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Strategies in fed-batch cultivation on the production performance of *Lactobacillus salivarius* I 24 viable cells

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Abstract The potential use of fed-batch cultivation (FBC) for improvement of the production of *Lactobacillus salivarius* I 24 biomass for subsequent use as probiotics was studied using a 2-L stirred-tank bioreactor. Three different constant feeding rates (0.1, 0.05, and 0.033 L/h) were applied in FBCs and their effect on carbon metabolism was evaluated. The carbon flux for cell built-up with reduction in lactic acid synthesis was observed in the fed-batch as compared to the batch cultivation mode. The viable cell number obtained in the constant FBC (CFBC) operated at a feeding rate of 0.05 L/h was 8 times higher (10.7×10^{10} CFU/mL) than that recorded in the batch cultivation. This gave the viable cell yield based on glucose consumed for CFBC of 26 times higher (11.3×10^{12} CFU/$g_{Glucose}$) than the batch cultivation. This study demonstrated CFBC, which is simple with minimal use of process control equipment, has an industrial potential for improvement of probiotic production.

Keywords: fed-batch cultivation, *Lactobacillus salivarius*, lactic acid bacteria, probiotics, carbon metabolism

Introduction

Probiotics are live microorganisms that provide beneficial health effects to humans and animals when consumed in adequate amounts. In recent years, the incorporation of probiotics into various food products is becoming increasingly popular (1). This is influenced by the increase of consumer awareness on the active role of food in the well-being and prolongation of life. The most commonly used probiotic species belong to the genus of *Lactobacillus* and *Bifidobacterium* (2). *L. salivarius* is among the promising probiotic candidate that is well-characterized as a probiotic microorganism as well as bacteriocin producer, and this bacterium has been classified as "generally recognized as safe (GRAS)" (3,4).

The three basic operating modes widely employed for microbial cell biomass production are continuous, batch, or fed-batch. Although traditional batch fermentation seems to dominate the lactic acid bacterial fermentation industry today (5), much interest in developing more efficient methods of producing biomass or lactic acid exist. Meanwhile, in comparison to fed-batch, continuous fermentation is typically considered as the least productive mode as an exhaustion of the supply feed can only be noticed at rather low feed rates (6).

Fed-batch cultivation (FBC) wherein substrates and nutrients are fed intermittently or continuously into the fermenter is the commonly exploited mode of operation for achieving high cell densities and product concentration. In fed-batch fermentation, the bacterial growth rate can be controlled by growth-limiting feeding strategies (7,8). The utilization of FBC of microorganisms has been studied quite extensively, for instance, in the cultivation of both conventional and recombinant *Escherichia coli* for production of various proteins (9-11). Nevertheless, fewer reports of FBC of lactic acid bacteria in the literature as compared to those of batch and continuous cultivations exist (5). There are some processes where fed-batch operation exhibits an advantage over batch and continuous processes such as substrate inhibition, catabolite repression, amino acid production, extension of operating time, replacement of water loss by evaporation, and high cell density (12-15). Redirecting the carbon flux more toward cell built-up rather than for the synthesis of a byproduct such as lactic acid is the main target to achieve high cell yield in mass cultivation of lactic acid bacteria (16). In the cultivation of

auxotrophic mutant microorganism, the excess of nutrient supply may result in higher cell growth but low accumulation of the targeted product owing to either product repression or inhibition. To overcome this problem, nutrient concentration is kept at appropriate levels by controlling its feeding rate. This strategy is frequently used in industrial scale production of amino acid employing auxotrophic mutant strains (17).

The most common performance measurement of FBC is the fermenter's productivity and final product concentration. Utilization of fed-batch fermentation as compared to that of batch fermentation usually implies an increase in final product concentration and productivity, and a decrease in manufacturing cost (12) mainly because of the use of smaller fermenter size. In addition, in contrast to continuous operation, fed-batch cultures present less risk of contamination, and when used under quasi-steady state conditions, fed-batch cultures can closely approximate continuous culture mode without the risk of a washout (18).

The focus of this work was to assess the potential of using fed-batch fermentation system for the biomass production of *L. salivarius* I 24. The feasibility of using the CFBC mode, aimed at improving the biomass production by redirecting the carbon flux toward cell built-up instead of lactic acid production was investigated.

