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High-density cultivation of *Lactobacillus plantarum* NCU116 in an ammonium and glucose fed-batch system

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Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria which are found in diverse environments such as the human body, animals and plants. In this study, we reported a simple fed-batch process for *Lactobacillus plantarum* NCU116 with high cell density. It was found that the optimum initial glucose concentration for this strain was 5% (w/v). To reduce the effect of acid and starvation, the exponential fed-batch culture and ammonium fed-batch system were introduced. At the end of the exponential phase of fermentation, the cell concentration was up to 9.35×10^9 CFU/ml.

Key words: *Lactobacillus plantarum*, high cell density cultivation, exponential fed-batch, ammonium.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of bacteria which are found in diverse environments such as the human body, animals and plants. It is well known that these bacteria have been used to produce various fermented foods from animal and plant materials. LAB is drawing more and more consumers' interest because of their health benefits. Industrialization of food bio-transformations increased the economical importance of LAB. Although, LAB are a low cost ingredient in food transformation processes, they play a crucial role in the development of the fermented products industry.

The growth of LAB is characterized by the generation and accumulation of acidic end products in the environment. Bacterial growth is a self-limiting process, and is often sub-optimal in nature compared to the laboratory where the growth environment is strictly controlled. When the medium can no longer provide the necessary nutrients, or if the environment generates a substance inhibitory to

growth, bacteria stop multiplying and 'reset' internal conditions to adapt to the new conditions. In milk fermentations, LAB degrade lactose, resulting in lactate accumulation and consequent acidification of the media to as low as pH 4.0, which may inhibit further growth and metabolism.

Several researchers studied the buffer salt and chemical neutralization method, but the cell concentration was lower than 10^9/ml. Other researchers investigated cell cycle culture and dialysis culture. Hayakawa et al. (1990) obtained a cell density of 40 g/L with a production rate of 13 g/L/h and water consumption of 0.2 m³/kg. Suzuki (1996) obtained a high cell dry weight of 141 g/L by using ceramic filters, and the production rate was approximately 0.8 g/L/h. Srivastava et al. (1992) described an ion exchange resin extraction-type lactic acid fermentation, with a cell density of 30 g/L. Although, the cell cycle culture and dialysis culture are much better in cell density and productivity when compared with buffer salt and chemical neutralization, the equipment and techniques are costly. Therefore, it is necessary to find a new way to achieve high-density culture of LAB High cell-density culture is usually attained using fed-

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batch systems as this technique avoids high initial concentrations of a substrate that might be growth-inhibitory to the organism. In order to achieve a high cell density, the cell growth rate must be kept at a high level for a relatively long period during the cultivation. In this study, we developed a simple but effective feeding strategy as well as an optimal initial medium which supports the growth of *Lactobacillus plantarum* to maximize the cell density by a fed-batch system and ammonia.

MATERIALS AND METHODS

The strain used in this study (*L. plantarum* NCU116) was obtained from pickled vegetables in the State Key Laboratory of Food Science and Technology, Nanchang University, China. The culture was maintained by monthly transfer on yeast-malt extract agar and stored at 4°C.

The growth medium (unless specified) contained (per liter): peptone, 10 g; beef extract, 10 g; CH_3COONa, 5 g; yeast extract, 5 g; $C_6H_6O_7(NH_4)_2$, 2 g; K_2HPO_4, 5 g; $MgSO_4$, 0.2 g. Glucose was used as the sole carbon and energy source with an initial concentration of 50 g/L in the growth media. The composition of the seed culture medium is identical to the growth medium, except that the initial glucose concentration is 30 g/L.

Cell growth in batch fermentation

Fermentation was carried out in a fully instrumented and computer controlled 15-L stirred tank bioreactor (Guoqiang Shanghai, China), equipped with a pH probe and a dissolved oxygen (DO) probe. 240 ml of pre-cultured seed was inoculated into 8 L of MRS medium, and then cultured for 36 h. Agitation was provided by a standard six-blade impeller at 100 rpm. The pH change during cultivation was monitored by a pH probe attached to a PID controller. To control pH at a set level, NH_4OH and lactic acid was automatically added to the culture broth. The fermentation temperature was maintained using a re-circulating water bath at 37°C, and the initial pH was set at a given level. During the fermentation, the pH and DO were monitored on-line. Cell growth and residual glucose level were analyzed off-line by the method that would be described later.

