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Improvement of nattokinase production by *Bacillus subtilis* using an optimal feed strategy in fed-batch fermentation

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Abstract

Nattokinase is an enzyme with strong fibrinolytic activity that can be used for preventing hypertension and cardiovascular diseases. In this study, *Bacillus subtilis* K-C3 was cultured in fed-batch fermentation for the production of nattokinase. In a first step the enzyme production was optimized by testing substrates for their influence on the enzyme activity. Experiments revealed glycerol as the best substrate with the highest enzyme yield and less by-product formation compared to other substrates. The optimal process parameters were then applied to fed-batch fermentation. An optimal feed strategy for fed-batch that allowed for maximum enzyme production was designed based on fed-batch kinetic model. During fed-bath operation a specific growth rate of 0.2 $h^{-1}$was controlled using a glycerol feed rate predetermined from the model. With the designed feed strategy, a high cell density of 73.47±2.63 g/l and a nattokinase activity of 9,198.51±268.09 units/ml were achieved at 28 hours of cultivation. An overall enzyme yield of 61.36±1.80 units/g was obtained. Productivity of nattokinase in fed-batch fermentation was 545.95±27.88 units/h, a 20-fold higher than that in batch fermentation. High titer and high productivity of nattokinase achieved in this study has indicated the efficiency of the designed feed strategy used for fed-batch process. The same strategy may also be applied to other processes to increase production of other enzymes.

Keywords: Nattokinase, Culture optimization, Fed-batch kinetic model, Fed-batch culture

1. Introduction

Nattokinase has been of considerable interest because of its capacity to digest fibrin in blood vessels (1, 2, 3, 4). The enzyme is currently considered in pharmaceutical industry as a promising drug for thrombolytic therapy. Using nattokinase as dietary supplement canhelp preventing cardiovascular diseases, primary hypercholesterolemia and hypertension (5,6). Nattokinase can be produced by *Bacillus ssp.* (7). For a commercial use of nattokinase, it is, therefore, necessary to develop an efficient fermentation process that results in maximum production of the enzyme at high yield, high titer and high productivity.

Type of substrates is crucial factor for an efficientproduction of nattokinase. We optimizedthe substrate factor such that the production of nattokinase by *B. subtilis* at high yieldcan be achieved.Different substrates were investigated in batch fermentationsfor their effects on nattokinaseproduction. The optimal substrate for maximal nattokinase yield was identified. Batch fermentation often experiences low titer and low productivity because of the inhibitory effect of high initial concentration of substrates as well as accumulation of inhibitory by-products such as acetic acid (8, 9, 10, 11).Thus, to overcome these inhibitions we pplied fed-batch fermentation strategy for reaching high titer and high productivity of nattokinase.Balanced and sufficient feed of substrate is critical to the success of fed-batch culture because it affects cell growth and nattokinase formation. Overfeeding of substrate may result in an accumulation of toxins and low enzyme activity. A fed-batch kinetic model was, therefore, developed and applied to ensure a sufficient, balanced addition of substrate for effective nattokinase formation during fed-batch operation. Using the kinetic model for designing substrate feed rate, a concentration of substrate can be maintained at low concentration throughout the culture and a specific cell growth rate can be controlled at its optimal for enzyme production. Here the performance of fed-batch fermentation for production of nattokinase by *B. subtilis* was reported.

2. Materials and Methods

Bacillus subtilis K-C3 (Biotec, Thailand) was used throughout this study. The experiments were performed in both shake flasks and bioreactors. The medium used for routine culture maintenance consisted of 5g/l yeast extract, 10g/l peptone, and 5 g/l NaCl.

2.1 Batch shake flask experiment. All experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml of culture medium. The experiments were conducted aerobically at 30°C on a 200 rpm shaking incubator. To investigate the effect of substrates on nattokinase production, cells were grown in the culture medium containing 5g/l yeast extract, 10g/l soy peptone, 5g/l NaCl and 10g/l substrate as specified. Soy peptone and yeast extract were used since they were reported as the optimum medium composition for nattokinase production by *B. subtilis* (12). Each substrate was sterilized separately and then added to the medium. Inoculum was prepared overnight with the same medium. Cultivations were initiated by adding the inoculum to give an initial OD_{600} of approximately 0.1-0.2. Samples were collected after 24 h for measuring concentration of cell, nattokinase, substrate and by-products.

