Journal of Microbiology and Biotechnology
Vol. 5. No. 5, 285-291, 1995

Fermentation Conditions for the Production of Cell Mass and Comparison of Saccharide Utilization in *Bifidobacterium longum* and *B. breve*

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Saccharide utilizations for the growth by *Bifidobacterium longum* and *Bifidobacterium breve* were compared. *B. longum* fermented glucose more rapidly than lactose as a carbon source whereas *B. breve* fermented lactose at a rate higher than that of glucose. The highest cell concentration, in the case of *B. longum*, was obtained when cultivated in a jar fermentor that contained modified MRS medium that half the beef extract was replaced by the same amount of tuna extract, and that pH was controlled at 6.0. *B. breve* showed the best growth when grown in a jar fermentor containing the MRS medium with lactose instead of glucose, controlled at pH 6.0. The optimal concentration of peptone in MRS medium for the growth of *B. breve* was 5 g/l.

Intestinal flora are composed of 100 trillion bacteria comprising 100 species, which have influence beneficially or deleteriously on the host's health. Bifidobacteria which are Gram-positive, non-motile, and strictly anaerobic bacteria with a variety of shapes ranging from short rods to Y-shaped or clubbed rods, constitute 5 to 10% of the total flora in the faeces of children and adults (11).

They have been extensively investigated due to the significant beneficial roles in human and animal health (6, 13). The health and nutritional benefits of bifidobacteria in the intestine can be proposed to: (a) prohibit the growth of many pathogenic and putrefactive bacteria by controlling the intestinal pH through the liberation of lactic and acetic acids (9); (b) correct abnormal conditions such as antibiotic-associated diarrhea (8); (c) enhance anticarcinogenic activity by the direct or indirect removal of procarcinogens such as nitrosamines or by stimulation of the immunological response (5, 6); (d) synthesize B-complex vitamins which are absorbed into the body (2); and, (e) may reduce the level of cholesterol in serum (3).

In addition to the potential benefits of bifidobacteria, the fact that the population of bifidobacteria in human intestine decreases or disappears with the age but the population of pathogenic bacteria increases has led to the development of commercial products containing viable cells of bifidobacteria or bifidogenic substances (4, 6, 12). The purpose of this study, therefore, is to develop commercial medium and fermentation processes optimal for the mass production of viable cells of bifidobacteria.

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Key words: *Bifidobacterium longum, Bifidobacterium breve*, fermentation

MATERIALS AND METHODS

Bacterial Strains, Chemicals and Media

B. longum HP1 and *B. breve* HP2 were isolated from faeces of a human adult and an infant, respectively and were identified according to the procedures described by Mitsuoka (10). Organisms were cultivated in Lactobacilli MRS Broth (Difco Lab, Detroit, MI, USA) or in LPY medium consisting of lactose 20 g, peptone 10 g, yeast extract 10 g, sodium acetate 3 g, KH_2PO_4 1 g, K_2HPO_4 2 g and cysteine·HCl 0.3 g per liter of distilled water. Tuna extract, a waste product in tuna fish-processing factories was purchased from Dong-Won Co. Ltd (Seoul, Korea). All other chemicals used were of reagent grade.

Cultivation Conditions

Organisms were maintained by anoxic and stringent culture techniques (7), and routinely grown at 37°C in anaerobic pressure tubes (Bellco Glass, Inc., Vineland, NJ, USA) containing 10 ml of MRS broth with a nitrogen gas headspace. The cultures

were mixed with 30% glycerol at a ratio of 1:1, frozen, and stored in a deep freezer. Experimental cultures were grown without shaking in N_2-gassed 125 ml Wheaton serum bottles containing 50 ml of MRS or LPY medium and 2% of the carbohydrate to be tested. Fermentation time course studies were conducted in a 5 liter-jar fermentor (Korea Fermentor Co., Incheon, Korea) that contained 2.5 liters of modified MRS medium or LPY medium. Fermentors were agitated at 200 rpm, gassed initially and at the time of sampling with N_2 gas, and controlled at pH 6.0 with 10% sodium hydroxide solution.

