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ORIGINAL PAPER

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A repeated batch process for cultivation of *Bifidobacterium longum*

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Abstract A repeated batch process was performed to culture *Bifidobacterium longum* CCRC 14634. An on-line device, oxidation-reduction potential (ORP), was used to monitor cell growth and uptake of nutrients in the culture. The ORP of the culture medium decreased substantially during fermentation until nutrients were depleted. Six cycles of batch fermentation using ORP as a control parameter were successfully carried out. As soon as ORP remained constant or increased, three-quarters of the broth was removed, and the same volume of fresh medium was fed to the fermenter for a new cycle of cultivation. Average cell concentrations of 1.9×10^9 and 3.4×10^9 cfu ml^{-1} for repeated batch fermentation in MRS (Lactobacilli MRS broth) and WY (containing whey hydrolyzates, yeast extract, L-cysteine) medium, respectively, were achieved. Cell mass productivities for batch, fed-batch and repeated batch fermentation using MRS medium were 0.51, 0.41, and 0.64 g l^{-1} h^{-1}, respectively, and those for batch and repeated batch using WY medium were 0.76, 0.99 g l^{-1} h^{-1}, respectively. The results indicate a possible industrial process to culture *Bifidobacteria* sp.

Keywords *Bifidobacterium longum* · Oxidation-reduction potential · Repeated batch fermentation

Introduction

Bifidobacteria are Gram-positive, non-motile bacteria that grow anaerobically. They are natural inhabitants of the gut of man and warm-blooded animals [7]. They play a significant role in controlling the pH in the large intestine and colon through the liberation of lactic and acetic acids, which in turn restrict the growth of many potential pathogens and putrefactive bacteria [11]. Bifidobacteria are also believed to have anticarcinogenic properties [6]. Due to their therapeutic effects, bifidobacteria have been widely used in cultured milk, beverages, cheese products, cookies, health foods [3, 4], and powdered milk [8].

Bifidobacteria metabolize carbohydrates using the fructose-6-phosphate phosphoketolase (F6PPK) pathway, which cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate [13]. The so-called bifid shunt pathway metabolizes carbohydrates to its final products of lactate and acetate at a molar ratio of 2:3. Lactate and acetate are inhibitory metabolites that retard growth of bifidobacteria and lactic acid bacteria in fermentation. Attempts have been made to remove these inhibitory metabolites by dialysis fermentation using microfiltration in the case of *Bifidobacterium longum* [16] or ultrafiltration with *Candida tropicalis* [14] and *Streptococcus salivarius* subsp. *thermophilus* [10] to achieve high cell concentration. However, frequent washing due to cake-like accumulation of bacterial cells in the hollow fiber or in channels of the filtration module is essential for dialysis fermentation using membrane technology. It is not easy to maintain optimum performance of a used membrane compared to a new one. Microfiltration modules are made of an organic polymer that is not heat sterilizable, and ultrafiltration modules cannot produce high filtration efficiency. Taniguchi et al. [16] employed a ceramic filter for continuous separation of inhibitory metabolites. The ceramic filter may be sterilized with steam or washed with acid and alkali. However, the small filtration area is the primary disadvantage of ceramic filters, and bacterial cells often become deposited in channels of the filtration module. Furthermore, dialysis fermentation often removes essential nutrients from the culture medium.

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The oxidation-reduction potential (ORP) is a measure of the activity of electrons involved in oxidation-reduction within an aqueous environment. The exact values of the ORP reflect all factors contributing to electron activity, such as chemical constituents of the system, variety of biological activities, pH, and temperature [9].

In this study, we found that ORP decreases substantially during cultivation of bifidobacteria until nutrient depletion. The ORP ceased to decline or even increased as soon as the carbon source in the culture medium was depleted. If more carbon source was added, however, it again decreased. Based on this behavior, the present investigation employed ORP to monitor nutrient consumption and cell growth during cultivation of *B. longum*. Repeated batch fermentation was performed to simulate continuous production of *B. longum*. Repeated batch processes have been used in systems that produce inhibitory products or metabolites [12, 15].

Materials and methods

Strains, media and culture conditions

Two kinds of media were used in the experiments. Lactobacilli MRS medium (Merck) contained (g/l): peptone 10, yeast extract 5, beef extract 10, dextrose 20, polysorbate-80 1, ammonium citrate 2, sodium acetate 5, magnesium sulfate 0.1, manganese sulfate 0.05, dipotassium phosphate 2, L-cysteine 0.5.