Equipments and control system

The fermentation was conducted in a 15-L stirred tank bioreactor with on-line pH and DO detection and control. The whole control system is composed of a central computer and control panel, showing the weight and pH values. The system consists of weighing, flow control and oxygen components.

Analytical methods

The cell concentration (bacterium number) was determined by the dilution-plate method. The 10-fold dilution was made by diluting 25 ml of sample with 225 ml of physiological saline. Further, 10-fold serial dilutions, ranging from 10^{-2} to 10^{-8}, were prepared and the microbial counts were determined according to the pour plate method. Each dilution was incubated on three plates. Total viable counts were determined after the MRS agar plates were incubated in an anaerobic condition at 37°C for 2 days. Glucose concentrations

were analyzed by using a glucose oxidase method.

RESULTS

Optimum initial glucose concentration

NCU116 was grown in shake-flask cultures in the MRS medium. The experiments were carried out with 1-L Erlenmeyer flasks containing 500 ml of fermentation medium. The fermentation medium was inoculated with 3% (v/v) of inoculums. The flasks were incubated at 37°C in an orbital shaker at 100 rpm for 16 to 20 h. No pH control was employed during the experiments, but initial pH was set at 6.0 for each flask.
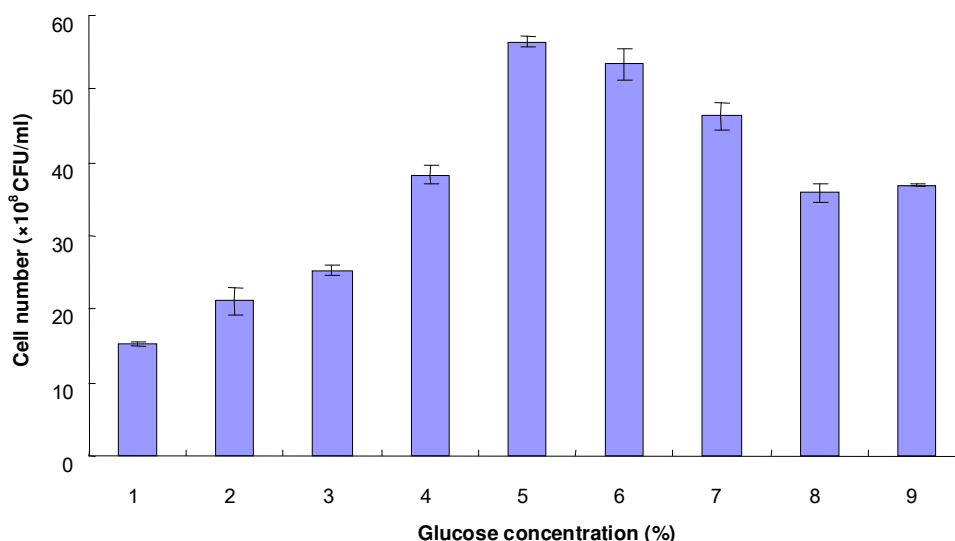
To determine the effect of initial glucose concentration on growth profile of NCU116, batch experiments were performed in flasks with glucose concentrations ranging from 1 to 9% (w/v). Figure 1 shows the cell concentration of NCU116 with different glucose concentrations. The result indicated that NCU116 grew well with glucose as the sole source of carbon and energy at a concentration of up to 5% (w/v). When the glucose concentration reached 7%, the cell growth was repressed and more severe inhibitory effects were observed with higher glucose concentrations.
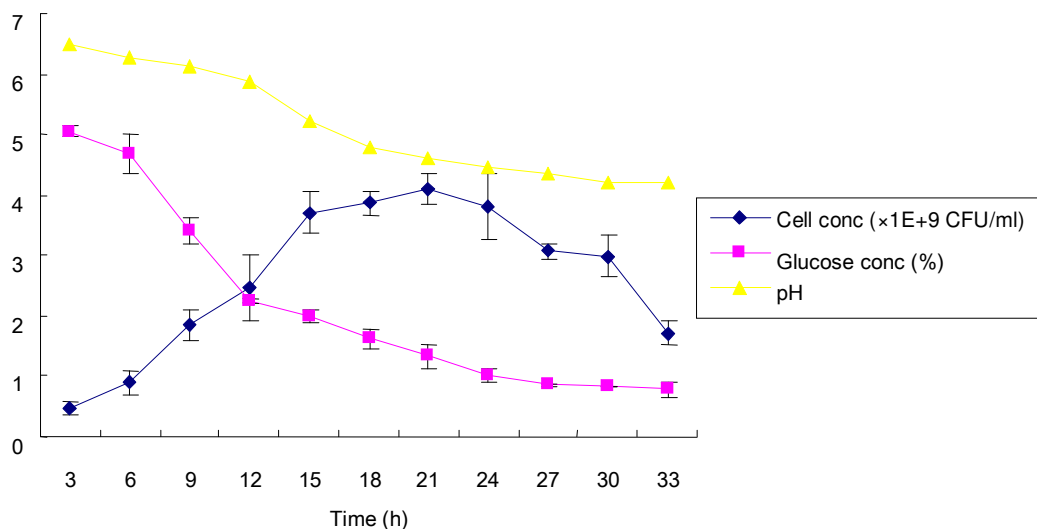
Growth curves of NCU116