2.2 Fed-batch bioreactor experiment. All fed-batch bioreactor experiments were carried out using 2 l B. Braun bioreactor (B. Braun Biotech International, Germany). Fed-batch experiment was carried out as batch culture initially with a working volume of 0.6 lat 30°C and a 200 rpm stirring rate.The medium used for bioreactor experiments was the same as that used

for shake flask experiments with 5g/l of glycerol for the initial growth of cell. Inoculum was a 50ml of an overnight grown cell culture in shake flask which gave a start OD_{600} of approximately 0.2-0.4. pH was controlled at 7.0 using 40%(v/v) NH_4OH and 40%(v/v) H_3PO_4. Sterilized antifoam solution was used for elimination of foaming. The batch fermentation was operated at 4 vvm aeration rate to maintain aerobic condition. For a controlled dissolved oxygen (DO%), stirring speed was set initially at 200 rpm and was adjusted via a feedback loop control to maintain DO level above 20%. Feed solution used in fed-batch culture contained 33.1g/l yeast extract, 370.1g/l soy peptone, 150g/l glycerol and 10ml/l trace metal solution. Trace metal stock solution which was composed of 200g/l $MgSO_4.7H_2O$, 2g/l $MnSO_4.7H_2O$, 1g/l $FeSO_4.7H_2O$, 1g/l $CoCl_2.6H_2O$, 200g/l $CaCl_2.2H_2O$, 1g/l $ZnCl_2$, 1g/l $CuSO_4.5H_2O$ and 20g/l KCl was autoclaved separately and added to the feed medium prior to use. Compositions of feed were calculated according to the balanced ratio of soy peptone to glycerol observed in batch operation as follows:

$$C_{sp,F} = 1.65.C_{gly,F} \quad (1)$$

where $C_{sp,F}$ and $C_{gly,F}$ are concentration of soy peptone and glycerol (g/l) in the feed solution. The soy peptone to yeast extract ratio used in this experiment was 11.19 which was an optimal value for the production of nattokinase based on fractional factorial design (12).

After 6 h of batch operation or the OD_{600} reaches approximately 3, feed medium was fed to the fermenter exponentially at a feed rate as determined based on the kinetic model of fed-batch (see next section for details). The calculated feeding profile was entered into the reactor controller which connected to feed pump for controlling substrate feed rate.Aeration was oxygen-enriched air (1:1 air-oxygen ratio). To ensure sufficient supply of oxygen to the culture, the DO% content of the culture was controlled by changing agitation speed (200–900rpm) and airflow rate (2-4l/min) for maintaining DO% within 5-20%. Temperature was set at 30°C. The pH was controlled at 7.0 with NH_4OH and H_3PO_4. Samples were periodically collected for cell, nattokinase, substrate and fermentation by-products measurements.

2.3 Design of feed rate based on fed-batch kinetic model. The model describes a balance of limiting substrate, glycerol, and cell growth. For a fed-batch bioreactor under non-limiting growth conditions, the mass balance of the cells and glycerol is represented by

$$\frac{d(x \cdot V)}{dt} = \mu \cdot x \cdot V \quad (2)$$

$$\frac{d(C_{gly} \cdot V)}{dt} = f \cdot C_{gly,F} - \left(\frac{\mu}{Y_{x/gly}} + \frac{q_{natto}}{Y_{natto/gly}} + m \right) \cdot x \cdot V \quad (3)$$

where x is cell concentration (g/l); V is culture volume (l); μ is cell growth rate (h^{-1}); f is feed flow rate (l/h); C_{gly} is glycerol concentration in the culture (g/l); $C_{gly,F}$ is glycerol concentration in the feed (g/l); $Y_{x/gly}$ is cell yield on glycerol (g-cell/g-glycerol); q_{natto} is specific production rate of nattokinase (unit/g-cell-h); $Y_{natto/gly}$ is nattokinase yield on glycerol (units/g-glycerol); m is specific maintenance coefficient (g-glycerol/g-cell-h).

