2.5-l fermenter containing 1 liter of TPY (2.5% glucose) broth to which 2% (v/v) of fresh inoculums had been added. The pH of the culture media was maintained at 5.5 with 2 N NaOH, 2 N KOH, 20% (w/v) Na_2CO_3, and 20% (v/v) NH_4OH solutions.

The $CaCO_3$ beads buffer system was as follows: Fresh 30% (w/v) $CaCO_3$-alginate beads were suspended in a fermenter with 1,000 ml TPY broth without K_2HPO_4 to prevent bead disruption from phosphate. Operating condition was similar to the above, except for agitation at 200±10 rpm.

Analytical Procedures

Viable cell counts were carried out by the spot counting method [2]. Ten ml of the culture broth was diluted serially in sterilized physiological saline solution and 200 μl of the diluted solutions were dropped onto the surfaces of TPY agar (1.5%) plates. The plates were incubated anaerobically at 37°C for 48 h.

During the cultivation, growth pattern was recorded by optical density at 600 nm using a spectrophotometer (Perkin-Elmer, Lambda 3B, Shelton, CT, U.S.A.) and the value of pH was also determined with a pH meter (Methrom Ltd., Swiss). Cell mean volume was determined within the range of 1 and 10 fl using the Coulter Counter (Model Z2, Coulter Electronics, Inc., Hialeah, FL, U.S.A.) with 50 μm aperture tube.

After the high cell density cultivation of *B. longum* with various buffers, bacterial cultures were stored in 1.5-l bottles at 4°C and divided into 1.5 ml microtubes to freeze at −20°C. Frozen samples were thawed at room temperature for 10 min before plating.

The morphology of Gram-stained *B. longum* after high cell density cultivation was examined with an optical microscope (Alphaphot-YS, Nikon, Japan).

The Media Used and Cell Growth

The viable cells grown under 12 different culture conditions and reference medium were enumerated. TPY can be considered conveniently as a reference. Skim milk [10% (w/v)] (Maeil Co., Korea) and 10% (w/v) whey permeate (Jiwon Technical Co., Ltd., Korea) based media were prepared by adding various carbon and nitrogen sources. After 20 h of incubation, viable cell counts were carried out as mentioned above.

RESULTS AND DISCUSSION

Effect of $CaCO_3$ Beads on High Cell Density Cultivation of *B. longum*

The various buffers were used for high cell density cultivation of *B. longum* (Table 1). Both strains cultivated well with all buffers, however, non-pH controlled cultures failed to raise viable cell counts, which were below 10^9 cfu/ml. Bifidobacterial cell cultivation with NaOH was poor to cultivate the cell. After 20 h of cultivation, viable cells were 4.25×10^9 cfu/ml of *B. longum* ATCC 15707 and 8.75×10^9 cfu/ml of *B. longum* HLC 3742. In the case of

Table 1. Effect of various buffers on high cell density cultivation of *Bifidobacterium longum* after culture at 37°C for 18 h.

Strains	Buffers	Maximum OD_{600}	Viable cell counts (log cfu/ml)[a]	Specific growth rate (h^{-1})
B. longum ATCC 15707	None	4.30	8.765±0.252	0.694
	$CaCO_3$ beads	12.40	10.347±0.298	1.310
	NaOH	9.10	9.628±0.203	0.940
	KOH	11.65	9.768±0.452	1.224
	Na_2CO_3	11.10	9.564±0.362	1.162
	NH_4OH	11.40	9.723±0.420	0.868
B. longum HLC 3742	None	3.15	8.935±0.223	0.452
	$CaCO_3$ beads	13.71	10.594±0.355	1.458
	NaOH	10.50	9.942±0.064	0.653
	KOH	12.10	10.041±0.198	1.212
	Na_2CO_3	11.20	9.881±0.534	1.306
	NH_4OH	10.35	9.851±0.653	1.874

[a]Data are mean values±standard deviations (n=3).

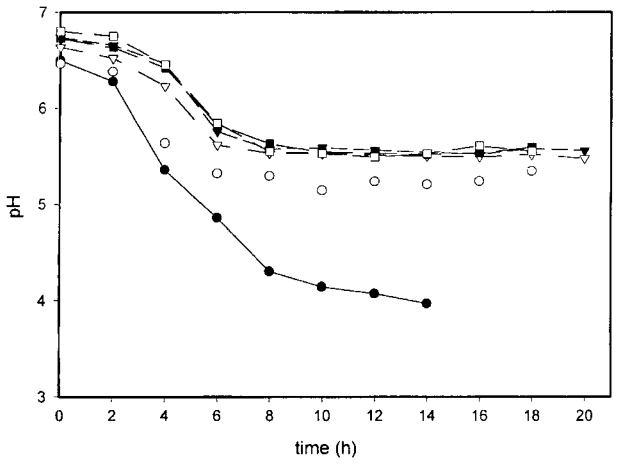

Fig. 1. pH changes in high cell density cultivation of *Bifidobacterium longum* HLC 3742 with various buffer solutions. Symbols: ●, none; ○, $CaCO_3$ beads; ▽, NaOH; ▼, KOH; □, Na_2CO_3; ■, NH_4OH.