The time course of cell growth in the batch culture of NCU116 without controlling the pH is shown in Figure 2. It was observed that the pH value of the culture broth decreased from its initial value of 6.5 to 4.5 in 24 h during the fermentation and the cell growth increased with the consumption of glucose. The decrease of the pH was likely due to the large amount of lactic acid during the fermentation process. After the maximum cell growth was achieved, the cell died rapidly because of glucose depletion and the extremely low pH value. Cell growth then decreased rapidly, indicating that cell lysis and subsequent cell death occurred. From the earlier mentioned observation, it was deduced that the pH value in the culture broth was critical in the cell growth. Hence, by proper control of pH in the fermentation, the cell growth may be prolonged and cell concentration might be increased.

Determination of neutralizer

Experiments have been conducted in order to determine the most appropriate concentration of glucose for NCU116 fermentation; we compared the neutralizing effect of Na_2CO_3 with NH_4OH in controlling the pH at 6.0. These experiments were conducted in a bioreactor at 37°C. The cultivation medium was inoculated with 3%

Figure 1. Effect of initial glucose concentrations on cell growth of NCU116.

Figure 2. The growth curves with batch cultivation of NCU116 without pH control.   , cell concentration; ▲, pH; ■, glucose concentration.

(v/v) of inoculums. Figures 3 and 4 shows the different growth curves of NCU116 cultured in MRS medium with pH control at 6.0 by using Na_2CO_3 and NH_4OH, respectively.

From Figures 3 and 4, it was obvious that the concentration of cells in the culture broth fed with neutralizer was higher than those without neutralizer, and the concentration reached 6.8×10^9 CFU/ml, with ammonia being better than Na_2CO_3. However, the curves of glucose consumption in the presence of ammonia and Na_2CO_3 were similar, as shown in Figure 4. Feeding of neutralizer was important to the high cell density cultivation of NCU116, whose growth and transition to stationary phase was accompanied by the accumulation of lactic acid, resulting in acidification of the media, arrest of cell multiplication and possible cell death.

