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# Culture Conditions and Growth Characteristics of *Bifidobacterium longum*

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A simple and low-cost medium was developed for the growth of *Bifidobacterium longum* KFRI 977. Of three bifidobacterial strains, *B. longum* KFRI 977 (ATCC 15707) showed the best growth in MRSC broth containing 0.3% oxgall, grew well in partially anaerobic condition, exhibited highest β-galactosidase activity, and was inhibitory against *Clostridium perfringens* KFRI 434. Of three developed media, the population of *B. longum* KFRI 977 was highest ($1.9\times10^9$/ml) in ISP based medium. The composition of ISP based medium is ISP (5%), glucose (1%), L-cysteine HCl (0.05%), Trypticase peptone (0.5%), yeast extract (0.5%), $MgSO_4$ (0.05%), Tween-80 (0.1%), and phosphate buffer (pH 7.0). Hydrolysis of ISP by Protease A was unnecessary, and the use of phosphate buffer (pH 7.0) prevented the formation of protein precipitate. Associative culture of *B. longum* KFRI 977 with *Lactobacillus acidophilus* KFRI 233 was proven to be deleterous to the growth of *B. longum* KFRI 977.

The genus Bifidobacteria are Gram positive anaerobic, asporogenous bacteria which inhabit the large intestine of animals. Since the discovery of the health promoting effects of bifidobacteria (16), extensive research has been carried on how to use them as a dietary adjunct or as a health food. Mainly, they are being incorporated in fermented milk and fresh milk products (8, 11). However, trials for industrial production of bifidobacterial probiotics have been hampered by their fastidious nature, i.e. anaerobiosis, growth factors. Specifically, little research has been done on developing low-cost medium and culture conditions for intensified growth of bifidobacterial strains of human origin. In this paper, we selected *B. longum* KFRI 977 after the examination of physiological characteristics, which are a prerequisite for human probiotics, and developed a low-cost medium for the industrial production of the selected strain.

## MATERIALS AND METHODS

### Microorganisms, Culture Media

All bifidobacterial strains, *B. longum* KFRI 977 (ATCC 15707), *B. infantis* KFRI 974 (ATCC 15697), and *B. bifidum* KFRI 973 (ATCC 11863) are human origin and were maintained as glycerol stocks in a -72℃ deep freezer. Before experiments, each culture was thawed and transferred to MRS broth supplemented with 0.05% L-cysteine HCl (MRSC broth), and incubated in GasPak jar (BBL) at 37℃ for 24 h. The morphology of strains was checked under the microscope and their genuineness was finally confirmed by a sugar fermentation test.

### Growth in Anaerobic, Partially Anaerobic, and Aerobic Conditions

The growth of strains were examined in different atmospheric conditions to see whether they could grow in reasonable numbers without the aid of an anaerobic culture system. Anaerobic growth was achieved by growing cells in GasPak jars, partially anaerobic growth was prepared by covering MRSC broth with liquid paraffin (MRSCP broth), and aerobic growth was done by growing cells in MRSC broth without GasPak and liquid paraffin. All strains were inoculated in capped bottles containing 100 ml MRSC broth. Approximately $3\times10^7$ CFU of 24 h grown cells were inoculated to each broth and the cultures were incubated at 37°C for 24 h. Enumeration of cells was done by pour plating using dilution buffer (0.45% $KH_2PO_4$, 0.6% $Na_2HPO_4$, 0.05% L-cysteine HCl, 0.05% Tween-80, pH 7.2) and BL agar (Difco). Plates were incubated in GasPak jar at 37℃ for 48 h. All experiments were repeated three times and the mean population was calculated.

### Growth of Cells in Bile

To simulate the conditions of the large intestine, oxgall (Difco) was added to MRSCP broth to make 0.3% (w/v). Every 30 min, a portion of cells were drawn and the

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time to reach $OD_{600}$ (Varian series 634, 1 cm cuvette) of 0.3 was recorded.

**Beta-galactosidase Activities**

The microassay method for determination of β-galactosidase activities of bifidobacterial strains was carried out by the method described previously (13).