KOH solution, both strains showed a similar specific growth rate, which was 1.224 h^{-1} of *B. longum* ATCC 15707 and 1.212 h^{-1} of *B. longum* HLC 3742. Na_2CO_3 buffer solution was efficient to increase cell density during cultivation of *B. longum* HLC 3742. Also, NH_4OH buffer solution increased the HLC 3742 strain, however the specific growth rate of ATCC 15707 strain was very low in the present study. This was most likely due to the reason that kinds of buffers influenced the growth of bacterial cells. Cell cultures with $CaCO_3$ beads indicated a maximum optical density of 12.40 for *B. longum* ATCC 15707 and 13.71 for *B. longum* HLC 3742 after 18 h of incubation. After 20 h of incubation, viable cells were enumerated to be 2.22×10^{10} cfu/ml of *B. longum* ATCC 15707 and 3.93×10^{10} cfu/ml of *B. longum* HLC 3742. These results indicated the highest cell counts than any other buffers tested. *B. longum* HLC 3742 is generally superior to *B. longum* ATCC 15707 in batch cultivation.

Changes of pH of the culture medium are shown in Fig. 1. $CaCO_3$ beads could automatically control the pH at 5.3 until the end of batch cultivation. Calcium carbonate reacted with organic acids such as lactic acid and acetic acid [9], therefore, CO_2 was produced by a side reaction, which provided the anaerobic condition for growth of *B. longum* (Fig. 2).

$$CaCO_3+CH_3CHOHCOOH+CH_3COOH$$
$$\downarrow\uparrow$$
$$CaCO_3+CH_3CHOHCOO^-+CH_3COO^-+2H^+$$
$$\downarrow$$
$$CH_3CHOHCOO\text{-}Ca\text{-}OOCCH_3+H_2O+CO_2(\uparrow)$$

Fig. 2. Reaction mechanism of calcium carbonate with the organic acids.

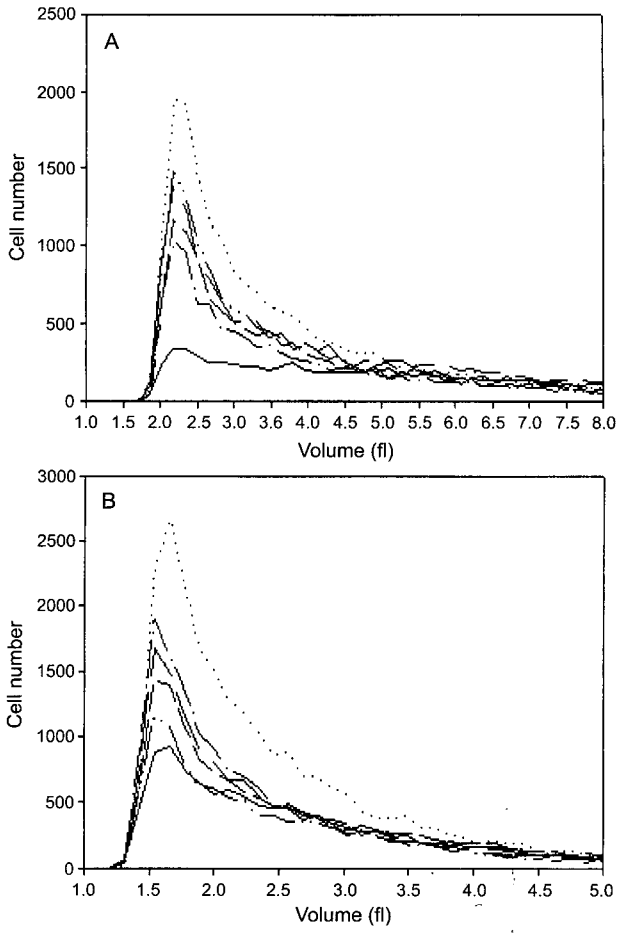

Fig. 3. Bifidobacterial cell size distribution after high cell density cultivation with various buffers.
(A) *B. longum* ATCC 15707; (B) *B. longum* HLC 3742. Symbols: ——, control; ·····, $CaCO_3$ beads; -----, NaOH; —·—, KOH; -·-·-, Na_2CO_3; —··—, NH_4OH.