Effect of pH control on cell growth

To examine the effect of pH on cell growth, experiments were conducted in a stirred tank bioreactor with the pH values at 7.5, 7.0, 6.5, 6.0, 5.0 and 4.0, respectively. Before the cultivation, the pH was adjusted to the earlier
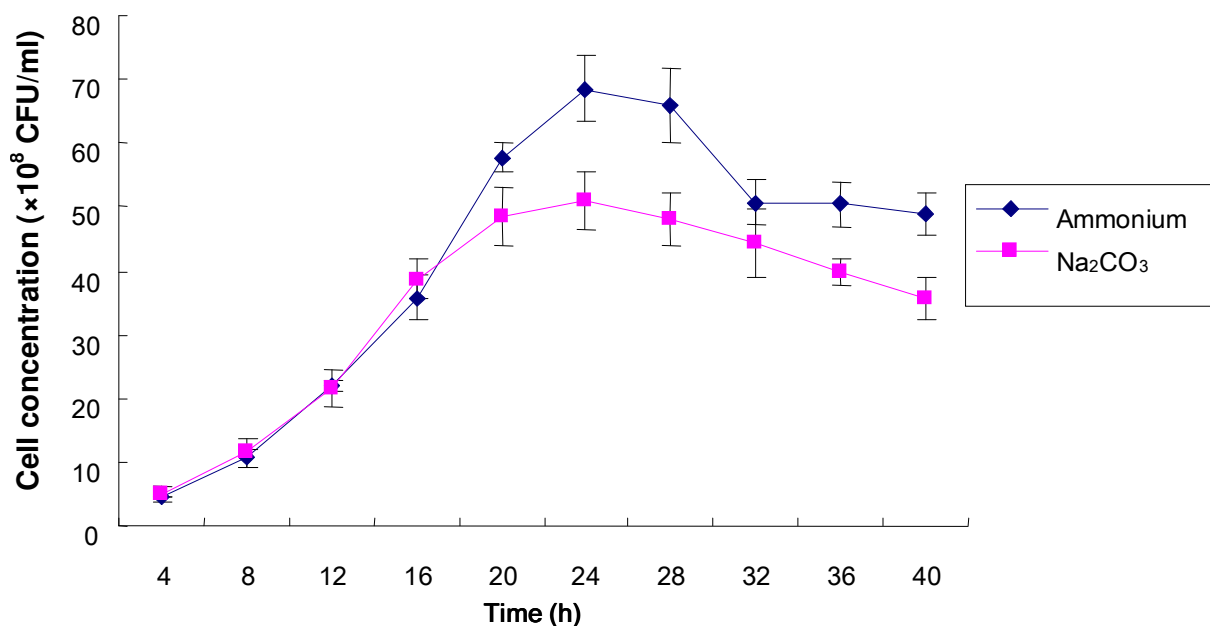
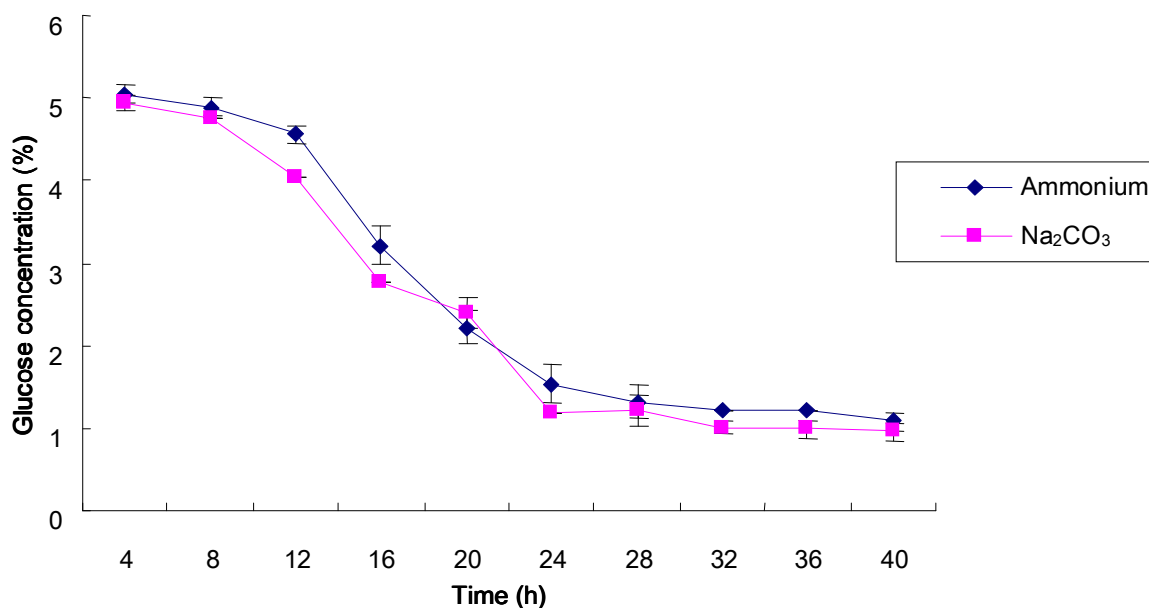**Figure 3.** Effects of neutralizers on cell concentration of NCU116.

Figure 4. Effects of neutralizers on glucose concentration of NCU116.

mentioned values with the NH_4OH or lactic acid, the pH was then maintained unchanged until the end of cultivation. As shown in Figure 5, the cell growth was strongly dependent on the pH of the medium. The highest cell concentration obtained was 6.28×10^9, 4.52×10^9, 5.22×10^9, 4.0×10^9, 3.3×10^9 and 1.18×10^9 CFU/ml when the pH was maintained at 6.5, 7.5, 7.0, 6.0, 5.0 and 4.0,

respectively. After the cell growth reached its maximum, it remained constant up to 30 h in prolonged cultivation when the pH was maintained at 6.5. In contrast, it was rapidly degenerated when the pH was maintained at other values.