**Antimicrobial Effect Against *Clostridium perfringens***

The antimicrobial effect of bifidobacterial strains against *C. perfringens* was examined by associative culture (13). Bifidobacterial strains and *C. perfringens* KFRI 434 were inoculated in 100 ml Reinforced Clostridial (RC) medium at the ratio of $10^4$:1 (v/v), approximately $3\times10^7$ and $3\times10^3$ CFUs, respectively. The control was obtained by inoculating only *C. perfringens*. Associative cultures and the control were incubated at 37°C in GasPak jars for 48 h, and the number of *C. perfringens* was enumerated by pour plating with Tryptose Sulfite Cycloserine (TSC) agar, a selective medium for clostridia. The anticlostridial effect of each bifidobacterial strain was calculated using the following equation and was expressed as % inhibition: (CFU/ml in control) - (CFU/ml in associative)/(CFU/ml in control) × 100

**Growth in ISP Based Medium**

Commercially sold isolated soy protein (ISP, 5%) was dissolved in both 100 ml distilled water and in 0.1 M phosphate buffer (pH 7.0). Protease A (Amano, Japan) was added at 790 U/g to both solutions, which were then covered with liquid paraffin, and the medium was incubated at 45℃ for 17 h. The control was ISP solution dissolved in phosphate buffer without enzyme. Filter sterilized (0.45 μM) glucose (1%) and L-cysteine HCl (0.05%) were added to pre-autoclaved (121℃, 15 min) ISP solutions. This was regarded as a base medium. To examine the cumulative effect of each growth promoter, pre-sterilized stock solutions of Trypticase peptone (BBL), yeast extract, $MgSO_4$, and Tween-80 were sequentially added to the base medium to make final concentrations of 0.5%, 0.5%, 0.05%, 0.1%, respectively. The concentration of growth promoters at this step was regarded as 1 × strength. At every step, approximately $3\times10^7$ CFU of overnight grown cells were inoculated and incubated at 37°C for 24 h. The effect of growth promoters with increased concentrations (2 − 5 × strength) was also examined.

**Growth in Whey Based Medium**

Commercially sold whey and L-cysteine HCl were dissolved in 100 ml distilled water at 5 and 0.05%, respectively, liquid paraffin was added and this was regarded as base medium. All four growth promoters (1 × strength) as mentioned above and 0.25% $CaCO_3$ were sequentially added and autoclaved (121℃, 15 min). At every step, approximately $3\times10^7$ CFU of overnight grown cells were inoculated and incubated at 37°C for 24 h.

**Growth in Soymilk**

Soybean, produced in Korea, and hot distilled water were mixed at 1:1 (w/v) ratio and soaked for 2 h. Nine x volume of hot distilled water was added and the sample was blended and filtered. The sample was divided into three groups (100 ml each); Protease A added, Protease S (Amano) added, and Protease A+S added. The concentration of both enzymes was 790 U/g. In case of adding both enzymes, the sample was digested by Protease A (45℃, 17 h) first and subsequently Protease S was added and digestion (70°C, 17 h) was allowed to take place. Liquid paraffin was added and samples were autoclaved (121°C, 15 min). The size of inoculum and incubation conditions were as above.

**Mixed Culture with *Lactobacillus acidophilus* KFRI 233**

The same number ($3\times10^7$ CFU) of *L. acidophilus* KFRI 233 and *B. longum* KFRI 977 were added to whey (5% whey + 0.25% $CaCO_3$ + growth promoters), ISP (Protease A untreated + phosphate buffer + growth promoters) and soymilk (Protease A + S). The volume of each medium was 100 ml and incubation continued at 37℃ for 24 h. The selective plating medium for *B. longum* was Reinforced Clostridial Agar on which *L. acidophilus* KFRI 233 appeared as pin point colonies, while *B. longum* KFRI 977 produced large colonies.

## RESULTS AND DISCUSSION

**Growth in Bile**

Kim (12) reported that bile resistance is one of the critical prerequisite for probiotics. Gilliland and Walker (6, 21) also stated that a strain with bile resistance shows better growth and colonization in the intestine than one without bile resistance. The concentration of bile in the human intestine is approximately 0.6% and it becomes diluted in the large intestine (10). *B. longum* KFRI 977 showed the fastest growth in MRSC broth containing 0.3% oxgall (Table 1), in which bile concentration is close to that of the large intestine. Clark and Martin (1) also stated that *B. longum* is most bile resistant among bifidobacteria. Compared to the bile resistance of *L. acidophilus* (13), the value of *B. longum* KFRI 977 was not inferior.

**Table 1.** Time (h) to reach $OD_{600}$ of 0.3 in MRSC broth[a] containing 0.3% oxgall.

| Strains | Time |
|---|---|
| *Bifidobacterium longum* KFRI 977 | 3.5 |
| *Bifidobacterium infantis* KFRI 974 | 5 |
| *Bifidobacterium bifidum* KFRI 973 | 6 |

[a]MRS broth supplemented with 0.05% L-cysteine HCl.