Quantification of Growth and Fermentation Substrates

For the determination of culture turbidities, culture broths were appropriately diluted with distilled water and the optical densities were measured at 660 nm using a Gilford spectrophotometer. To measure viable cell concentrations, culture broths were appropriately diluted with a diluent consisting of KH_2PO_4 4.0 g, Na_2HPO_4 6.0 g, L-cysteine·HCl 0.5 g, Tween80 0.5 g, agar 1.0 g and NaCl 8.5 g per liter of distilled water, plated on Lactobacilli MRS agar medium, placed in an anaerobic jar with anaerobic system for generating H_2 and CO_2 (Difco Lab. Dtroit, MI, USA), filled with N_2 gas, and incubated at 37°C for 2 days. Correlation ratio between optical density and viable cell concentration was almost constant during growth phase, and 1.0 of optical density corresponded to 2.4×10^9 cells per ml for *B. breve* and 1.8×10^9 cells per ml for *B. longum*. Glucose was enzymatically determined using Sigma Diagnotics glucose reagents (Sigma, St, Louis, MO, USA). Lactose was estimated either by the dinitrosalicylic acid method (1) or by the Somogy-Nelson method (15).

RESULTS AND DISCUSSION

Growth Characteristics on Saccharides

Growth properties of *B. longum* and *B. breve* on a variety of saccharides were compared to discover the proper carbon sources for the production of cell mass, and to identify the species. As shown in Table 1, both species grew well on glucose, maltose, lactose, melibiose, raffinose, maltotriose, and isomaltose and did not grow on mannan. It is, however, of interest that *B. longum* showed rapid growth on fructose and xylose, poor growth on arabinose, sucrose, palatinose and turanose, and no growth on mannose, sorbitol, mannitol, cellobiose, trehalose, maltitol, glycogen and starch, while *B. breve* showed the rapid growth on sorbitol, mannitol, sucrose, trehalose, palatinose, turanose, maltitol and starch, the less rapid growth on fructose, mannose, cellobiose and glycogen, and no growth on arabinose and melezitose.

The fact that *B. longum* utilizes arabinose as a carbon source but *B. breve* does not coincides with the characteristics distinguishing the two species as described in Bergey's Manual of Systematic Bacteriology (14). Consequently, summarizing the results in Table 1, the additional characteristics distinguishing those species are that *B. breve* can utilize mannose, sugar alcohols such as sorbitol, mannitol and maltitol, polysaccharides such as glycogen and starch, disaccharides such as cellobiose and trehalose, whereas *B. longum* cannot utilize those substrates but can utilize the xylose and melezitose that *B. breve* can't.

Optimization of Medium Composition in Sealed Serum Bottles

In general, bifidobacterial species require complex growth factors, and therefore, they should be grown on complex medium such as MRS medium and LPY medium. Accordingly, the modification of MRS medium or LPY medium to be used as a commercial medium for the mass production of viable cells in *B. longum* and *B. breve* was investigated because they

Table 1. Saccharide utilization by *B. longum* and *B. breve*[a].

Addition of saccharide	*B. longum*		*B. breve*	
	Growth ($O.D_{660}$)	Final pH	Growth ($O.D_{660}$)	Final pH
No addition	0.4	5.92	0.9	5.44
Glucose	3.6	4.06	3.1	4.41
Xylose	2.2	4.07	0.8	5.53
Fructose	3.6	4.18	1.7	4.91
Mannose	0.4	5.70	1.6	4.86
Arabinose	1.1	5.10	0.9	5.44
Sorbitol	0.4	5.73	2.9	4.19
Mannitol	0.4	5.99	3.4	4.13
Sucrose	0.9	5.41	3.6	4.17
Maltose	3.0	4.31	4.0	4.06
Lactose	1.9	4.70	4.3	4.02
Cellobiose	0.4	5.90	1.4	4.94
Trehalose	0.4	6.00	2.0	4.63
Melibiose	2.2	4.38	3.3	4.15
Palationse	1.3	4.88	2.5	4.46
Raffinose	2.9	4.14	4.1	4.05
Melezitose	3.2	4.11	0.9	5.35
Turanose	1.3	4.73	2.5	4.44
Maltitol	0.4	5.91	2.2	4.50
maltotriose	3.4	4.17	5.3	4.07
Isomaltotriose	2.8	4.09	3.3	4.11
Glycogen	0.4	5.94	1.8	4.74
Starch	0.5	5.81	2.7	4.52
Mannan	0.4	5.79	0.8	5.50