Materials and Methods

Microorganism and inoculum preparation *L. salivarius* I 24, which was isolated from the chicken intestine (19), was used throughout this study. The bacterial cells were grown in De-Man, Rogosa, and Sharpe (MRS) broth, harvested under aseptic conditions by centrifugation for 5 min at 12,857× *g*, and were washed twice using 0.02 M, pH 7.2 phosphates buffer. The cell pellet was then resuspended in the same volume of 20% (w/v) sterilized skim milk. The suspension was transferred into a sterile universal bottle and chilled at 4°C for 2 h. Subsequently, it was frozen for at least 24 h at 80°C and freeze-dried for 24 h in a freeze-dryer (Epsilon 1-8D; Martin Christ, Osterode am Harz, Germany). The bacterium was sub-cultured in the MRS medium for subsequent use throughout the experiments. For inoculum preparation, the stock culture was sub-cultured onto MRS agar plates and incubated for 48 h at 37°C. Single colonies picked from countable plates were inoculated into Schott bottles containing a desirable volume of the MRS broth. The culture bottles were incubated at 37°C for 16-18 h to obtain an initial cell concentration of approximately 10^7 CFU/mL.

Batch cultivation Batch cultivation was performed facultatively in a 2-L stirred-tank fermenter (BIOSTAT, B. Braun Biotech International, Melsungen, Germany) containing 1.5 L of fermentation medium. The optimized medium for growth of *L. salivarius* I 24 used in this study consisted of 33.24 g/L glucose, 43.1 g/L yeast extract, 2 g/L K_2HPO_4, 0.02 g/L $MgSO_4$, and 1 mL/L Tween 80. The pH of the culture was maintained at 6.10 using 5 M NaOH. The temperature was set at 37°C, and the culture was agitated at impeller tip speed (6-bladed turbine impeller) of 0.42 m/s. The fermenter was inoculated with 10% (v/v) inoculum.

CFBC CFBC was performed in a 2-L stirred-tank bioreactor in two phases. In the first phase, *L. salivarius* I 24 was grown in an initial batch mode with a 0.8 L working volume of a medium with composition as described for batch cultivation. Upon glucose exhaustion, which corresponded to approximately 8 h of the initial batch fermentation, the second phase was initiated. In this phase, the fed-batch mode was operated by continuously adding 0.5 L of concentrated feeding medium (medium FM A and FM B, which were 2.5- and 5-times concentrated, respectively, than the medium used in batch fermentation) into the fermenter at three different constant feeding rates (0.1, 0.05, and 0.033 L/h) to a total volume of 1.3 L.

Analytical procedures Serial decimal dilutions of each sample (10^5-10^9) were prepared and plated onto MRS agar plates and incubated at 37°C for 48 h. The number of viable cells was calculated from the total number of colonies counted on a plate multiplied by the dilution involved and was expressed as colony forming units per mL (CFU/mL) and $\log_{10}$ CFU.

A sample with a known volume was filtered through a pre-weighed 0.2 μm cellulose nitrate membrane filter (Whatman, Maidstone, UK) and washed twice with 0.5% NaCl. The filter with cells retained was then dried in an oven at 95°C until a constant weight was achieved. The dry cell weight (DCW) was calculated as follows:

$$DCW\ (g/L)=(W_f-W_i)/V$$

where;

W_f=total weight of filter paper and cell

W_i=weight of filter paper

V=volume of the liquid medium

Lactic acid was determined using HPLC (Waters Corporation, Milford, MA, USA) with a 996 photodiode array detector and a refractive index detector. The separation of organic acids was achieved by using a Waters Shodex KC-811 packed column (8×300 mm) as the stationary phase. The mobile phase was 3 mM H_2SO_4. The flowrate of the mobile phase and column temperature was controlled at 1 mL/min and 60°C, respectively. The samples were prepared by filtering the fermentation broth with Whatman nylon filter paper (0.2 μm pore size, 13 mm diameter).