The earlier mentioned findings indicated that the cell growth was favorable and the cells remained stable when
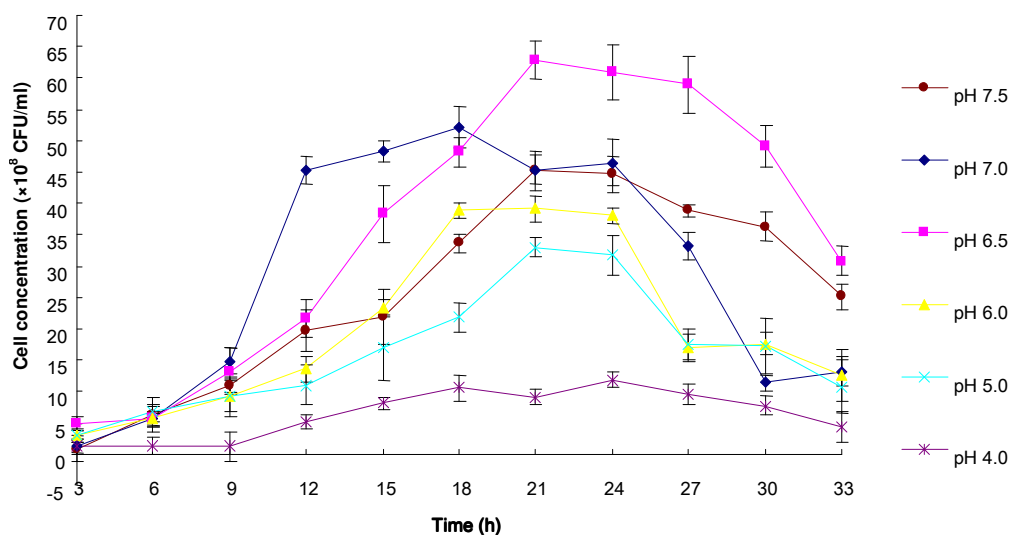
Figure 5. Effect of pH on cell growth of NCU116.

the pH was kept at 6.5. When the pH was kept at 4.0, the cell growth was degenerated at the beginning of cultivation. The cells started growing quickly after 9 h during cultivation (Figure 5). Survival in acid conditions was likely due to the expression of specific sigma factors of *Lactobacillus* (Lee et al., 1995), or adaptation to low pH (Foster and Hall, 1991). However, how the pH affects the growth of NCU116 remains to be investigated.

Effect of fed-batch culture on NCU116

From the experiments discussed earlier, it can be concluded that the higher cell concentration using the best neutralizer can be obtained at pH 6.5 during the fermentation. Two strategies of fed-culture were attempted to achieve higher cell density. One strategy was constant fed-batch culture and the other was exponential fed-batch culture.

As shown in Figures 6 to 7, the high cell growth rate was accompanied by a high rate of glucose consumption during the exponential phase (8 to 20 h). At the point of glucose depletion, the cell concentration was attained at 5.65×10^9 and 6.5×10^9 CFU/ml, respectively. The glucose concentration was monitored during the fermentation and a pre-determined amount of nutrient feeding solution was added before the end of the exponential phase. Therefore, the glucose feeding rate of constant fed-batch culture was controlled at 40 g/h during the fermentation, which led to a glucose concentration at approximately 20 g/L. The feeding rate of exponential fed-batch culture is shown in Figure 8.

As shown in Figure 6, the cell concentration increased from 1.64×10^9 to 5.65×10^9 CFU/ml during the course of

glucose constant fed-batch culture and from 1.23×10^9 to 6.5×10^9 CFU/ml during the course of exponential fed-batch culture. In contrast, the highest cell concentration was 4.67×10^9 CFU/ml with non-fed-batch as shown in Figure 6. From the aforementioned, it was concluded that the exponential fed-batch culture was better than constant fed-batch culture and non-fed-batch culture.

Glucose, initially at 50 g/L, was readily utilized during the fermentation (Figure 2) and was completely consumed by 30 h. As no further glucose was added into the fermentation medium, the growth then ceased due to the glucose depletion. However, with the exponential fed-batch culture, glucose was added after 10 h at the concentration of 300 g/L, which only increased the cell concentration from 4.67×10^9 to 6.5×10^9 CFU/ml after further 20 h cultivation. So, the exponential fed-batch culture must be combined with pH control. As shown in Figure 9, the cell concentration was up to 9.35×10^9 CFU/ml, and the glucose concentration was kept at a stable level (3.5%).