**Growth in Different Atmospheric Conditions**

Selection of a bifidobacterium strain that grows well without the aid of an anaerobic system would be helpful for the industrial production of probiotics. *B. longum* KFRI 977 could grow in partially anaerobic simple conditions (i.e. MRSCP broth) and there was not much difference between growth in GasPak jars and growth under partially anaerobic conditions (Table 2). This was in accordance with the data made by Shimamura et al. (20) who reported that *B. longum* have certain degree of oxygen tolerance.

**Beta-galactosidase Activities**

More than 90% of oriental people are reported to be lactose intolerance, and the application of bifidobacterium in milk as a dietary adjunct has been tried (2). Among three tested strains, *B. longum* KFRI 977 showed the highest β-galactosidase activity (Table 3), and this was in agreement with the data reported by Desjardins (3). In this respect, consumption of strain 977 may help in the alleviation of lactose maldigestion problem.

**Table 2.** The number (CFU/ml) of bifidobacterial strains grown in MRSC broth incubated in different atmospheric conditions.

| Strains | without paraffin[a] | with paraffin[b] | GasPak[c] |
|---|---|---|---|
| *B. longum* KFRI 977 | $1.2\times10^8$ | $2.9\times10^8$ | $3.1\times10^8$ |
| *B. infantis* KFRI 974 | $7.1\times10^7$ | $7.9\times10^7$ | $8.1\times10^7$ |
| *B. bifidum* KFRI 973 | $6.9\times10^7$ | $7.1\times10^7$ | $8.4\times10^7$ |

[a]Aerobic condition, [b]Partially anaerobic condition, [c]Anaerobic condition.

**Table 3.** β-galactosidase activities (μmole ONP/μg protein) in bifidobacterial strains.

| Strains | β-galactosidase activity |
|---|---|
| *B. longum* KFRI 977 | 326.6 |
| *B. infantis* KFRI 974 | 265.7 |
| *B. bifidum* KFRI 973 | 301.6 |

**Table 4.** % inhibition of bifidobacterial strains against *Clostridium perfringens* KFRI 434 in associative culture[a].

| Media | CFU/ml of *B. longum* 977 | |
|---|---|---|
| | In control | In associative |
| whey[a] | $8.3\times10^8$ | $4.8\times10^8$ |
| ISP[b] | $1.9\times10^9$ | $5.1\times10^8$ |
| soymilk[c] | $5.8\times10^8$ | $3.9\times10^8$ |

[a]5% whey+0.25% $CaCO_3$+growth promoters (1×strength), [b]ISP based medium+growth promoters (1×strength), [c]Soymilk+Protease A+S (both at 790 U/g).

**Inhibitory Effect Against C. *perfringens***

It is well known that the population of *C. perfringens* increases as people get older, and *vice versa* (16). This may indicate that there lies relationship between aging and clostridial population. *C. perfringens* is also known for causing food poisoning. *B. longum* KFRI 977 remarkably inhibited the growth of *C. perfringens* (Table 4). At this point, it is not clear what is responsible for the inhibition. In general, undissociated acetic acid and lactic acid are believed to play a role in this phenomenon (11). However, Gibson and Wang (5) reported that *B. longum* are able to exert an inhibitory effect against *C. perfringens* not necessarily related to acid production. More detailed research conducting in this field will be an attractive subject for further investigation.

**Growth in ISP Based Medium**

Well known bifidogenic factors, i.e. human milk whey, lactulose, and sugars containing N-acetylglucosamine, are effective in stimulating the growth of bifidobacteria, however, they are expensive. Therefore, a low-cost substitute for these materials is required for the industrial production of bifidobacteria. According to the literature (9, 15, 18, 19), the addition of Trypticase peptone and yeast extract is almost equivalent to the effect of bifidogenic factors. This was true in this experiment. In case of strain 977, the addition of Trypticase peptone increased the population by 170.8%, yeast extract resulted in 329.2% increase, and after adding four growth promoters, the population increased by 546.6% (Table 5). To investigate the effect of growth promoters, its strength was varied (2-5 x). However, increased strength of growth promoters did not result in any remarkable growth increase (data not shown). The Protease A treated group showed lower population ($1.3\times10^9$ CFU/ml) than the protease untreated group ($1.9\times10^9$ CFU/ml). It might be that Protease A treatment resulted in extra digestion of soy protein in such a way that hydroyzed peptides are not usable for strain 977. Forming protein precipitate after autoclaving medium prevents effective cell harvest. This problem could be avoided when ISP based medium was prepared by phosphate buffer (pH 7.0), while, ISP dissolved in distilled water generated heavy precipitate.