WY medium contained (g/l): whey 50, yeast extract 10, L-cysteine 0.5. Whey in the WY medium was hydrolyzed by protease (0.1 g l^{-1}, Flavourzyme; Novo Nordisk, Denmark) at 45°C for 20 h. The hydrolyzate was clarified by centrifugation (7,000 *g* for 20 min at 4°C). The supernatant was supplemented with yeast extract (50 g l^{-1}) and L-cysteine (0.5 g l^{-1}).

B. longum CCRC 14634 was obtained from the Culture Collection and Research Center, Food Industry and Development, Hsinchu, Taiwan. A loop of inoculum was transferred into 150-ml MRS medium and incubated anaerobically using a GasPak culture system (Becton Dickinson, Sparks, Md.) for 24 h at 37°C. The resultant culture was mixed with glycerol to a final concentration of 50% (v/v) and stored at −70°C.

The fermentation seed culture (150 ml) was incubated anaerobically at 37°C for 20 h. The medium of seed culture (WY or MRS) was the same as that for the batch, fed-batch or repeated batch fermentation. Batch fermentation was performed in a 5-l jar fermenter (CMF-5, Chin-Chi, Taiwan) with a working volume of 1.5 l at 37°C, and agitation speed of 10 rpm. The pH value was controlled at 6.0 using 5 *N* NaOH. Mineral oil was overlaid on the top of medium in the fermenter for sterilization in order to maintain anaerobic conditions during fermentation.

Computer control

The fermentation process was controlled by the software "Genie" (Advantech, Taiwan). Measured signals of pH (InPro 3030/325; Mettler Toledo, Greifensee, Switzerland) and ORP (Mettler Toledo, Pt4805-DPAS-SC-K8S/325) are connected to a PCLD 8115 wiring terminal board (Advantech), connected with a PCL-818 L data acquisition card communicating with a Genie program. The control signal was connected as output to a PCLD-885 power relay board in order to control NaOH and glucose addition. An IBM-compatible personal computer was used for data processing and recording.

The ORP decreased substantially during fermentation until nutrient depletion. In fed-batch fermentation, as soon as ORP ceased to decline, or even increased, glucose was added to a concentration of 1.5 g l^{-1}. In repeated batch fermentation, again, as soon as ORP ceased to decline or even increased, three-quarters of the medium was removed, and the same volume of fresh medium was fed to the fermenter for a new cycle of culture. Both the fed-batch fermentation and repeated batch fermentation started with the same conditions as batch fermentation.

Analytical methods

The carbon sources (glucose or lactose), lactic acid and acetic acid in the culture broth were analyzed by HPLC under the following conditions: column, Supelcogel H (300 ×7.8 mm); mobile phase, 0.1 *N* H_2SO_4; detector, a Waters 410 RI detector; temperature, 30°C [5]. Cell concentration was determined by measuring the optical density at 600 nm (OD_{600}). Dry cell weight was determined by filtering the culture broth through a 0.2 μm membrane filter (Advantec, Pleasanton, Calif.). The cell slurry on the membrane was washed by distilled water, followed by drying at 80°C to constant weight.

Results

Batch fermentation

In a batch cultivation of *B. longum* in MRS medium (pH 6.0), carbon source (glucose) was consumed to a very small concentration (2 g l^{-1}) at 8 h after inoculation (Fig. 1). The maximum cell concentration was 1.3×10^9 cfu ml^{-1} (OD_{600} 8.1 or 4.1 g l^{-1}) and the final concentration of lactic acid was 12.3 g l^{-1}. The concentration of acetic acid was approximately the same as that of lactic acid (data not shown). It was noted that the ORP decreased substantially during the exponential growth phase until a residual glucose level of 2 g l^{-1} remained in the medium, and the cell density decreased, possibly due to self-lysis. If cells were harvested at the maximum cell concentration (8 h after inoculation), the productivity of the cell mass was 0.51 g l^{-1} h^{-1}.