Glucose was determined using HPLC with a refractive index detector. Sample injection was performed using a sample loop valve equipped with a 25 mL loop. The stationary phase was a pre-packed Merck NH_2 column (Waters Corporation). The mobile phase was an isocratic mixture of acetonitrile and water (80:20, v/v). The flowrate of the mobile phase was 1 mL/min and was kept at 30°C. The

samples were prepared using the same procedure described above for the organic acid determination.

Biomass productivity (Q_b) and lactic acid productivity (Q_p) were calculated using the following equations:

$$Q_b=V*X/t$$
$$Q_p=V*LA/t$$

where,

*V*X*=total dry cell weight (g) or total viable cell count (CFU)

*V*LA*=total lactic acid produced (g)

t= total fermentation time (h)

The biomass ($Y_{x/s}$) and lactic acid ($Y_{p/s}$) yield were calculated using the respective equations:

$$Y_{x/s}=V*X/S$$
$$Y_{p/s}=V*LA/S$$

where;

V*X=total dry cell weight (g) or total viable cell count (CFU)

V*LA=total lactic acid produced (g)

S=total glucose consumed (g)

Statistical analysis All experiments were performed in triplicate (*n*=3) and data were expressed as means±standard error of means. Analyses of variances for data were performed using SAS (Version 9.0, SAS Inc., Cary, NC, USA). Duncan multiple range test was used to determine significance among treatment means. Significance was declared at $p<0.05$.

Results and Discussion

Influence of feed rate on CFBC The maximum cell densities of both FM A and FM B media were increased with increasing feeding rate from 0.033-0.05 L/h but decreased at 0.1 L/h (Table 1). Nevertheless, the maximum cell densities obtained using FM B were much higher than those of FM A in all the feeding rates tested. The highest cell density (10.7×10^{11} CFU/mL) in cultivation with FM B medium, which corresponded to dry cell weight of 7.11 g/L, was obtained at a feeding rate of 0.05 L/h. From Fig. 1D, it can be seen that the residual glucose concentration was kept at a very low level (<0.10 g/L) during the CFBC with the feeding rate of 0.05 L/h. This observation suggested that a quasi-steady state was achieved between the glucose consumption rate and the propagation of cells because the rate of cell growth (dX/dt)=rate of substrate consumption (dS/dt)=0 for 8 h. This phenomenon occurred when the substrate was instantaneously consumed by the microorganism as it was fed into the culture (20). As soon as the feeding stopped, the cells seemed to function as resting cells. However, the culture continued to produce lactic acid until a declining phase was achieved at around 22 h of incubation.

The constant addition of nutrients at a feeding rate of 0.033 L/h resulted in lower cell density attained at the end of cultivation, i.e., 5.2 and 6.86 g/L for FM A and FM B, respectively (Table 1), even though the residual glucose profiles were similar (Fig. 1A and 1B). This might be because of the lower growth rate where the growth was restricted by the slow addition of nutrient or the growth was severely inhibited by lactic acid accumulated in the culture (8). This assumption was supported by the cultivation data, which showed that the cell growth reached a stationary phase even though fresh medium was still fed into the culture. Reduction in the cell density was observed when nutrient feeding was stopped.

The lowest cell density was observed in CFBC with the fastest feeding rate (0.1 L/h) (Table 1). The reduced growth might be because of the quick addition of substrate that caused the dilution of cells in the bioreactor. Glucose was accumulated immediately when the fed-batch phase started (Fig. 1F), indicating that the glucose uptake capacity by *L. salivarius* I 24 was reduced when the glucose inflow exceeded the propagation activity, which in turn, inhibited the growth.

Effect of substrate concentration in the feed on CFBC Preliminary studies had shown that *L. salivarius* I 24 failed to grow in a batch culture using FM A medium owing to substrate inhibition. High osmotic pressure created by the presence of the high concentration of glucose in the culture reduced the cell viability (21). Likewise, a few studies have reported on the inhibition of microbial growth and

Table 1. Effects of feeding rates using FM A and FM B on cultivation performance of *L. salivarius* I 24 in CFBC

