Influence of pH and Impeller Tip Speed on the Cultivation of *Bifidobacterium pseudocatenulatum* G4 in a Milk-Based Medium

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Abstract Biomass production of *Bifidobacterium pseudocatenulatum* G4 in a milk-based medium was carried out in a 2- and 10-L stirred tank fermenters. The effects of impeller tip speed (0.28, 0.56, and 0.83 m/s) and pH control (6.0, 6.5, and 7.0) on the biomass production were investigated. The growth performance in the 2-L fermenter was significantly improved when the impeller tip speed was held constant at 0.56 m/s and the pH was controlled at 6.5. These conditions yielded a maximum biomass of 1.687×10^9 cfu/mL, a maximum specific growth rate of 0.504 h^{-1}, a biomass productivity of 9.240×10^7 cfu/mL·h, and a biomass yield of 9.791×10^{10} cfu/g lactose. The consumption of milk lactose resulted in the accumulation of 7.353 g/L acetic acid and 6.515 g/L lactic acid, with an acetic:lactic ratio of 1.129. Scale-up of the fermentation process to a 10-L fermenter based on a constant impeller tip speed of 0.56 m/s yielded reproducible results with respect to biomass production and cell viability. © KSBB

Keywords: *bifidobacteria*, *biomass*, *pH*, *impeller tip speed*

INTRODUCTION

Since the suggestion that the longevity of Bulgarian peasants was related to their high consumption of fermented milk by Metchnikoff in 1908 [1], probiotic organisms, such as bifidobacteria and lactic acid bacteria, have become increasingly incorporated into dairy products such as yogurt or cultured milk drinks. As a result, probiotic dairy products have emerged as one of the most developed functional food products in the European market [2].

The mass production of a probiotic culture is commonly carried out using a milk-based medium in the controlled environment of a fermenter [3]. In addition to the importance of using a good growth medium that is capable of supporting optimal cell production, microbial growth also depends on environmental factors, such as pH and agitation rate. It was reported that the optimum pH for the growth of *Bifidobacterium bifidum* ranged between 6.0 and 7.0. No growth was observed at a pH of 5.5 or less [4]. During the fermentation of bifidobacteria, the pH of the medium decreases drastically due to the ac-cumulation of organic acids. Therefore, many researchers con-trol the probiotic fermentation processes at specific pH values. For instance, in one study, the pH of the medium was held constant at 6.2 during the cultivation of *B. longum* [5]. In other studies, a pH of 6.5 was used to grow *Lactobacillus* strains in a milk-yeast extract medium [6], and the growth of *Lactococcus lactis* was carried out in a milk medium added with yeast extract and bacto-peptone [7]. Meanwhile, a pH of 6.65 was used for the biomass production of *Bacillus coagulans* [8].

The effect of mixing is another important environmental factor that affects the growth performance or probiotic organisms. In addition to the uniform distribution of nutrients and heat in the fermenter, good mixing is also necessary to prevent cells from being subjected to fluctuations in pH due to the intermittent action of pH control [9]. For instance, an agitation speed of 200 rev/min was used for the cultivation of *B. longum* in glucose or lactose-based media [10] as well as for the cultivation of *L. lactis* in milk-based media [7]. An agitation speed of 100 rev/min was used during lactobacillus

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fermentation in 10% skim milk supplemented with yeast extract [6]. Her *et al*. used an agita-tion speed as low as 10 rev/min to cultivate *B. longum* in De Man, Rogosa and Sharpe (MRS) broth and whey-based medium [11].

With the optimization of process variables in small-scale studies, one major concern is the scale-up of fermentation processes to a larger vessel. Various factors, such as unequal mass, heat, or oxygen transfer, may affect microbial growth after scale-up. The applicability of different scale-up strategies is dependent upon the process conditions. For example, scale-up based on the volumetric coefficient of oxygen transfer, k_La, might be an appropriate criterion for scaling-up aerobic fermentation processes. A constant power per volume ratio can be used in almost every scale-up problem, with the exception of problems related to mixing. However, it is known that scale-up on this basis will increase the shear in the fermenter [12].

Mixing time, the time required to reach a particular mixing intensity at a given scale, is another option [13]. However, this method is known to be costly because large-scale reactors require more power [14]. A simple technique that takes the shear of the impeller tip into consideration is scale-up based on constant impeller tip speed. If scale-up is attempted based on this technique, the power consumption per unit volume will decrease [15]. The amount of shear due to agitation should be kept at an appropriate level since higher shear rates may physically damage the cells, thereby affecting the microorganisms [16]. Therefore, researchers should thoroughly consider the different aspects of the various scale-up methods for different microorganisms.