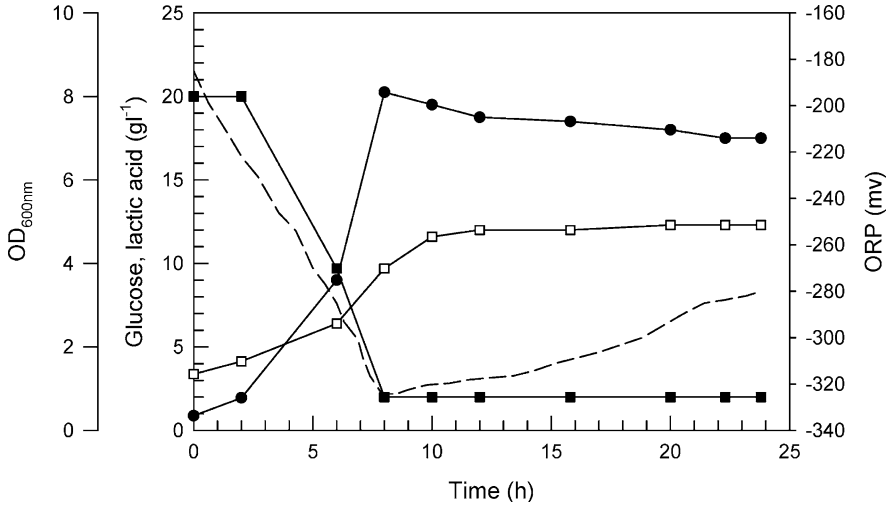

Fig. 1 Time-course batch fermentation of *Bifidobacterium longum* in MRS medium. *Closed circles* OD_{600nm}, *closed squares* glucose, *open squares* lactic acid, *dotted lines* oxidation-reduction potential (ORP). Fermentation was carried out at 37°C and pH 6.0

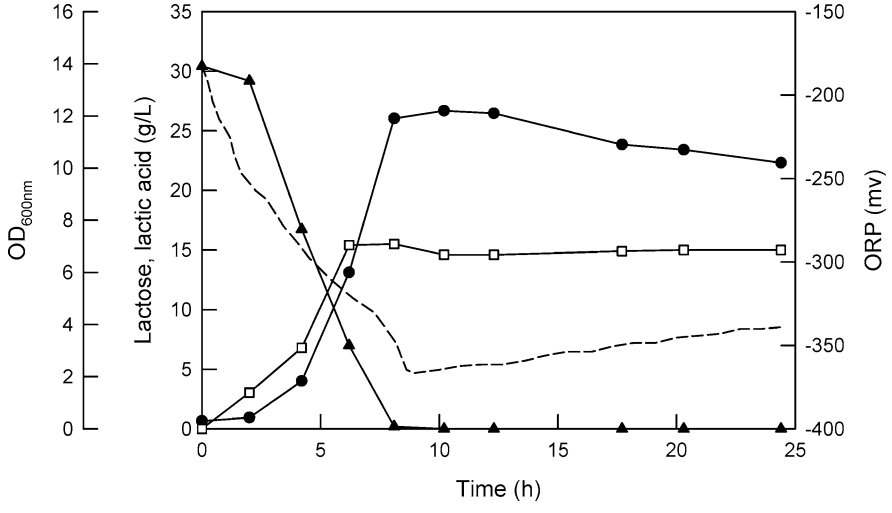

Fig. 2 Time-course batch fermentation of *B. longum* in WY medium. *Closed circles* OD_{600nm}, *closed triangles* lactose, *open squares* lactic acid, *dotted lines* ORP. Fermentation was carried out at 37°C and pH 6.0

Another batch fermentation was performed using WY medium, the carbon source (lactose) being depleted at 8 h as shown in Fig. 2. The maximum cell concentration was 3.5×10^9 cfu ml^{-1} (OD_{600} 12.2 or 6.1 g l^{-1}) and the final concentration of lactic acid was 15.5 g l^{-1}. Again, the ORP decreased substantially in the exponential growth phase until a maximum cell concentration was achieved (Fig. 2). WY medium was more