DISCUSSION

During the high cell density culture of LAB, growth will be arrested and enter into stationary phase. This can be provoked by numerous stress conditions like cold, heat, osmotic, oxidative or acid stress, or starvation. Among these conditions, nutrient starvation is one of the most common causes and bacterial growth itself contributes to nutrient exhaustion and subsequent starvation of one or more compounds. Moreover, some extreme environmental stress conditions may provoke a deprivation of one or several components, apart from their direct effects
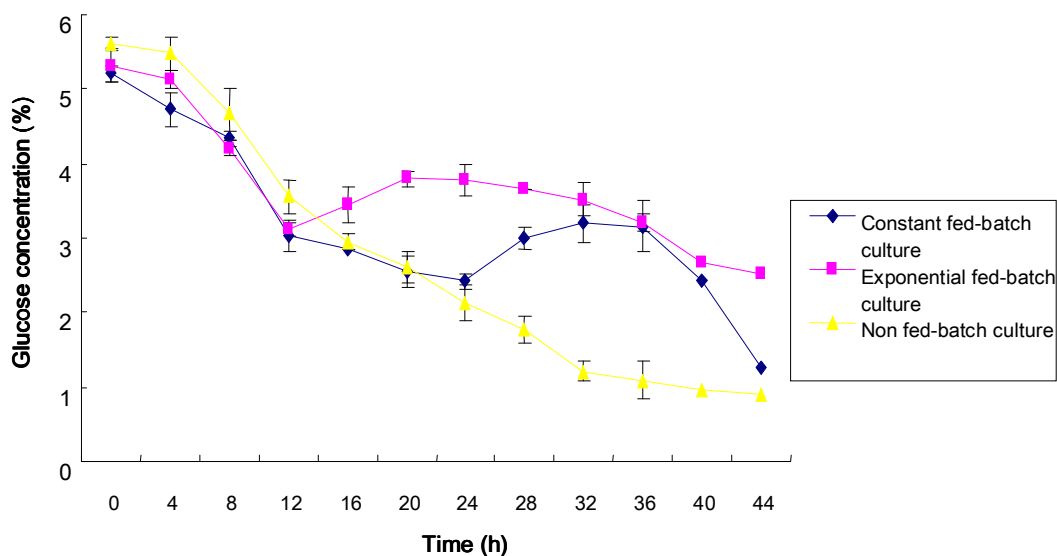
Figure 6. Effect of fed-batch mode on glucose concentration of NCU116. Time courses of constant fed-batch ( ), exponential fed-batch (■) and non fed-batch (▲).
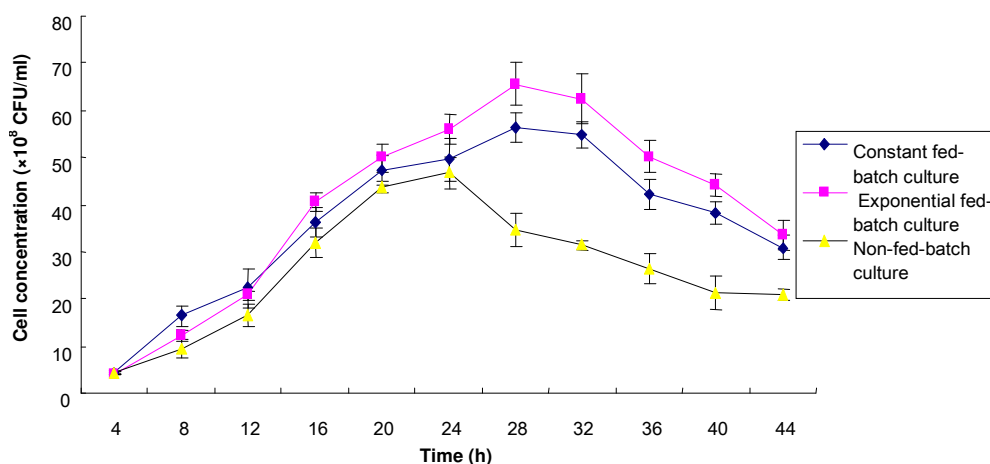

Figure 7. Effect of fed-batch mode on cell concentration of NCU116. Time courses of constant fed-batch ( ), exponential fed-batch (■) and non fed-batch (▲).