There are no reports on the cell mass production of bifidobacteria currently available in the literature. Therefore, information on the kinetics of bifidobacteria fermentation is required. In view of the lack of information on the process of cultivating bifidobacteria in a milk medium, this study aimed to improve the growth performance of *B. pseudocatenulatum* G4 in a 2-L fermenter. The effect of impeller tip speed and strategies for controlling the pH of the medium were investigated in the present study. This was followed by the scale-up of the fermentation process to a 10-L fermenter based on constant impeller tip speed.

MATERIALS AND METHODS

Microorganism, Inoculum, and Medium Preparation

B. pseudocatenulatum G4 was obtained from Probiotic Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia. Previous studies reported that this strain, which was isolated from infant stools, displayed probiotic characteristics such as bile tolerance and the deconjugation of bile acids [17], antibacterial activity, antimicrobial susceptibility, and adherence properties [18]. The strain was stored at –20°C in TPY:glycerol (20:80). For the propagation of the inoculum, a pure colony of the strain was transferred from Trypticase Phytone Yeast (TPY) agar (Scharlau-Chemie, Barcelona, Spain) to TPY broth and incubated anaerobically at 37°C for 24 h. The culture was further propagated once in TPY broth by 10% inoculation to obtain an approximate initial biomass concentration of 10^7 cfu/mL. Cells were harvested by centrifugation at 3,000 rev/min for 15 min at 4°C in order to minimize the carryover of the previous media during inoculation. The cells were resuspended in a separately-sterilized yeast extract solution that was used as the inoculum.

The optimized medium for the biomass cultivation of this strain suggested by Stephenie [19] was used in this study. The medium contained 28 g/L of skim milk and 22 g/L of yeast extract. The yeast extract solution was autoclaved separately and then aseptically added into the fermenter. This was done in order to prevent the production of undesirable precipitation in the medium. The fermentation medium was flushed with oxygen-free nitrogen gas in order to create an anaerobic environment during the 2-L and 10-L scale fermentation processes.

Fermentation

All fermentation studies were carried out at 37°C. Approximately 5 mL of sample was withdrawn every 2 h for analysis. A 2-L stirred tank fermenter (Biostat B, B. Braun, Germany) with a working volume of 1,500 mL was used. The geometric details of the fermentation process are shown in Table 1. In order to study the effect of impeller tip speed on biomass production, fermentation was carried out using tip speeds of 0.28, 0.56, and 0.83 m/s, which corresponded to agitation speeds of 100, 200, and 300 rev/min, respectively. The culture pH was not controlled during fermentation. Impeller tip speed was calculated using the following equation:

$$\text{Impeller tip speed (m/s)} = \frac{\pi ND}{60} \quad (1)$$

where $\pi = 3.142$
N = Agitation speed (rev/min)
D = Impeller diameter (m)

The effect of pH control (pH 6.0, 6.5, and 7.0) on the growth performance of this strain was investigated by the automatic addition of 10 M NaOH or 3 M malic acid. Fermentation without pH control was carried out for comparison. The agitation speed was fixed at 200 rev/min throughout the fermentation process.

A 10-L stirred tank fermenter (Biostat C, B. Braun) with a working volume of 10 L was also used in the present study. The geometric details of the fermenter are shown in Table 1. The cultivation conditions were as follows: impeller tip speed, 0.56 m/s; agitation speed, 143 rev/min; and pH, 6.5.

As a control, TPY broth was used as the fermentation medium in the 10-L stirred tank fermenter. The geometric details and cultivation conditions were the same as those used in the 10-L scale fermentation (Table 1).

Microbiological Analysis

For the enumeration of viable cells, the samples were seri-

Table 1. Geometric details and operating conditions of 2- and 10-L stirred tank fermenters

Dimension/operating condition	Fermenter scale	
	2 L	10 L
Impeller tip speed (m/s)	0.56	0.56
Agitation speed N (rpm)	200	143
Working volume (L)	1.5	10
Number of impeller (Rushton turbine)	2	3
Liquid height, H_L (m)	0.110	0.452
Impeller height, H_i (m)	0.020	0.080
Vessel diameter, D_t (m)	0.130	0.185
Impeller diameter, D_i (m)	0.053	0.075
Impeller blade width, W_i (m)	0.010	0.010
Impeller blade length, L_i (m)	0.015	0.017

ally diluted in 0.1% (w/v) sterile peptone water (Merck, Darmstadt, Germany) and plated in duplicate onto TPY agar. The plates were incubated anaerobically at 37°C for 3 days. An anaerobic condition was achieved by placing the plates in anaerobic jars containing Anaerocult A (Merck). Cell viability was expressed as cfu/mL.