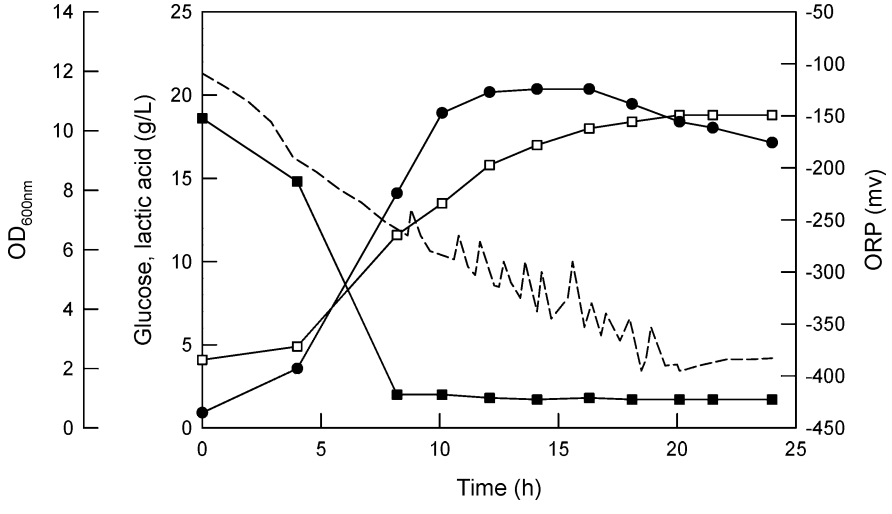

Fig. 3 Time-course fed-batch fermentation of *B. longum* in MRS medium. *Closed circles* OD_{600nm}, *closed squares* glucose, *open squares* lactic acid, *dotted lines* ORP. Fermentation was carried out at 37°C and pH 6.0

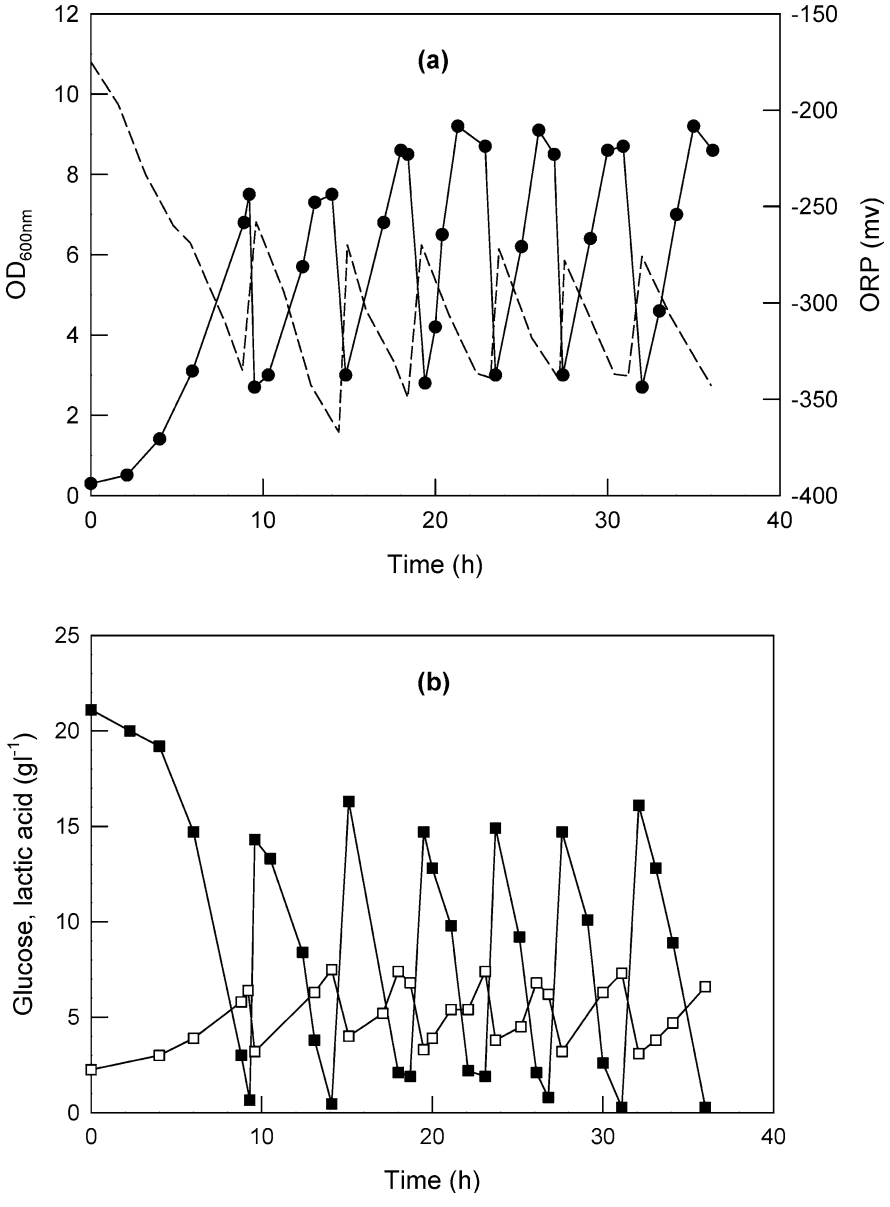

Fig. 4a, b Time-course repeated batch fermentation of *B. longum* in MRS medium. **a** *Closed circles* OD_{600nm}, *dotted lines* ORP; **b** *closed squares* glucose, *open squares* lactic acid. Fermentation was carried out at 37°C and pH 6.0

favorable for cell growth than MRS medium. A higher cell concentration and more metabolites (lactic acid and acetic acid) were achieved in WY medium. If cells were harvested at 8 h of fermentation (end of the exponential phase), the productivity of the cell mass was 0.76 g l^{-1} h^{-1}.