The model can be used to determine an optimal feed rate of glycerol for maintaining a constant growth rate and enzyme production. During a fed-batch process, no accumulation of the glycerol concentration is assumed. Thus, combination of Eq (2) and Eq (3) yields an optimal, balanced glycerol feed rate as

$$f(t) = \frac{\left(\frac{\mu}{Y_{x/gly}} + \frac{q_{natto}}{Y_{natto/gly}} + m \right) \cdot x_0 \cdot V_0 \cdot \exp[\mu \cdot (t - t_0)]}{C_{gly,F}} \quad (4)$$

where $f(t)$ is the volumetric feed rate of glycerol (l/h); x_0 is initial cell concentration before feeding (g/l); V_0 is initial culture volume before feeding (l); t_0 is time of feeding start (h); t is processing time (h).

2.4 Cell concentration measurement. The concentration of cells was measured through optical density (OD) at 600 nm (spectrophotometer DR/2500, Hach Company, Singapore). The OD_{600} was converted to dry cell weight per liter using a standard curve which showed one unit of OD_{600} corresponded to 0.456 g of cell dry weight/l based on experimental results. Dry cell weight was determined by collecting cell culture, centrifuged and washed once with deionized water. The sample was then transferred to a pre-weighed tube and dried at 100°C for 24 h in a glass test tube. The dry cell weight was determined by subtracting the sample mass from the mass of the empty tube.

2.5 Substrate and product quantification. Concentration of substrate and fermentation products was measured using a HPLC system (Shimadzu Asia Pacific, Singapore) equipped with a HPX-87H column (Bio-Rad Laboratories, Thailand), and a refractive index detector. The column was run in an isocratic mode at 65°C and 0.5 ml/min using a mobile phase of 5 mM H_2SO_4. The concentration of metabolites was determined from the standard curve correlating peak area to known metabolite concentration.

2.6 Nattokinase assay. Activity of nattokinase was determined from the hydrolysis of fibrin clot according to method developed byAnson (13). Briefly, 25 µl of fibrinogen solution was mixed with 5 µl of thrombin solution. The formed clots were digested with 25 µl of crude enzyme in 20mM sodium phosphate buffer (pH 7.2). The reaction was incubated at 37ºC for 20 min and was stopped by adding 250 µl of 10% (w/v) trichloroacetic acid. After centrifuged at 4,000 rpm for 10 min. 100 ml of the supernatant was mixed with 1 ml of Reagent A (50 ml of 2% (v/w) Na_2CO_3 in 0.1 M NaOH mixed with 1 µl of 0.5% (v/w) $CuSO_4.5H_2O$ in 1% (v/w) sodium citrate) and incubated for 10 min. Then, the solution was mixed with 100 µl of Folin reagent and incubated for 30 min. The absorbance at 750 nm was measured. For negative control, 10% (w/v) trichloroacetic acid was added before adding crude enzyme. The enzyme activity was calculated from the difference of absorbance between sample and negative control solution using predetermined standard curve that converted OD_{750} to the amount of tyrosine equivalent. One unit of nattokinase activity is defined as the amount of enzyme to produce 1 mmol of tyrosine per milliliter per minute at 37ºC.