Determination of Lactose

Lactose consumption was determined using a modification of the High Performance Liquid Chromatography (HPLC) method described by Hou *et al*. [20]. One mL of sample was centrifuged at 13,000 rev/min for 10 min. The supernatant fraction was filtered through a 0.2-μm nylon membrane filter and injected onto an HPLC system (Jasco Co., Tokyo, Japan) equipped with a 250 mm × 4.6 mm Alltech Prevail Amino 5-μm column (Alltech Associates Inc., Deerfield, USA). The mobile phase used was 80% (v/v) acetonitrile (HPLC grade) (Fisher Scientific, New Jersey, UK). The flow rate was held constant at 2.0 mL/min, and the analysis was carried out at ambient temperature with an RI detector (Jasco RI-1530, Jasco Co.). Lactose quantification was carried out using an external standard method. The retention time for lactose was 17.15 min.

Determination of Organic Acids

Organic acids were analyzed using a modification of the method of Marsili *et al*. [21], as described by Narvhus *et al.* [22] and Ostlie *et al*. [23]. One gram of sample was added to 0.2 mL of 0.5 M H_2SO_4 and 8 mL of acetonitrile (Fisher Scientific) and mixed well. This was followed by centrifugation at 5,000 rev/min for 10 min and filtration through a 0.2-μm nylon membrane filter. The filtrate (20 μL) was analyzed using a 300 × 7.8 mm Aminex HPX-87H HPLC column (Bio-Rad Laboratories, Richmond, USA) held at 34°C. The HPLC system used in this study was a Shimadzu LC-10AS Liquid Chromatograph (Shimadzu, Kyoto, Japan) with a Shimadzu SPD-10AV UV-VIS detector set at 210 nm. The mobile phase used was 0.01 N H_2SO_4 at a flow rate of 0.7 mL/min. Quantification of organic acids was carried out using an external standard method. The organic acids were separated at the following retention times: acetic acid, 12.63 min; lactic acid, 10.66 min; and formic acid, 11.60 min.

Statistical Analysis

One-way Analysis of Variance (ANOVA) was performed using MINITAB version 13 (Minitab Inc., United States). Data were expressed as the mean ± SD of two experiments. Statistical significance was accepted at a probability level of $P < 0.05$.

RESULTS AND DISCUSSION

2-L Stirred Tank Fermenter

Effect of Impeller Tip Speed

Fig. 1 shows the effect of impeller tip speed on the growth and lactose consumption of this strain. Generally, the growth and lactose consumption patterns were similar at different tip speeds. Growth was slow at the beginning, and rapid exponential growth was typically observed after 8 h of fermentation. Meanwhile, lactose was gradually consumed during the entire course of fermentation. Lactose, the primary carbon source for growth and acid production in milk, was measured to represent substrate consumption. The initial lactose content in the 2.8% skim milk medium was 14.659 ± 0.103 g/L. After 24 h of fermentation, 6.291 to 8.038 g/L lactose remained unused in the broth.

The performance and kinetic parameter values were compared at different impeller tip speeds, and the results are shown in Table 2. A low maximum biomass of 2.523×10^8 cfu/mL was obtained when no mixing was applied during fermentation. This was due to the presence of a major stagnant area, whereby nutrients and bacterial cells were not uniformly distributed. Improved growth performance was obtained when the culture was mixed at impeller tip speeds of 0.28, 0.56, and 0.83 m/s. The highest biomass production was achieved at an impeller tip speed of 0.56 m/s, which yielded a maximum biomass of 6.670×10^8 cfu/mL, maximum specific growth rate of 0.311 h^{-1} and biomass productivity of 3.590×10^7 cfu/mL·h. The poor performance at higher impeller tip speeds may be due to the high shear rate of bacterial cells at the tip of the impeller. On the other hand, insufficient mixing or mass transfer in the fermenter at lower tip speeds resulted in poor cell growth.

The metabolism of *B. pseudocatenulatum* G4 in milk media produced high concentrations of acetic and lactic acid. In general, higher concentrations and increased production of organic acids were observed with higher cell growth rates (Table 2). However, it was noted that there was no significant difference between the acetic acid yield ($Y_{A/s}$) and lactic acid yield ($Y_{L/s}$) per unit of lactose consumed at different impeller tip speeds.