[a]Cells were grown at 37°C for 24 h in a pressure tube containing 10 ml of MRS medium (initial pH 7.0) that each saccharide was added to be 2% instead of glucose, and cells grown in a medium containing each saccharide were used as an inoculum to the corresponding saccharide.

grow well on those media. Firstly, the possibility of whether expensive medium components, such as yeast extract and beef extract in MRS medium and LPY medium can be replaced by tuna extract, an inexpensive substrate in Korea, was tested based on the effects on their growth. Table 2 compares the final cell concentrations when *B. longum* was grown on MRS media in which beef extract or yeast extract was replaced by tuna extract.

The results indicate that either beef extract or yeast extract can be completely replaced by tuna extract without any effects on growth, but both components can't be replaced at once. Consequently, the effects of the partial replacement of beef extract or yeast extract by tuna extract on the growth of *B. longum* were investigated. When the 50% of beef extract in MRS medium was replaced by the same amount of tuna extract, or when 70% of yeast extract was replaced by the same amount of tuna extract, higher cell concentrations were obtained (Table 3).

In the case of *B. breve*, whether or not yeast extract could be replaced by beef extract or tuna extract was tested using LPY medium that contained twice the amount of peptone but did not contain beef extract, and this was compared to MRS medium. As shown in Table 4, the best growth was obtained in LPY medium that contained the same amount of tuna extract instead of yeast extract. In the preliminary experiments, the removal of peptone from MRS and LPY medium resulted in poor growth in *B. longum* and *B. breve* (data not shown), and therefore, the optimal concentration of peptone to maximize the final cell concentration could be determined later using a jar fermentor.

Table 2. Effects of replacement of medium components in MRS medium by tuna extract on the growth of *B. longum*[a].

Concentrations of medium components			Growth ($O.D_{660}$)	Final pH
Beef extract	Yeast extract	Tuna extract		
-	-	-	0.42	5.49
-	-	10 g/l	1.22	4.73
-	5 g/l	10 g/l	2.66	4.4
10 g/l	-	10 g/l	2.65	4.50
10 g/l	5 g/l	-	2.67	4.32

[a]Experiments were conducted in sealed 125 ml-Wheaton serum bottles containing 50 ml of MRS medium modified as indicated, which were incubated for 18 hours. - indicates no addition.

Table 3. Comparison of growth when beef extract or beef extract in MRS medium were partially replaced by tuna extract in *B. longum*[a].

Concentration of medium components			Growth ($O.D_{660}$)	Final pH
Beef extract (g/l)	Yeast extract (g/l)	Tuna extract (g/l)		
6.0	5.0	4.0	3.01	4.22
5.0	5.0	5.0	3.42	4.15
4.0	5.0	6.0	3.13	4.18
10.0	3.5	1.5	2.71	4.31
10.0	2.5	2.5	2.68	4.28
10.0	1.5	3.5	3.61	4.05
10.0	5.0	0.0	2.67	4.32

[a]Experiments were conducted in sealed 125 ml - Wheaton serum bottles containing 50 ml of MRS medium modified as indicated, which were incubated for 18 hours.

Differential Utilization of Saccharides

MRS medium contains glucose but LPY medium contains lactose as a carbon source, and therefore the effects of glucose and lactose on the growth of *B. longum* and *B. breve* were investigated in both MRS and LPY medium. The results, shown in Table 5, illustrate that *B. breve* prefers lactose to glucose as a sole carbon source, in contrast to *B. longum* that shows the highest growth when grown on MRS medium containing glucose. In addition to this, *B. breve* appears to grow better on MRS medium than on LPY medium.