3. Results and Discussion

3.1 Effect of substrates on nattokinase production. Batch shake flask experiments of *B. subtilis* K-C3 culturing on four different substrates for nattokinase production were examined. Of all substrates, glycerol gave the highest nattokinase yield of 47.4±1.2 unit-enzyme/g-glycerol(Fig. 1). This finding is in agreement with the previous report showing the use of glycerol resulted in a high production of proteins (14). K-C3 grew at a slower rate on glycerol than other substrates. K-C3 also produced less acetate and succinate when grow on glycerol compared to other substrates. Accumulation of organic acids including acetate and succinate often occurs at a high specific growth rate (15,16). The higher growth rate likely generates a higher flux of carbon through glycolysis and an overflow towards pyruvate which leads to an increasing drain of the acids. Thus, the slower specific growth rate of K-C3 in glycerol-grown culture may explain the less accumulation of acids observed. Reduced amount of acetate, while growing on glycerol, was also found in other studies (17). Since glycerol yields a superior performance with higher

enzyme yield and lower by-product formations than other substrates, it is considered the optimal substrate and is chosen for later use in fed-batch fermentation of K-C3for nattokinase production.

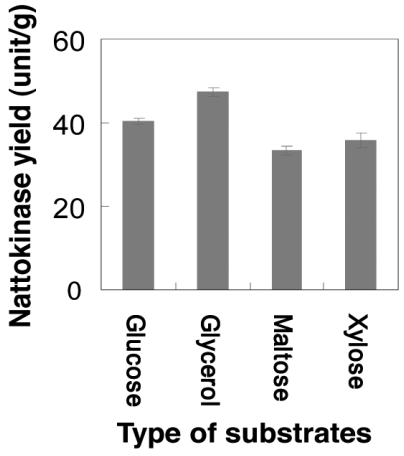

Figure 1. Effect of different substrates on nattokinase production of *B. subtilis* K-C3. Nattokinase yield by K-C3 growing on four substrates: glucose, xylose, glycerol, and maltose in shake flask batch fermentations. The results reveal the highest nattokinase yield is achieved on glycerol. Yield is based on total substrate consumed.

3.2 Design of fed-batch fermentation. A controlled fed-batch strategy was applied to increase titer and productivity of nattokinase. To ensure a balanced growth, enzyme production and glycerol utilization, and avoiding accumulation of by-products, the feed profile was designed based on fed-batch kinetic model by assuming glycerol as the only limiting substrate (Eq 2 and 3). Specific cell growth rate is one important variable for the design of feed. A fed-batch culture that is operated at an optimal specific growth rate ensures a maximum productivity of products (18, 19). In this work, the specific growth rate was set constantly at 0.2 h^{-1} during fed-batch since this was reported as an optimal growth rate of *Bacillus* for maximum enzyme production (20,21). This rate is approximately 51% of the maximum specific growth rate as observed in batch fermentation. The cell growth rate can be controlled during fed-batch operation via an exponential feed rate of glycerol (Eq (4)). Other kinetic parameters including cell yield, nattokinase yield and the specific rate used for estimation of feeding rate were obtained experimentally from the batch culture. Feed medium composition for fed-batch was designed based on batch fermentation. During batch culture cell growth ceased after 23 h, indicating that a certain component became growth-limiting. At this time point, only 64% of glycerol was utilized, implying that glycerol was not the growth-limiting substrate. In this case soy peptone, an organic nitrogen source, is the growth-limiting compound. The results allowed for an estimation of the balanced ratio between soy peptone and glycerol to be 1.65 g-soy peptone/g-glycerol (Eq (1)). This ratio was then used for designing feed medium during fed-batch culture. To ensure glycerol as the only growth-limiting substrate during fed-batch operation, soy peptone was added 50% in excess of the computed value in the feed solution. Oxygen is also found to be critical for nattokinase synthesis (result not shown). Thus, a feedback loop control of dissolved oxygen by variation of agitation speed was applied. Such control loop was to increase the dissolved oxygen concentration in the culture by increasing of agitation rate. Once the agitation speed reached its maximum limit, oxygen-enriched air was then used to increase oxygen transfer rate to the culture and aeration rate was increased to help keeping DO at the controlled level for ensuring oxygen sufficient condition throughout the fed-batch process.