A similar finding was reported by Brosseau and Zajic [24] who reported that the activity of the anaerobic organism

Table 2. Kinetic parameter values from batch fermentation of *B. pseudocatenulatum* G4 in a 2-L stirred tank fermenter at different impeller tip speeds

Kinetic parameter values	Impeller tip speed (m/s)			
	0	0.28	0.56	0.83
Biomass				
Maximum biomass, x_{max} (cfu/mL)	2.523×10^{8a}	4.027×10^{8b}	6.668×10^{8c}	4.130×10^{8b}
Time taken to reach maximum biomass, t (h)	20	20	18	18
Maximum specific growth rate, μ_{max} (h^{-1})	0.174 ± 0.014^{a}	0.241 ± 0.011^{b}	0.311 ± 0.014^{c}	0.287 ± 0.020^{bc}
Biomass productivity, P_x ($\times 10^7$ cfu/mL·h)	1.145 ± 0.021^{a}	1.913 ± 0.096^{b}	3.590 ± 0.293^{c}	2.181 ± 0.122^{b}
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)	4.791 ± 0.704^{a}	5.825 ± 0.117^{a}	9.146 ± 0.607^{bc}	6.902 ± 0.518^{ac}
Organic acids				
Maximum acetic acid concentration, A_{max} (g/L)	2.018 ± 0.229^{a}	2.490 ± 0.123^{ab}	2.851 ± 0.233^{b}	2.704 ± 0.174^{ab}
Acetic acid productivity, P_A (g/L·h)	0.084 ± 0.010^{a}	0.125 ± 0.006^{bc}	0.119 ± 0.010^{bc}	0.113 ± 0.007^{ac}
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.307 ± 0.023^{a}	0.357 ± 0.025^{a}	0.346 ± 0.022^{a}	0.434 ± 0.090^{a}
Maximum lactic acid concentration, L_{max} (g/L)	2.387 ± 0.169^{a}	2.902 ± 0.395^{ab}	3.850 ± 0.207^{b}	3.487 ± 0.307^{ab}
Lactic acid productivity, P_L (g/L·h)	0.099 ± 0.007^{a}	0.145 ± 0.020^{ab}	0.160 ± 0.009^{b}	0.145 ± 0.013^{ab}
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.364 ± 0.012^{a}	0.416 ± 0.064^{a}	0.467 ± 0.042^{a}	0.558 ± 0.102^{a}
Acetic:lactic ratio	0.845	0.858	0.741	0.775

Results were reported as mean ± S.D. of two experiments.
[a,b,c]Mean values in the same row with different letters differ significantly ($P < 0.05$).
Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

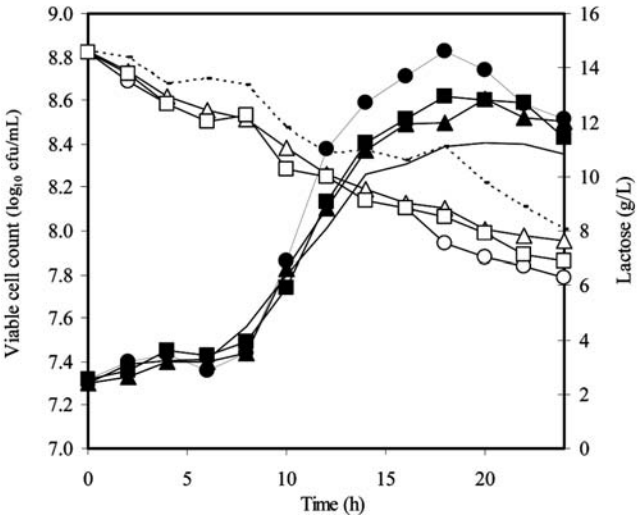

Fig. 1. Effect of impeller tip speed on the growth and lactose consumption of *B. pseudocatenulatum* G4 in a 2-L stirred tank fermenter. (——,·····) 0 m/s, (▲,△) 28 m/s, (●,○) 0.56 m/s, (■,□) 0.83 m/s. Closed symbols represent the biomass count, and the open symbols represent the rate of lactose consumption.

Citrobacter intermedius was much higher at 700 rev/min than at 400 rev/min; however, a further increase to 940 rev/min led to a reduction in the growth rate. This was because the agitation in the bioreactor creates shear forces, which can damage the structure of the cell or cause variations in growth rate and product formation [25]. In addition to the possibility of causing mechanical damage to microorganisms, increased agitation would also result in higher rates of energy consumption, which subsequently increases the production cost [26]. Mixing during anaerobic fermentation processes can be carried out at much lower tip speeds because the issue of aeration efficiency can be ignored. Thus, the major role of mixing in anaerobic fermentation is to provide sufficient substrate and heat transfer throughout the culture broth [24].

During fermentation, the accumulation of cells or biosynthesized products leads to the continuous modification of the rheological characteristics of the medium. This produces heterogeneous regions in the fermenter. Therefore, the optimum hydrodynamic condition for the bioreactor must be established [13]. Agitation is a critical factor in larger fermenters and must be taken into consideration in order to maintain culture homogeneity, mass and heat transfer [25]. The results of the present study showed that an impeller tip speed of 0.56 m/s, which corresponded to an agitation speed of 200 rev/min, resulted in the best performance in the biomass production of *B. pseudocatenulatum* G4.