In order to confirm if *B. longum* and *B. breve* metabolize glucose and lactose differentially, the time courses for the growth of *B. longum* and *B. breve* when grown on MRS medium containing glucose or lactose were studied. Fig. 1 and Fig. 2 show the time courses for the fermentation of glucose or lactose by *B. longum*

Table 4. Effects of replacement of yeast extract in LPY medium by beef extract or tuna extract on the growth of *B. breve*.[a]

Medium components added			Growth ($O.D_{660}$)
Yeast extract (g/l)	Beef extract (g/l)	Tuna extract (g/l)	
-	-	-	1.35
10	-	-	4.19
-	10	-	4.05
-	-	10	4.35

[a]Experiments were conducted in sealed Wheaton serum bottles containing 50 ml of LPY medium modified as indicated which were incubated for 24 hours. - indicates no addition.

Table 5. Comparison of growth on MRS medium and LPY medium containing glucose and lactose as a carbon source in *B. longum* and *B. breve*[a].

Medium	Carbon source	Growth ($O.D_{660}$)	
		B. longum	*B. breve*
LPY	Lactose	3.83	4.19
LPY	Glucose	3.61	3.03
MRS	Lactose	2.90	5.53
MRS	Glucose	4.55	3.14

[a]Cells were cultivated in sealed serum bottles containing 50 ml of medium for 23 hours.

and *B. breve*, respectively. It is of great interest that *B. breve* displays a higher growth rate, higher final cell concentration and higher substrate consumption rate when grown on lactose rather than on glucose. On the other hand, *B. longum* showed the opposite result, that is, a higher growth rate, higher final cell concentration, and higher substrate consumption rate on glucose rather than on lactose. *B. breve* is the most common bifidobacteria isolated from infants with *B. infantis* but is not found in the intestines of children and adults. *B. longum* is occasionally isolated from infants but found in high numbers in the intestines of children, adults and elderly persons (11). Therefore, the fact that *B. breve* prefers lactose to glucose while *B. longum* prefers glucose to lactose suggests that *B. longum* could be abundant in the intestines of children and adults fed mainly with starch-foods, but *B. breve* could be abundant in infants fed with milk containing lactose as a major carbohydrate. The fundamental biochemical basis for the differential metabolism of saccharides by bifidobacteria are now under investigation in our laboratory.

Optimization of Medium Composition in Jar Fermentors

Based on the fundamental studies performed in sealed serum bottles for the optimization of medium composition, the fermentation conditions to obtain the maximum cell concentration in the two bifidobacterial species were tested in a jar fermentor. Before conducting the experiments, the effects of initial pH on the growth of bifidobacteria were studied because pH may be a major factor preventing further growth in the stationary growth phase. As shown in Table 6, *B. longum* and *B. breve* could grow from pH 4.0 to 7.0, and from

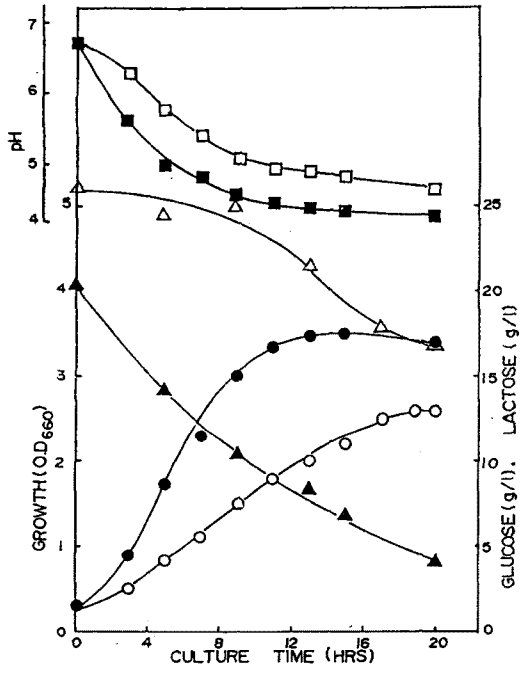

Fig. 1. Fermentation time courses of *B. longum* when grown in MRS medium containing glucose or lactose as a C-source.

● and ○ indicate the cell concentrations ($O.D_{660}$) on glucose and lactose, respectively. ▲ and △ indicate glucose and lactose concentration, respectively. Experiments were perfomed in a 500 ml-Erlenmeyer flask containing 200 ml of medium.