on the cells constituents. Poolman et al. (1987) and Konings et al. (1997) showed that the stress conditions can indirectly provoke starvation or energy depletion, irrespective of the extra cellular amount of the substrates. These conditions of nutrient depletion could be delete-rious for long-term cell viability. Most species of LAB are neutrophiles, and the effect of acid stress on bacterial physiology is not known in detail. However, it is well established that acids can passively diffuse through the cell membrane and rapidly dissociate into protons and charged derivatives to which the cell membrane is impermeable after entry into the cytoplasm (Presser et al., 1997). Several reviews in this field are available, for

example Kolter (1993), Hall (1995), Georgopoulos and Welch (1993) and Hecker et al. (1996).

Fed-batch cultivation modes have been widely applied for high cell density culture. Evans and Ratledge (1983) studied *Candida curvata* D growing on glucose and xylose in a continuous fermentation process and obtained a cell density of approximately 14 g/L. Pan et al. (1986) obtained a cell density of 185 g/L in an 84 h fed-batch culture of *Rhodotorula glutinis* aerated with oxygen-enriched air. Yamauchi et al. (1983) obtained a cell density of 153 g/L using fed-batch culture of *Lipomyces starkeyi* for 140 h. Meesters et al. (1996) used glycerol as a carbon source in fed-batch fermentation with *Candida*
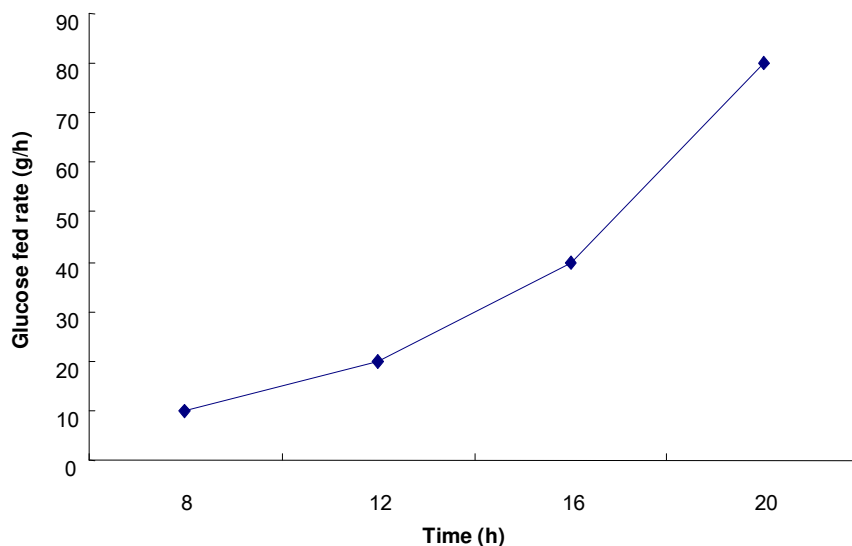
Figure 8. Time course of glucose fed rate with exponential fed-batch culture. The initial glucose fed rate was 10 g/L and the highest glucose fed rate was controlled at 80 g/L. Cells were grown for 20 h.
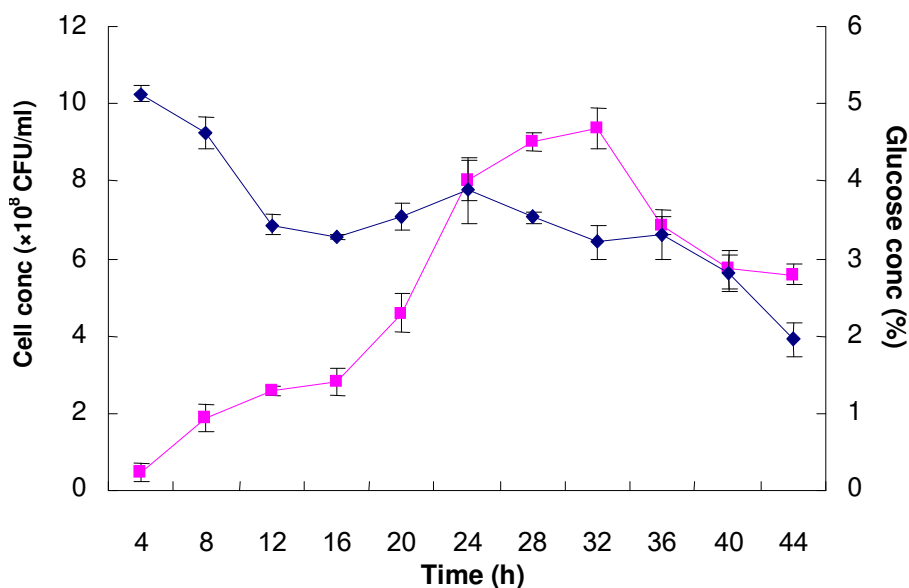
Figure 9. Effect of exponential fed-batch culture on cell growth of NCU116. The pH value was controlled at 6.5 with ammonium. Time course of cell concentration ( ) and glucose concentration (■).