**Table 5.** Growth[a] of bifidobacterial strains in ISP based media.

| Formulae | CFU/ml of strains(% increase) | | |
|---|---|---|---|
| | *B. longum* 977 | *B. bifidum* 973 | *B. infantis* 974 |
| ISP[b] | $2.4\times10^8$ (0) | $2.3\times10^7$ (0) | $2.1\times10^7$ (0) |
| ISP+P | $4.1\times10^8$ (170.8) | $4.1\times10^7$ (178.3) | $2.9\times10^7$ (138.1) |
| ISP+P+Y | $7.9\times10^8$ (329.2) | $6.3\times10^7$ (273.9) | $5.1\times10^7$ (242.9) |
| ISP+P+Y+M | $8.5\times10^8$ (354.1) | $7.5\times10^7$ (326.1) | $6.9\times10^7$ (328.6) |
| ISP+P+Y+M+T | $1.3\times10^9$ (541.6) | $8.1\times10^7$ (352.2) | $7.9\times10^7$ (376.2) |

[a]Incubation time was 24 h at 37℃, [b]ISP (5%), phosphate buffer (pH 7.0), glucose (1%), L-cysteine HCl (0.05%), Protease A treated.

**Table 6.** Growth of *B. longum* KFRI 977 in whey based media and soymilk.

| Formulae | CFU/ml |
|---|---|
| 5% whey+0.25% $CaCO_3$+growth promoters[a] | $8.4\times10^8$ |
| 5% whey+0.25% $CaCO_3$ | $1.9\times10^8$ |
| 5% whey | $1.4\times10^8$ |
| untreated soymilk | $8.6\times10^7$ |
| Protease A treated soymilk | $1.4\times10^8$ |
| Protease S treated soymilk | $3.5\times10^8$ |
| Protease A+S[b] treated soymilk | $5.8\times10^8$ |

[a]1 x strength of growth promoters were used, [b]Both enzymes were added at 790 U/g.

**Table 7.** Associative growth of *B. longum* KFRI 977 and *L. acidophilus* KFRI 233 in different media.

| Media | CFU/ml of *B. longum* 977 | |
|---|---|---|
| | In control | In associative |
| whey[a] | $8.3\times10^8$ | $4.8\times10^8$ |
| ISP[b] | $1.9\times10^9$ | $5.1\times10^8$ |
| soymilk[c] | $5.8\times10^8$ | $3.9\times10^8$ |

[a]5% whey+0.25% $CaCO_3$+growth promoters (1×strength), [b]ISP based medium+growth promoters (1×strength), [c]Soymilk+Protease A+S (both at 790 U/g).

**Growth in Whey Based Medium**

From the data of β-galactosidase activity, it was expected that strain 977 should grow well in whey. The addition of growth promoters resulted in remarkable growth increase, while $CaCO_3$, which was added to neutralize pH, showed only a slight growth increase (Table 6). The population obtained from whey based medium ($8.4\times10^8$ CFU/ml) was higher than that of MRSC ($3.1\times10^8$ CFU/ml, Table 2). In addition, the protein precipitate was slightly formed after incubation of the medium (data not shown).

**Growth in Soymilk**

Soymilk is used to supplement deficient diets or as a substitute for cow's milk in lactose intolerant people. Despite their advantages, however, they are not widely accepted as they have non-digestible galactooligosaccharides such as stachyose and raffinose which cause abdominal flatulence. However, *B. longum* are reported to possess α-galactosidase, the enzyme that hydrolyze stachyose and raffinose (4, 14, 17). The highest growth was observed from the Protease A + S treated group (Table 6). Again, this data is higher than the growth observed from MRSC (Table 2 and 6). When ISP based medium was digested by Protease A, lowered population was observed; however, Protease A and S digestion of soymilk generated opposite result. At present, we do not know the reason why such diference has occurred.

**Associative Culture with *L. acidophilus***

Associative culture of bifidobacteria and *L. acidophilus* often results in increasing bifidobacterial population due to the hydrolysis of protein by *L. acidophilus* (7). However, the opposite results were obtained in this experiment (Table 7). The generation of lower population after enzyme treatment of ISP based medium and like the report of Desjardins (3), strain 977 is presumed to have proteolytic activity, which forced competitive growth, thereby resulting in lower population of strain 977.

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