	FM A			FM B		
Kinetic parameter	0.033 L/h	0.05 L/h	0.1 L/h	0.033 L/h	0.05 L/h	0.1 L/h
Final viable cell concentration ($\times10^{11}$CFU/mL)	1.70±0.20^{b}	1.93±0.20^{a}	1.65±0.03^{b}	9.89±1.00^{d}	10.70±0.95^{d}	7.98±0.50^{e}
Final biomass concentration (g/L)	5.20±0.05^{b}	5.46±0.02^{a}	5.06±0.05^{c}	6.86±0.25^{e}	7.11±0.05^{d}	6.20±0.20^{f}
Final lactic acid concentration (g/L)	39.78±0.20^{b}	37.70±0.50^{c}	40.39± 0.10^{a}	65.26±0.20^{e}	58.18±0.50^{f}	65.78±0.20^{d}
Viable cell yield ($\times10^{12}$ CFU/$g_{Glucose}$)	2.69±0.05^{b}	2.90±0.10^{a}	2.53±0.03^{c}	9.09±1.15^{d}	11.3±1.00^{d}	8.21±1.00^{e}
Biomass yield ($g_{DCW}/g_{Glucose}$)	0.08±0.02^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.07±0.01^{d}	0.07±0.01^{d}	0.06±0.01^{d}
Lactic acid yield ($g_{LA}/g_{Glucose}$)	0.61±0.03^{a}	0.58±0.01^{b}	0.62±0.02^{a}	0.71±0.01^{d}	0.61±0.05^{f}	0.68±0.01^{e}
Viable cell productivity ($\times10^{9}$ CFU/mL·h)	9.04±0.50^{c}	10.70±0.50^{b}	12.70±0.50^{a}	46.10±4.00^{d}	59.40±3.50^{d}	39.90±1.50^{e}
Biomass productivity (g_{DCW}/L·h)	0.25±0.04^{c}	0.30±0.02^{b}	0.39±0.05^{a}	0.31±0.01^{f}	0.39±0.10^{d}	0.31±0.01^{e}
Lactic acid productivity (g/L·h)	1.73±0.05^{c}	2.09±0.10^{b}	3.11±0.10^{a}	3.83±0.05^{d}	3.23±0.05^{e}	2.99±0.05^{f}

[1)a-f]Mean values in the same row with different superscripts are significantly different from each other ($p<0.05$).