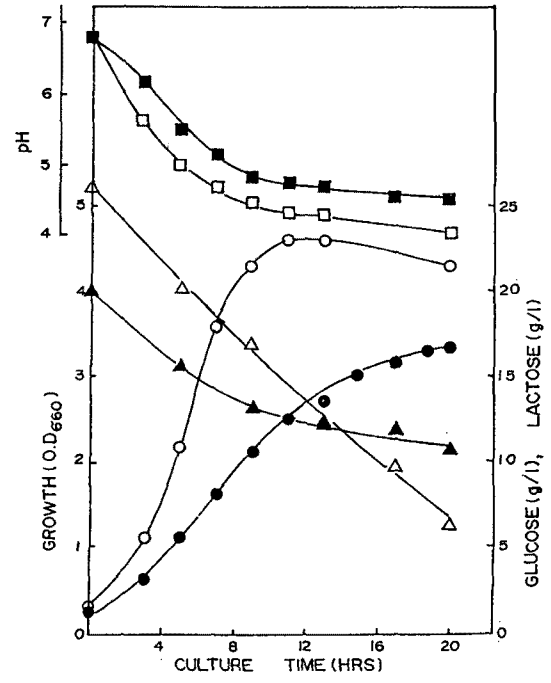

Fig. 2. Fermentation time courses of *B. breve* when grown in MRS medium containing glucose or lactose as a C-source.

● and ○ indicate the cell concentrations ($O.D_{660}$) on glucose and lactose, respectively. ▲ and △ indicate glucose and lactose concentration, respectively. Experiments were performed in a 500 ml-Erlenmeyer flask containing 200 ml of medium.

Table 6. Effects of initial pH on the growth of *B. longum* and *B. breve*[a].

Initial pH of medium	Growth ($O.D_{660}$)	
	B. longum	*B. breve*
2.0	0.0	0.0
3.0	0.0	0.0
3.5	0.0	0.0
4.0	0.1	0.5
4.5	1.6	1.4
5.0	2.6	2.0
6.0	3.2	2.9
7.0	1.9	1.8
8.0	0.0	0.3
9.0	0.0	0.0
10.0	0.0	0.0

[a]Cells were cultivated for 48 hours in a pressure tube containing MRS medium for *B. longum* and LPY medium for *B. breve*, respectively.

pH 4.0 to 8.0, respectively, and in both species, it was found pH 6.0 optimal for their growth.

The experiments, therefore, to optimize the medium composition were carried out in a 5 liter-jar fermentor that was controlled at pH 6.0 with 10% NaOH solution, agitated at 200 rpm, and not gassed. Fig. 3 shows the effects of replacement of beef extract by tuna extract in MRS medium on the growth of *B. longum*. When beef extract was completely replaced by the same amount of tuna extract, the growth rate was not affected but the final cell concentration was severely affected and found to be much lower than that on standard MRS medium. In the case of replacement of half the amount of beef extract by an equivalent amount of tuna extract, both growth rate and final cell concentration were hardly affected. This may indicate that beef extract contains growth factors not present in tuna extract, and therefore, it