Effect of pH-Controlled Fermentation

The performance and the kinetic parameter values at different pHs were compared and the results are summarized in Table 3. Batch fermentation was also carried out without pH control for comparison. The results showed that the growth of this strain was greatly influenced by pH. A low biomass count and decreases in milk lactose consumption and organic acid production were observed when the pH was left uncontrolled during fermentation. The growth performance was dramatically improved when the culture pH was held con-

Table 3. Kinetic parameter values from batch fermentation of *B. pseudocatenulatum* G4 in a 2-L stirred tank fermenter when the pH was controlled at various values

Kinetic parameter values	Culture pH			
	No pH control	6.0	6.5	7.0
Biomass				
Maximum biomass, x_{max} (cfu/mL)	6.668×10^{8a}	1.698×10^{9b}	$1.687 \times 10^{9\ b}$	3.443×10^{8a}
Time taken to reach maximum biomass, t (h)	18	20	18	24
Maximum specific growth rate, μ_{max} (h^{-1})	0.311 ± 0.014^a	0.486 ± 0.004^b	0.504 ± 0.007^b	0.375 ± 0.014^c
Biomass productivity, P_x ($\times 10^7$ cfu/mL·h)	3.590 ± 0.293^a	8.450 ± 0.099^b	9.240 ± 0.085^b	1.377 ± 0.250^c
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)	9.146 ± 0.607^a	9.589 ± 0.255^a	9.791 ± 0.086^a	3.865 ± 0.692^b
Organic acids				
Maximum acetic acid concentration, A_{max} (g/L)	2.851 ± 0.233^a	7.359 ± 0.072^b	7.353 ± 0.182^b	1.892 ± 0.093^c
Acetic acid productivity, P_A (g/L·h)	0.119 ± 0.010^a	0.307 ± 0.003^b	0.409 ± 0.004^c	0.079 ± 0.004^d
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.346 ± 0.022^a	0.508 ± 0.005^b	0.499 ± 0.017^b	0.220 ± 0.011^c
Maximum lactic acid concentration, L_{max} (g/L)	3.850 ± 0.207^a	6.481 ± 0.267^b	6.515 ± 0.047^b	2.350 ± 0.098^c
Lactic acid productivity, P_L (g/L·h)	0.160 ± 0.009^a	0.270 ± 0.011^b	0.362 ± 0.003^c	0.098 ± 0.004^d
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.467 ± 0.042^a	0.447 ± 0.018^a	0.443 ± 0.022^a	0.273 ± 0.011^b
Acetic:lactic ratio	0.741	1.135	1.129	0.805

Results were reported as mean ± S.D. of two experiments.
[a,b,c]Mean values in the same row with different letters differ significantly ($P < 0.05$).
Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

stant at 6.0 and 6.5. A maximum biomass of 1.698×10^9 cfu/mL and maximum specific growth rate of 0.486 h^{-1} were achieved when the pH was maintained at 6.0, whereas a pH of 6.5 contributed to a maximum biomass of 1.687×10^9 cfu/mL and a maximum specific growth rate of 0.504 h^{-1}. Even though the maximum biomass obtained was equally high at both pH 6.0 and 6.5, maximum biomass was observed at 18 h at a pH of 6.5. Thus, it is recommended that the pH be controlled at 6.5 during fermentation in order to cultivate this strain. On the other hand, controlling the pH at 7.0 during the fermentation process resulted in a long lag phase of 14 h, which led to low maximum biomass (3.443×10^8 cfu/mL), maximum specific growth rate (0.375 h^{-1}), biomass productivity (1.377×10^7 cfu/mL·h) and biomass yield (3.865×10^{10} cfu/g lactose).

Fig. 2 shows the typical time course of *B. pseudocatenulatum* G4 fermentation when the pH is controlled at various levels. When fermentation was carried out without pH control and at pH 7.0, 6.290 and 6.018 g/L lactose remained unconsumed after 24 h, respectively. On the other hand, when fermentation was carried out at a constant pH of 6.5, lactose was efficiently utilized after 18 h, and the biomass count and productivity rates were high. Microbial growth moved into the stationary phase as lactose was depleted at 18 h. Similarly, at pH 6.0, the maximum biomass was obtained at 2 h after lactose was completely consumed (22 h). The lactose utilization pattern can directly reflect the activity of lactase, the enzyme that hydrolyzes lactose from milk. The lactase activity of *B. bifidum* was optimal at a pH of 6.5. The enzyme activity was reduced by approximately 30% at pH 5.0 and was negligible at alkaline pH (8.0) [27]. Hsu *et al.* [28] also discovered that the enzyme activity was highest at an initial pH of 6.5. Therefore, in this study, it was believed that lactose was completely consumed at pH 6.5 due to the optimal enzyme activity observed at this pH.