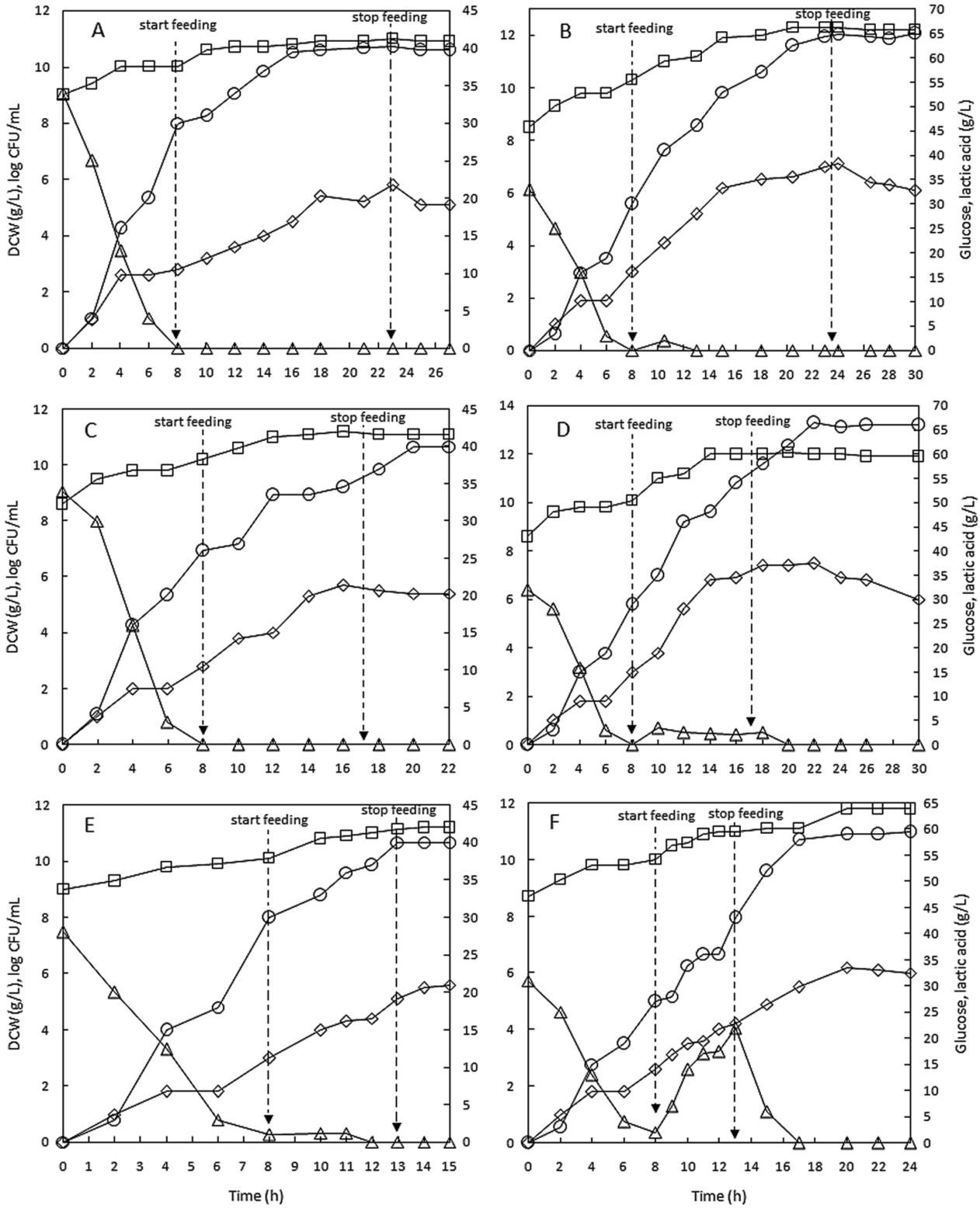

Fig. 1. The time course of CFBC at 0.03 L/h using (A) FM A and (B) FM B; 0.5 L/h using (C) FM A and (D) FM B; and 0.1 L/h using (E) FM A and (F) FM B. Symbols represent (◇) DCW, (□) CFU/mL, (○) lactic acid, and (△) glucose.

product yield because of high concentrations of the substrate in the culture during batch fermentation (22,23). However, when operated as a fed-batch culture, improvements in cell viability and dry cell weight were observed even when FM B, which was 2-times concentrated than FM A, was used (Table 1).

The fed-batch fermentation using FM B also showed better

biomass yield and biomass productivity when compared to FM A at all feeding rate tested in this study. In comparison with FM A, the use of a higher concentration of nutrient by feeding with FM B (2-times concentrated) provided significant improvement in terms of a viable cell count (384, 454, and 482%), biomass yield (225, 290, and 238%), and biomass productivity (214, 455, and 410%) at various feeding rates (0.1, 0.05, and 0.033 L/h), respectively. These results suggested that it was possible to have higher cell viability merely by increasing the concentration of nutrients in the feed medium.

Generally, lactic acid production was much higher when FM B was used (Table 1), which implied that the volumetric lactic acid productivity was dependent on the total biomass presence in the culture. Hence, conditions that provide high cell density also favor high lactic acid accumulation. However, when CFBC operated at a feeding rate of 0.1 L/h, was allowed to proceed as a batch wise after the feeding phase was stopped at the 13th h, the productivity of lactic acid on FM B (2.99 g/L.h) was lower than that on FM A (3.11 g/L.h). This could be explained by the fact that FM B was a more concentrated feed medium, and it would require a longer time for *L. salivarius* I 24 to consume all the residual glucose remaining in the culture to achieve the maximum lactic acid accumulation.