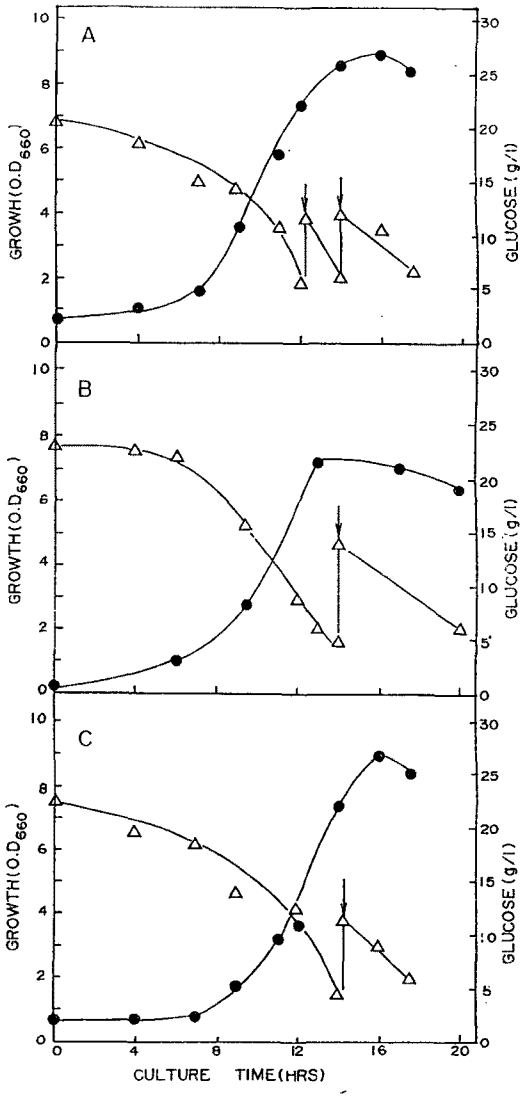

Fig. 3. *Comparison of fermentation time courses in B. longum* when grown in a fermentor containing MRS medium (A) and the modified media that beef extract was completely replaced by tuna extract (B) and that a half amount of beef extract was replaced by the same amount of tuna extract (c).

The arrows indicate the addition of glucose solution (50%. 100 ml) to the fermentor containing 2.5 l of medium. ●; Cell concentration. △; Glucose concentration.

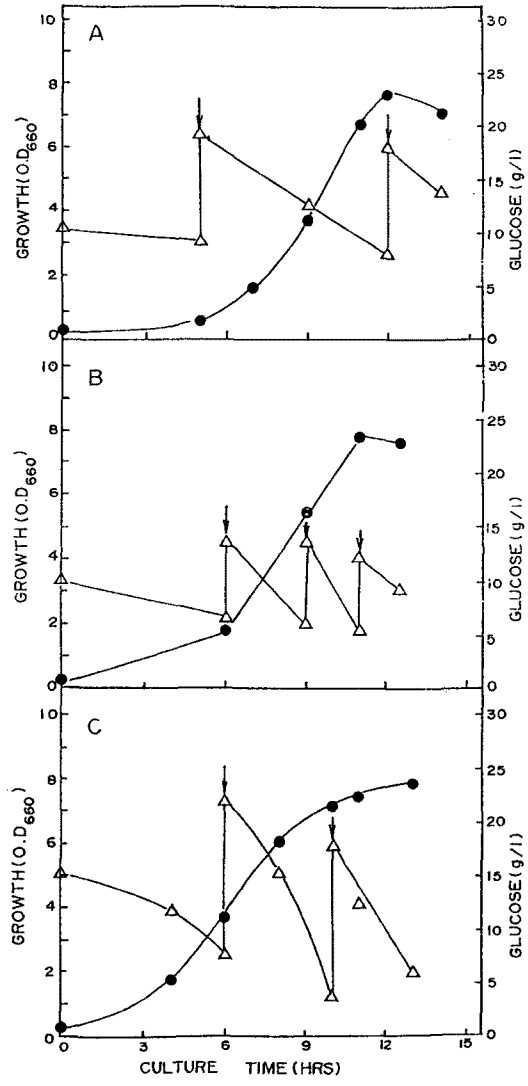

Fig. 4. Fermentation time courses of *B. longum* when grown on MRS media that yeast extract was partially replaced by tuna extract, and that peptone concentration was reduced. A; MRS medium was modified to contain 1.5 g/l of yeast extract and 3.5 g/l of tuna extract. B; MRS medium was modified to contain each 2.5 g/l yeast extract and tuna extract. C; Peptone concentration of MRS medium was reduced to a half.

The arrows indicate the addition of glucose solution (50%, 100 ml) to the fermentor containing 2.5 l of medium. ●; Cell concentration. △; Glucose concentration.

was concluded that beef extract could be partially replaced by tuna extract.

In the fundamental experiments to replace yeast extract, the partial replacement of yeast extract by tuna extract resulted in better growth in *B. longum* (Table 3), and therefore, the effects of partial replacement of yeast extract were studied in a jar fermentor. According to the results shown in Fig. 4, by contrast with the results obtained from the experiment in sealed serum bottles, lower final cell concentrations were obtained when 50% or 70% of the yeast extract in MRS medium was replaced by tuna extract (Fig. 4), in comparison with standard MRS medium (Fig. 3A). Also, Fig. 4C shows that the fermentation time course of *B. longum* when the concentration of peptone in MRS medium was reduced from 10 g/l to 5 g/l. The data indicate that the final cell concentration becomes somewhat decreased but the growth rate was almost unaffected. The effect on the growth of *B. longum* when MRS medium was supplemented with other medium components such as Tween 80 and vitamines (e.g. nicotinic acid, thiamine) was also tested but growth was nearly unaffected (data not shown).