curvatus with a cell density of 118 g/L.

To develop a simple and improved culture method for high cell density culture of NCU116, batch flask cultures were carried out to determine the suitable substrate concentration of the initial medium. It was found that the optimum initial glucose concentration was 5% (w/v). To reduce the effect of acid and starvation, the exponential fed-batch culture and ammonium fed-batch system were introduced. At the end of the exponential phase of fermentation experiment, the cell concentration was up to 9.35×10^9 CFU/ml.

In conclusion, we described a simple fed-batch process for a *L. plantarum* NCU116 with high cell density. The process features a nutrient-rich initial medium and sole

carbon source feeding, which is convenient for large-scale operation. This strategy will be useful in further engineering of cost effective microbial agent production process.

ACKNOWLEDGEMENTS

REFERENCES

Evans CT, Ratledge C (1983). A comparison of the oleaginous yeast Candida curvata, grown on different carbon sources in continuous and batch culture. Lipid, 18: 623-629.

Foster JW, Hall HK (1991) Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. 173: 5129-5135.

Georgopoulos C, Welch WJ (1993) Role of the major heat shock proteins as molecular chaperones. Ann. Rev. Cell. Biol. 9: 601-634.

Hall HK, Karem KL, Foster JW (1995) Molecular responses of microbes to environmental pH stress. Adv. Microbial. Physiol. 37: 229-272.

Hayakawa K, Sabsawa H, Nagumeme T (1990). High density culture of *Lactobacillus casei* by cross flow culture flow culture method based on kinetic properties of the microorganism . Ferment. Bioeng. 70: p. 404.

Hecker M, Schumann W, Vtlker U (1996) Heat-shook and general stress response in *Bacillus subtilis*. Mol. Microbiol. 19: 417-428.

Kolter R (1993). The stationary phase of the bacterial life cycle. Annu. Rev. Biochem. 47 : 855-874.

Konings WN, Lolkema JS, Bolhuis H, van Veen HW, Poolman B, Driessen AJ (1997). The role of transport processes in survival of lactic acid bacteria. Energy transduction and multidrug resistance. Antonie Van Leeuwenhoek, 71: 117-128.

Lee IS, Lin J, Hall HK, Bearson B, Foster JW (1995). The stationary-phase sigma factors (RpoS) is required for a sustained acid tolerance response in virulent Salmonella typhimurium. Mol. Microbiol. 17: 155-167.

Meesters PAEP, Huijberts GNM, Eggink G (1996). High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Appl. Microbiol. Biotechnol . 45: 575-579.

Pan JG, Kwak MY, Rhee JS (1986). High density cell culture of *Rhodotorula glutinis* using oxygen-enriched air. Biotechnol. Lett. 8: 715-718.

Poolman B, Smid EJ, Veldkamp H, Konings W (1987) Bioenergeticconsequences of lactose starvation for continuously cultured *Streptococcus cremoris* and *Streptococcus lactis*. J. Bacteriol. 169: 1460-1468.

Presser KA, Ratkowsky DA, Ross T (1997). Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Appl. Environ. Microbiol. 63: 2355-2360.

Srivastava SC, Mausner LF, Mirzadeh S (1992). Improved specific activity of reactor produced 117mSn with the Szilard-Chalmers process. Int. J. Radiation Appl. Instrumentation. Part A. Applied Radiation and Isotopes, 9:1117-1122.

Suzuki T (1996). A dense cell culture system for microorganisms using a stirred ceramic filters. Ferment. Bioeng. 82: 264-271.

Yamauchi H, Mori H, Kobayashi T, Shimizu S (1983). Mass production of lipids by *Lipomyces starkeyi* in microcomputer-aided-fed-batch culture. J, Ferment. Technol., 61: 275-280.