Cell size distribution was investigated by using a Coulter Counter after cultivation (Fig. 3). Cell size cultivated with $CaCO_3$ beads was 2.3 fl of *B. longum* ATCC 15705 and 1.8 fl of *B. longum* HLC 3742, which was similar to cultures with other buffers. However, cell distribution cultivated with $CaCO_3$ beads was smaller than others. These results were further confirmed by observing their morphology (Fig. 4). The portion of mono-sized bifidobacterial cells indicated the highest value after the high cell density cultivation with $CaCO_3$ beads, which has been suggested to be favorable for freeze-drying, because crystalline of inner cell is limited by their small size during the freezing process. However, the relationship between cell size and freeze-drying should be further studied.

Skim Milk- and Whey Permeate-Based Media

To develop a cheap media for industrial *Bifidobacterium* spp. production, skim milk- and whey permeate-based media were tested (Table 2). TPY medium containing 2%

Table 2. Comparative growth of *Bifidobacterium longum* in different media without pH regulation.

Medium[a]	*B. longum* ATCC 15707		*B. longum* HLC 3742	
	Viable cell counts (log cfu/ml)[b]	PH	Viable cell counts (log cfu/ml)[b]	pH
TPY+2%G	9.685±0.120	4.23	9.887±0.095	4.09
TPY+2%L	9.463±0.037	4.25	9.654±0.173	4.12
10%SM	8.235±0.232	5.10	8.653±0.155	5.14
10%SM+2%G	9.165±0.142	4.98	9.544±0.224	5.02
10%SM+2%L	9.072±0.195	5.05	9.371±0.160	5.10
10%SM+2%G+1%YE	9.746±0.234	4.09	9.834±0.185	4.02
10%SM+2%L+1%YE	9.434±0.198	4.08	9.538±0.196	4.12
10%WP	8.162±0.032	4.38	8.633±0.172	4.25
10%WP+2%G	9.015±0.158	4.71	9.041±0.338	4.16
10%WP+2%L	8.964±0.332	4.52	9.322±0.453	4.33
10%WP+2%G+1%YE	9.366±0.285	4.05	9.681±0.152	3.98
10%WP+2%L+1%YE	9.256±0.231	4.12	9.415±0.052	4.05

[a]G; glucose, L; lactose, SM; skim milk, YE; yeast extract.
[b]Data are mean values±standard deviations (n=3).

glucose increased viable cells more than TPY containing 2% lactose. Skim milk-based media had high buffering capacity, which prevented the decrease of pH. Although whey permeate-based media had rich micronutrients, they did not stimulate the growth more than the skim milk-based one. When 1% yeast extract was supplemented with 10% skim milk containing 2% glucose, as a nitrogen source, both strains of ATCC 15707 and HLC 3742 increased up to 3.81×10^9 cfu/ml and 1.95×10^9 cfu/ml, respectively. Therefore, 10% skim milk supplemented with 2% glucose and 1% yeast extract was found to be a suitable medium, supporting the growth to 5.57×10^{10} cfu/ml for *B. longum* ATCC 15707 and 6.82×10^9 cfu/ml for *B. longum* HLC 3742. Skim milk has been suggested to be an economical and good cultivation medium despite of its nitrogen source [7]. However, it is technically difficult to monitor cell growth in both skim milk- and whey-based media because of their interfering turbidity.

Preservation of Bacterial Suspension

Viability of bacterial preparation with various buffers in 10% skim milk supplemented with 2% glucose and 1% yeast extract was examined during long-term storage (Table 3). Thus, each culture after harvesting at the exponential phase was spread on TPY agar medium for viable cells counting. High cell density cultivation with $CaCO_3$ beads had the highest number of viable cells, which was 2.80×10^{10} cfu/ml for *B. longum* ATCC 15707 and 6.28×10^{10} cfu/ml for HLC 3742, respectively. Both strains cultivated with $CaCO_3$ beads were the most stable during storage. After 180 days at 4°C, $CaCO_3$ beads culture had 2.24×10^8 cfu/ml of ATCC 15707 and 2.97×10^8 cfu/ml of HLC 3742. Also, after 180 days storage at −20°C, the cultures with $CaCO_3$ beads showed viable cells of 1.67×10^9 cfu/ml of ATCC 15707 and 2.58×10^9 cfu/ml of HLC 3742. The number of viable cells stored at −20°C was higher than that stored at 4°C. Other buffer solutions generally showed lower viability, however, storage of −20°C was more stable than 4°C storage. Consequently, the cultivation using $CaCO_3$ beads buffer is suggested to be advantageous not only to cultivate *B. longum* but also to preserve bifidobacterial cultures.

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