In case of *B. breve*, LPY medium and MRS medium that contained lactose instead of glucose was compared with respect to growth and substrate utilization in a jar fermentor. In a jar fermentor where pH was controlled at 6.0, *B. breve* showed a slow growth rate, low final cell concentration and low substrate consumption rate in LPY medium (Fig. 5A), but in MRS medium, it showed a higher growth rate, higher final cell concentration and higher substrate consumption rate (Fig. 5B). For *B. breve*, the effects of peptone on growth were also investigated in a jar fermentor. As shown Fig. 5C, growth was entirely unaffected when the peptone concentration of MRS medium was reduced from 10 g/l to 5 g/l. However, when peptone was reduced to a concentration lower than 5 g/l or completely removed, the growth of *B. breve* was severely affected (data not shown).

Other medium components such as beef extract and yeast extract were tested in a jar fermentor with respect to their effects on the growth of *B. breve* when they were partially or completely replaced by tuna extract. However, the growth rate and final cell concentration of *B. breve* were severely decreased by the replacements (data not shown). Consequently, MRS medium with half the amount of beef extract replaced by tuna extract appeared to be optimum for obtaining maximum cell concentrations in *B. longum*. However, *B. breve* showed the best growth in MRS medium modified to contain lactose as a carbon source instead of glucose, and to contain half the amount of peptone.

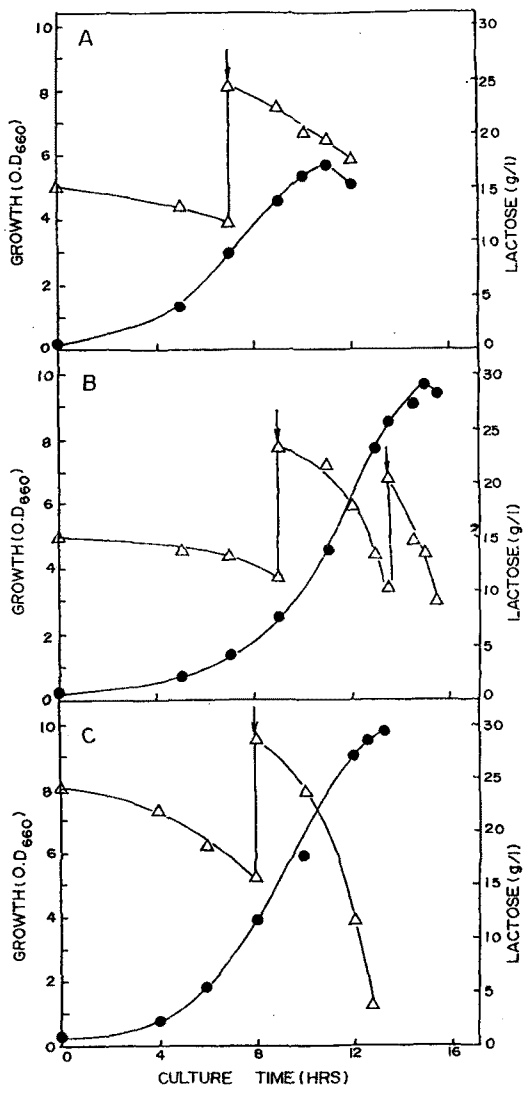

Fig. 5. Fermentation time courses of *B. breve* when cultivated in a pH-controlled fermentor containing LPY medium (A), MRS medium modified to contain lactose instead of glucose (B), and MRS medium that peptone concentration was reduced to a half in addition to the replacement of glucose by lactose (C).
The arrows indicate the addition of lactose solution (50%, 100 ml) to the fermentor. ●; Cell concentration. △; Lactose concentration.

Acknowledgement

This research is a result of G7 project supported by the Ministry of Science and Technology of Korea and Pulmuone Co. Ltd.

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(Received June 20, 1995)