Fed-batch fermentation

Fed-batch fermentation was performed using MRS medium as shown in Fig. 3. Feeding of medium was performed when ORP ceased to decline. Fermentation stopped after 21 h as the total volume of 4 l was reached. A maximum cell concentration of 5.2×10^9 cfu ml^{-1} was achieved after 14 h and the concentration of lactic acid was approximately 17 g l^{-1}. Metabolites completely inhibited cell growth after 14 h. The viable cells, however, consumed nutrients after 14 h, and excreted lactic acid and acetic acid continuously. Taniguchi et al. [16] reported that growth of *B. longum* YIT 4021 was completely inhibited in 17 g l^{-1} lactic acid and acetic acid. If cells were harvested at the maximum cell concentration (14 h after inoculation), the productivity of the cell mass was 0.41 g l^{-1} h^{-1}. The productivity is lower than that of batch fermentation (0.51 g l^{-1} h^{-1}) using MRS medium because of the strong growth inhibition resulted from the high concentration of acetic acid and lactic acid in the later stages (9–21 h) of fermentation.

ORP decreased substantially as the fed-batch fermentation proceeded (Fig. 3). This might be due to accumulation of chemical constituents that contribute electron activities involved in oxidation-reduction within the broth. Pulse increases in the decreasing ORP were due to feeding of glucose (Fig. 3) in the fed-batch fermentation.