The production of acetic and lactic acids corresponded to the growth of the organism. Both acids were only detected after the microorganism began to multiply during the exponential growth phase. The amount of acids produced varied according to the fermentation condition. Higher acidification activity was observed with higher cell growth rates. For instance, at pH 6.5 when growth was most desirable, *B. pseudocatenulatum* G4 produced up to 7.353 g/L of acetic acid and 6.515 g/L of lactic acid after 18 h of fermentation. Whereas at pH 6.0, the strain produced up to 7.359 g/L of acetic acid and 6.481 g/L of lactic acid after 24 h of batch cultivation. On the other hand, only 1.892 g/L of acetic acid and 2.350 g/L of lactic acid were produced when the maximum specific growth rate was low at pH 7.0. Meanwhile, formic acid, another metabolic end product of bifidobacteria, was only detected in the culture broth when the pH was controlled at 6.5 (101.10~286.34 g/L) and 6.0 (111.00 g/L). No formic acid was detected at pH 7.0 or without pH control (results not shown).

It is well known that bifidobacteria produce high amounts of acetic and lactic acid during fermentation, generally in the theoretical molar ratio of 1.5, causing the culture pH to drop drastically. It is interesting to note that the concentration of acetic acid exceeded that of lactic acid when growth was favorable at pH 6.0 and 6.5, with acetic acid:lactic ratios of 1.135 and 1.129, respectively (Table 3). The concentration of acetic acid was lower than that of lactic acid when the growth rate was low at pH 7.0 (ratio 0.805) and when no pH control was applied (ratio 0.741). Some researchers have