Comparison between fed-batch and batch fermentation on cell biomass production Based on the results obtained from FBC and batch cultivation of *L. salivarius* I 24, various kinetic parameters describing the performance of the two fermentation systems are summarized in Table 2. It is important to note that the biomass yield (0.07 $g_{DCW}/g_{Glucose}$) was similar for CFBC and batch fermentation but lactic acid yield for CFBC (0.61 $g_{LA}/g_{Glucose}$) was significantly lower than that for the batch fermentation (0.98 $g_{LA}/g_{Glucose}$). A previous study reported that although higher final cell concentration (9.45 g/L) was obtained in the fed-batch fermentation of *L. plantarum* with a total glucose supplied of 300 g/L as compared to the batch with 10 g/L glucose in the medium, the biomass yield obtained in the fed-batch (0.032 g/g) was much lower than that obtained in the batch (0.22 g/g) fermentation (24). In the meantime, the viable cell yield (11.3×10^{12} CFU/$g_{Glucose}$) for CFBC of *L. salivarius* I 24 was 26 times higher than that obtained in the batch fermentation (0.44×10^{12} CFU/$g_{Glucose}$). This data implies that the viability of *L. salivarius* I 24 cells was significantly improved in CFBC, where the glucose in the culture was maintained at a low level throughout the fermentation. Generally, the carbon flux in lactobacilli fermentation tends to switch to lactic acid production (20,25). In this study, when the cultivation was operated in CFBC, it was found that the carbon flux was enhanced to cell built-up with a reduction in lactic acid production. This result agrees with the finding of Liew (26) who reported on fed-batch fermentation that enhanced the *L. rhamnosus* biomass production and reduced lactic acid accumulation in the culture. A significant improvement in *L. plantarum* LP02 biomass production in fed-batch fermentation with controlled glucose feeding rate as compared to that in batch fermentation has also been reported (24).

Table 2. Comparison of batch and CFBC for biomass and lactic acid production by *L. salivarius* I 24

Kinetic parameter	Batch	CFBC[1)]
Final viable cell concentration (×10^{10} CFU/mL)	1.31±0.02[a2)]	10.70±0.95[b]
Final biomass concentration (g/L)	2.35±0.05[a]	7.114±0.05[b]
Final lactic acid concentration (g/L)	29.50±0.50[a]	58.18±0.50[b]
Viable cell yield (×10^{12} CFU/$g_{Glucose}$)	0.44±0.01[a]	11.30±1.00[b]
Biomass yield ($g_{DCW}/g_{Glucose}$)	0.07±0.01[a]	0.07±0.01[a]
Lactic acid yield ($g_{LA}/g_{Glucose}$)	0.98±0.01[a]	0.61±0.05[b]
Viable cell productivity (×10^{9} CFU/mL.h)	1.76±0.50[a]	59.40±3.50[b]
Biomass productivity (g_{DCW}/L.h)	0.28±0.02[a]	0.39±0.10[b]
Lactic acid productivity (g/L.h)	3.94±0.10[a]	3.23±0.05[b]

[1)]CFBC at 0.05 L/h using FMB

[2)a-b]Mean values in the same row with different superscripts are significantly different from each other (p<0.05).

Nonetheless, the authors did not monitor the accumulation of lactic acid during the fermentation in both modes.

The result from this study suggests that the FBC of *L. salivarius* I 24 may have favorable potential in the production of biomass since high final biomass concentration with low lactic acid accumulation could be obtained. In fed-batch fermentation, final biomass concentration and biomass yield could be further improved through the application of more complicated feeding strategies. For example, appropriate sensors in connection with the feeding pump may be used to accurately control the glucose level in the culture for improvement of carbon flux for cell build-up. A very high viable cell count, which was four times higher as compared to that in batch cultivation, was achieved in the fed-batch fermentation of *Bacillus sphaericus* with an accurate control of specific growth rate (27). The use of cyclic or repeated fed-batch mode to reduce the fermentation time and increase biomass productivity shall also be explored. For example, the biomass productivity for repeated FBC of *B. sphaericus* using glycerol as carbon source was increased by about six times compared to that obtained in the conventional batch cultivation (27).

For the efficient cultivation of lactic acid bacteria aimed at producing high cell biomass, low lactic acid accumulation in the culture is desirable to reduce the inhibitory effect to cell growth. The inhibition of growth of lactic acid bacteria with the presence of undissociated lactic acid in the culture is well-known. The trapping of the undissociated lactic acid as lactate salt during the fermentation has been used as an approach to partially overcome the inhibition effect of lactic acid, improving the fermentation efficiency (28). In addition, the acidic culture also needs to be neutralized with alkali to set the favorable pH for the growth of lactic acid bacteria. Reduced lactic acid accumulation in the culture would require less alkali for neutralization; thus, production cost in large scale process could also be further reduced.

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