3.3 Fed-batch fermentation for maximum nattokinase production. Time profiles for cell growth, enzyme production and residual glycerol of a controlled fed-batch culture using a designed feed of glycerol at a constant specific growth rate is shown in Fig. 2A. During

the batch process, cell grew exponentially, whereas the DO% decreased due to an increasing oxygen demand of the growing cell. Feeding of glycerol was initiated 6 h after inoculation. At this time, the feed medium was added at the predetermined exponential rates into the culture. Throughout fed-batch operation, glycerol concentration stayed below 2 g/l indicating a well-balanced feed of substrate and substrate consumption rate of the cell culture. The maximum acetate concentration was 6.1 g/l. Evaluation of feed strategy resulted in the specific growth rate of 0.20±0.01 h^{-1} during fed-batch culture which well matched with the determined value from the model. This indicates that the specific growth rate can be well controlled at the set point through an exponential feed strategy based on kinetic model (Eq (2-4)). The predetermined and measured values of glycerol feed rate and added volume are consistent as shown in Fig 2B.The dual control systems for dissolved oxygen by increasing agitation and aeration rates allowed for maintaining the DO at relatively 5–20% of total saturation of the medium. When DO in the culture dropped below 20%, the agitation rate was increased via a feedback loop control to maintain DO at above zero to ensure oxygen sufficiency. The aeration rate was also increased to guarantee the sufficient supply of oxygen demanded of the cell. A typical time course of the cultivation of K-C3 under application of the described control strategies was shown in Fig. 2C. With the optimized feed strategy based on kinetic model for fed-batch fermentation, the cell concentration reaches its maximum of 73.47±2.64 g-cdw/l (OD_{600} of approximately 160.88±5.77) at 24 h and high titer of nattokinase of 9198.51±268.10 units/ml was achieved. The volumetric nattokinase production rate in fed-batch cultivation was 545.95±27.88 units/ml-h which was 20 times higher than that in batch cultivation. The high titer and high productivity obtained indicated the efficiency of feed strategy used in fed-batch culture resulting in an optimal production of nattokinase. The growth characteristics and enzyme production phenotype are summarized in Table 1.

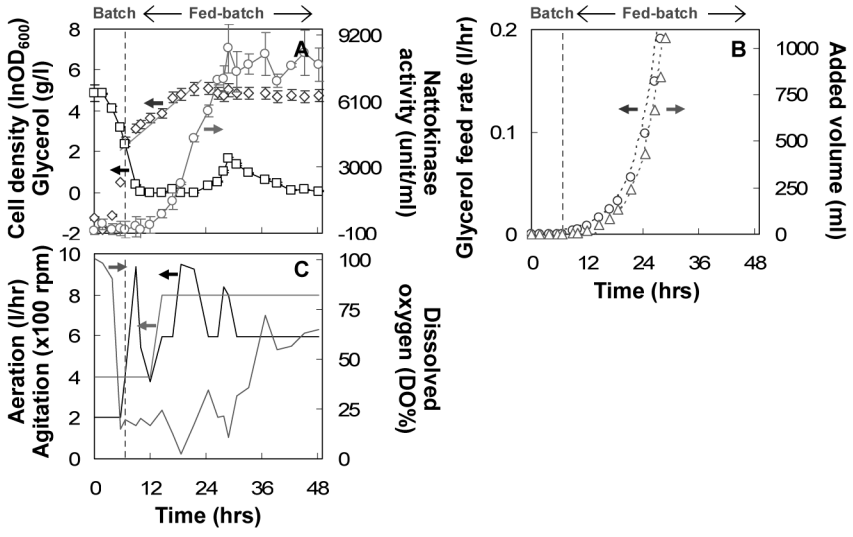

Figure 2. Fed-batch fermentation for production of nattokinase by *B. subtilis* K-C3 with glycerol as a feed substrate. (A) Time profiles of cell growth (◇), glycerol concentration (□), and nattokinase activity (○). Cell growth was maintained at a constant rate by a control of glycerol feeding. The specific growth rate was controlled at 0.2 h^{-1}. The observed growth rate matches well with the predicted values (-). (B) Dissolved oxygen (-), agitation rate (-) and aeration rate (-) in a controlled fed-batch fermentation. Dissolved oxygen was kept above zero by increasing agitation speed and aeration rate. (C) Glycerol feeding rate (○) and added culture volume (△) during fed-batch operation. The experimental values are in agreement with the computed values by the fed-batch kinetic model Eq (4) shown in dash lines.