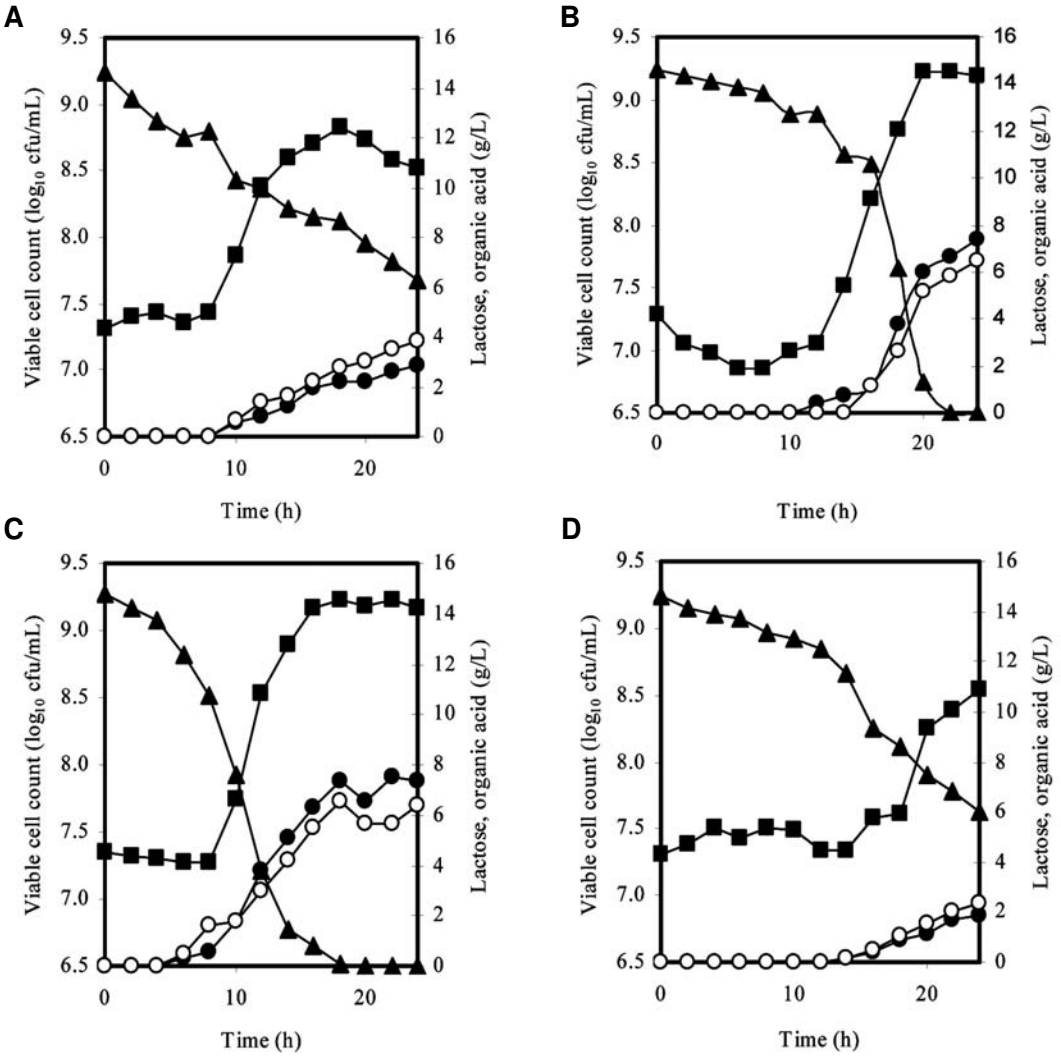

Fig. 2. Batch fermentation of *B. pseudocatenulatum* C4 in a 2-L stirred tank fermenter when the pH was controlled at various values. (A) No pH control, (B) pH 6.0, (C) pH 6.5, (D) pH 7.0. (■) Viable cell count; (▲) lactose concentration; (●) acetic acid concentration; (○) lactic acid concentration.

also reported deviations of the molar ratio from the theoretical value during bifidobacteria fermentation. For instance, Ostlie *et al*. [23] reported that *B. animalis* BB12 produced 6.901 g/kg acetic and 6.949 g/kg lactic acid after 48 h of fermentation in UHT milk supplemented with 0.5% tryptone, which corresponded to a ratio of 0.99. Samona *et al.* [29] also found that more lactic acid (45~290 mmol/L) than acetic acid (1~1.4 mmol/L) was produced during the metabolism of *B. adolescentis* in milk. Meanwhile, *B. longum* produced acetic:lactic ratios of 1.0 in milk [29], 0.93 in MRS-whey permeate media [30], 1.0 in MRS broth [11], and a ratio of 0.5 in soymilk fermentation [31]. These results show that the molar ratio of acetic and lactic acids produced by bifidobacteria varied with different strains, media and fermentation times [20].

An important factor that limits the growth bifidobacteria during batch cultivation of in milk is the depletion of essential nutrients in the milk medium [32]. Fermentation is also greatly affected by the low pH of the medium due to organic acid production from lactose metabolism. According to Her *et al*. [11], lactic acid and acetic acid are inhibitory metabolites that can slow the growth of bifidobacteria. Thus, fermentation is not limited by the amount of lactose available, but rather by the production of organic acids and the subsequent lowering of the pH. The low pH inhibits the growth of the organism even before lactose is depleted. The pH of the medium, which is a measure of the concentration of H^+ ions present, is a very important but often neglected environmental factor [25]. Narvhus *et al.* [22] suggested that fermentation stops as a consequence of pH rather than the amount of organic acid produced. Therefore, many studies have used an external buffering system, such as a vegetarian growth medium for the cultivation of bifidobacteria, during fermentation processes [33]. Effective media buffering is

Table 4. Batch cultivation of *B. pseudocatenulatum* G4 in different fermentation scales

Kinetics parameter values	Fermentation scale	
	2-L fermenter	10-L fermenter
Biomass		
Maximum biomass, x_{max} (cfu/mL)	1.687×10^{9a}	1.396×10^{9a}
Time taken to reach maximum biomass, t (h)	18	18
Maximum specific growth rate, μ_{max} (h^{-1})	0.504 ± 0.007^{b}	0.480 ± 0.017^{b}
Biomass productivity, P_x ($\times 10^7$ cfu/mL·h)	9.240 ± 0.085^{a}	7.704 ± 1.638^{a}
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)	9.791 ± 0.086^{a}	9.456 ± 1.387^{a}
Organic acids		
Maximum acetic acid concentration, A_{max} (g/L)	7.353 ± 0.182^{b}	7.412 ± 0.114^{b}
Acetic acid productivity, P_A (g/L·h)	0.409 ± 0.004^{b}	0.412 ± 0.006^{b}
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.499 ± 0.017^{b}	0.509 ± 0.026^{b}
Maximum lactic acid concentration, L_{max} (g/L)	6.515 ± 0.047^{b}	6.574 ± 0.248^{b}
Lactic acid productivity, P_L (g/L·h)	0.362 ± 0.003^{b}	0.365 ± 0.014^{b}
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.443 ± 0.022^{b}	0.451 ± 0.013^{b}

Results were reported as mean ± S.D. of two experiments.
[a,b,c]Mean values in the same row with different letters differ significantly ($P < 0.05$).
Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