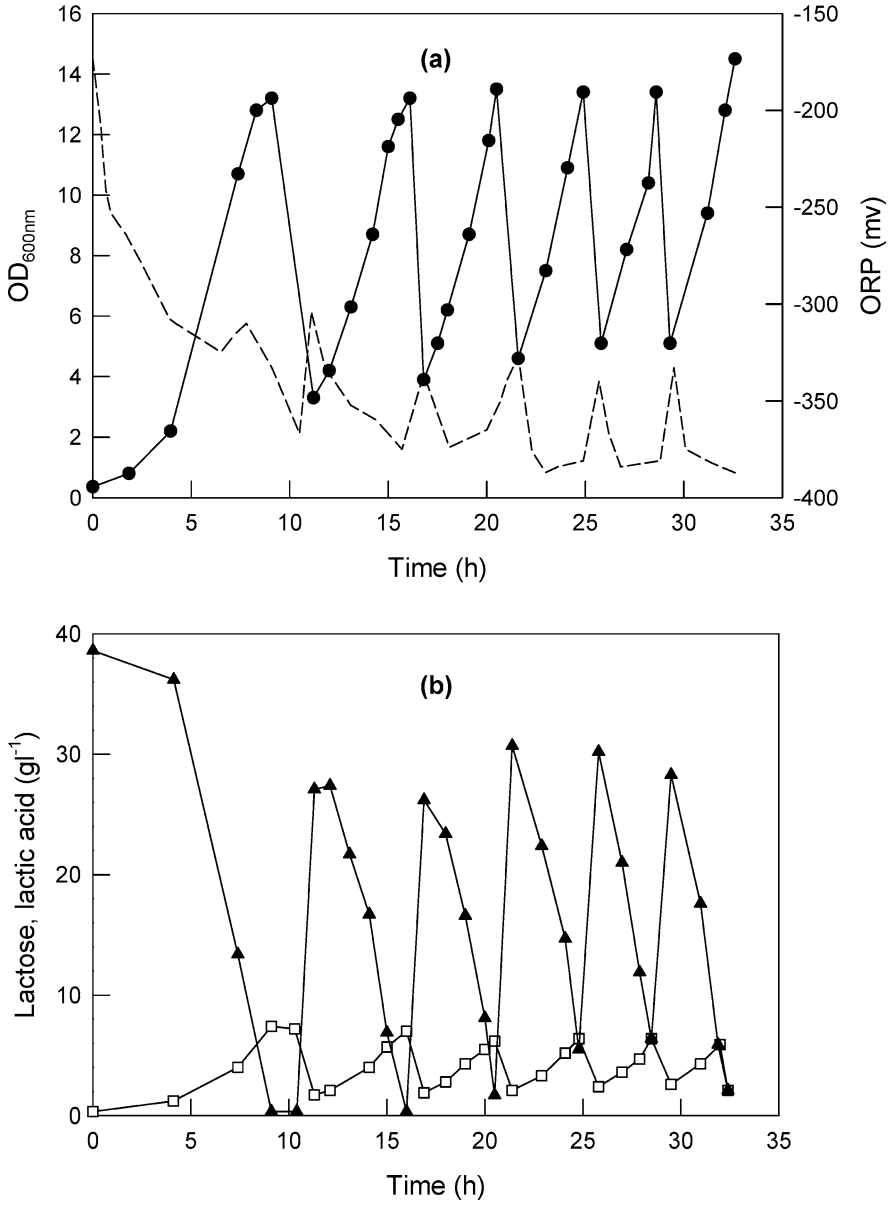

Fig. 5a, b Time-course repeated batch fermentation of *B. longum* in WY medium. **a** *Closed circles* OD_{600nm}, *dotted lines* ORP; **b** *closed triangles* lactose, *open squares* lactic acid. Fermentation was carried out at 37°C and pH 6.0

Repeated batch fermentation

Repeated batch fermentation started with the same conditions as batch fermentation. Figures 4a and b show a typical run of repeated batch fermentation of *B. longum* in MRS medium. The first batch was stopped as soon as the ORP ceased to decline at 8 h after inoculation. This is the same time as batch fermentation, when cells were harvested at the maximum cell concentration as shown in Fig. 1. A portion (75%, v/v) of broth was discharged and the same volume of fresh medium was fed. Feeding of fresh medium resulted in a sharp increase in ORP and decreases in lactic acid and acetic acid. As soon as the fresh nutrient was fed for each new cycle, cells could grow in exponential phase without an apparent lag phase. It took 4.5 h on average over six cycles as shown in Fig. 4b to complete a cycle. The average cell concentration was 1.9×10^9 cfu ml^{-1}, which is similar to the batch fermentation in Fig. 1. The productivity of cell mass based on the 37-h fermentation as shown in Fig. 4a was 0.64 g l^{-1} h^{-1}. The productivity was higher than that of batch fermentation (0.51 g l^{-1} h^{-1}) using MRS medium because of the elimination of the lag phase for each new cycle.

For repeated batch fermentation using WY medium (Fig. 5), again, it took nearly 4.5 h to complete a cycle. The average output cell concentration was 3.4×10^9 cfu ml^{-1}, which is almost equal to that in batch process (Fig. 2). Productivity of cell mass based on the 33-h fermentation was 0.99 g l^{-1} h^{-1}. Again, the productivity was higher than that of batch fermentation (0.76 g l^{-1} h^{-1}) using WY medium because of elimination of the lag phase for each new cycle.

The ORP was lower (Fig. 5a) in the repeated batch fermentation compared to that in batch fermentation (Fig. 2). This might be due to accumulation of some

chemical constituents involved in oxidation-reduction within the broth.

Discussion

In this work, the on-line device ORP has been successfully used to monitor cell growth and nutrient utilization. Changes in ORP during anaerobic fermentation of *B. longum* always reflect the presence of carbon source (glucose or lactose). This behavior has also been described in microaerobic fermentation of yeast *C. tropicalis* [14].

An industrial medium for cultivation of bifidobacteria must have the characteristics of low cost, ability to support bacterial growth, and availability of ingredients. Process facilities must be as simple as possible. The WY medium used was that of Corre et al. [1] with one modification, whey being hydrolyzed by protease. The average viable cell number was 3.4×10^9 cfu ml^{-1} in repeated batch fermentation compared with 9×10^8 cfu ml^{-1} as reported by Corre et al. [1]. In the latter report, whey was not treated with protease. Lack of free amino acids and small peptides resulted in poor growth of bifidobacteria in milk [2].

Taniguchi et al. [16] employed cross flow filtration to achieve a 7-fold increased cell density of *B. longum* compared with batch fermentation. The lactose concentrations in the filtrate and input medium reported by Taniguchi et al. [16] were 43.5 and 50 g l^{-1}, respectively. This indicates that 87% of the lactose was not consumed in the broth. The work of Taniguchi et al. employed fresh medium to dialyze inhibitory metabolites to achieve a very high cell density [16]. The repeated batch process in this study takes advantage of complete consumption of nutrients. The process is similar to continuous fermentation. The cells can be harvested by centrifugation or concentrated by membrane filtration following by freeze drying or spray drying.

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