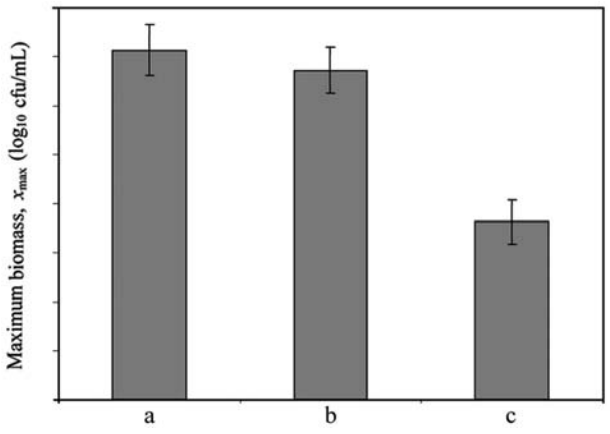

Fig. 3. Maximum biomass of *B. pseudocatenulatum* G4 achieved at different fermentation scales in milk-based medium. Growth in TPY broth was used as a control. a, Milk-based medium in 2-L stirred tank fermenter; b, milk-based medium in 10-L stirred tank fermenter; c, TPY broth in 10-L stirred tank fermenter.

desirable in order to prevent sublethal injury caused by exposure to the low pH during incubation. During free-cell fermentation of *L. lactis*, the population reached 8.1×10^9 cfu/mL under pH control, which was much higher than the populations obtained without pH control (1.9×10^9 cfu/mL). These results confirm the advantage of pH control during cell mass production [34].

10-L Stirred Tank Fermenter

In order to ensure that the fermentation conditions used with both the 2- and 10-L fermenters were as similar as possible, the impeller tip speed parameter was used as a criterion for process scale-up while all other fermentative conditions were equal. The impeller tip speed was maintained at 0.56 m/s, which corresponded to agitation speeds of 200 and 143 rev/min in the 2- and 10-L fermenters, respectively. The culture pH was held constant at 6.5 throughout the experiment.

The kinetic parameter values of different fermentation scales are shown in Table 4. The results showed that by keeping the impeller tip speed constant, the maximum biomass, maximum specific growth rate, biomass productivity and production of organic acids were statistically the same in the 2- and 10-L fermenters. Therefore, constant impeller tip speed was applicable to the scale-up of this organism. The milk-based medium and process conditions used to cultivate this strain yielded higher biomasses than those obtained using TPY commercial media (Fig. 3).

For successful scale-up to larger reactors, the process conditions are kept as close to the initial fermentation conditions as possible. Although satisfactory growth performance may be achieved in a small-scale flask, reproducibility in larger reactors becomes a real challenge. In this case, mixing and shear stress were considered to be potential factors affecting the biomass production when scaled-up to larger scales. Non-homogeneous mixing could be one of the main reasons for the difficulty in scale-up to larger vessels. When the scale is increased, the culture broth may become more heterogeneous, with unequal distribution of nutrients and cells in some areas of the reactor [35]. On the other hand, the maximum shear experienced in a fermenter is at the tip of the impellers. If the impeller speed is too fast, it can physically damage the cells; however, if the speed is too low, the culture broth may not be well mixed [16]. Therefore, it is neces-

sary to use a reasonable impeller tip speed for the fermentation process. Hence, scale-up based on a constant impeller tip speed was able to yield reproducible results in different scales of *B. pseudocatenulatum* fermentation.

CONCLUSION

When developing a fermentation process for biomass production, it is critical to ensure that the growth kinetics obtained are reproducible at different scales. In this work, the feasibility of scale-up based on a constant impeller tip speed was demonstrated. The best condition for the growth of this strain in a milk-based medium was obtained at an impeller tip speed of 0.56 m/s when the pH was controlled at 6.5. A tailor-made process condition is necessary to improve the process stability for large-scale fermentation processes.

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NOMENCLATURE

D	Impeller diameter (m)
N	Agitation speed (rev/min)
t	Time taken to reach maximum biomass (h)
A_{max}	Maximum acetic acid concentration (g/L)
L_{max}	Maximum lactic acid concentration (g/L)
P_A	Acetic acid productivity (g/L·h)
P_L	Lactic acid productivity (g/L·h)
P_x	Biomass productivity ($\times 10^7$ cfu/mL·h)
x_{max}	Maximum biomass (cfu/mL)
$Y_{A/s}$	Acetic acid yield (g acetic/g lactose)
$Y_{L/s}$	Lactic acid yield (g lactic/g lactose)
$Y_{x/s}$	Biomass yield ($\times 10^{10}$ cfu/g lactose)
μ_{max}	Maximum specific growth rate (h^{-1})
π	3.142

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