Table 1. Summary of cell growth and nattokinase kinetics of *B. subtilis* K-C3 in an O_2-controlled, glycerol feeding, fed-batch process. Standard deviation is from duplicate measurements.

Cell growth and Nattokinase production	
Cell density [OD_{600nm}]	160.875±5.777
Cell density [g-cdw/l]	73.472±2.638
Nattokinase activity [units/ml]	9198.51±268.097
Specific activity [units/ml-OD_{600nm}]	57.245±3.722
Cell growth rate [h^{-1}]	0.202±0.01
$R_{Nattokinase}$ [units/ml-h]	545.955±27.881
Y_X [g-cdw/g-glycerol]	0.49±0.017
$Y_{Nattokinase}$ [units/g-glycerol]	61.36±1.804
$Y_{Acetate}$ [g/g-glycerol]	0.041±0.000
$Y_{Succinate}$ [g/g-glycerol]	0.004±0.000

In summary, we described fermentation optimization in fed-batch process that resulted in high yield, high titer and high productivity of nattokinase by *B. subtilis* K-C3.Glycerol as substrate was suggested by batch fermentation results as the optimal cultivation for high yield of nattokinase synthesis. These optimal parameters were extended to fed-batch fermentation to improve titer and productivity of nattokinase by *B. subtilis*. Using a successful feed strategy in fed-batch fermentation, low concentration of substrate and by-products was maintained and good production of enzyme was achieved compared to the batch results. Using the feed strategy designed by kinetic model, the levels of nattokinase activity of 9198.51 units/ml was achieved which was the highest reported in literatures to date for the production of nattokinase in *B. subtilis* (12,22,23,24). The results shown here prove the feasibility of these strategies that can be used to achieve an efficient production at high yield, high titer and high productivity of other enzymes.

4. References

(1) Sumi, H., Nakajima, N., Mihara, H. In vitro and in vivo fibrinolytic properties of nattokinase. Thromb Haemost. 1992; 89:1267.

(2) Fujita, M., Hong, K., Ito, Y., Fujii, R., Kariya, K., Nishimuro, S. Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. Biol Pharm Bull. 1995; 18(10):1387-1391.

(3) Tai, M. Nattokinase for prevention of thrombosis. Am J Health Syst Pharm. 2006; 63:1121-1123.

(4) Hsia, C.H., Shen, M.C., Lin, J.S., Wen, Y.K., Hwang, K.L., Cham, T.M., Yang, N.C. Nattokinase decreases plasma levels of fibrinogen, factor VII, and factor VIII in human subjects. Nutr Res. 2009; 29(3):190-196.

(5) Kim, J.Y., Gum, S.N., Paik, J.K., Lim, H.H., Kim, K.C., Ogasawara, K., Inoue, K., Park, S., Jang, Y., Lee, J.H. Effects of nattokinase on blood pressure: a randomized, controlled trial. Hypertens Res. 2008; 31:1583-1588.

(6) Wu, D.J., Lin, C.S, Lee, M.Y. Lipid-lowering effect of nattokinase in patients with primary hypercholesterolemia. Acta Cardiol Sinica. 2009; 25:26-30.

(7) Hellmuth, K., Korz, D.J., Sanders, E.A., Deckwer, W.D. Effect of growth rate on stability and gene expression of recombinant plasmids during continuous and high cell density cultivation of *Escherichia coli* TG1. Biotechnol. 1994; 32:289-298.

(7) Peng, Y., Yang, X., Zhang, Y. Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo. Appl Microbiol Biotechnol. 2005; 69:126-132.

(8) Riesenberg, D. High-cell-density cultivation of *Escherichia coli*. Curr Opin Biotechnol. 1991; 2(3):380-384.

(9) Park, Y.S., Kai, K., Lijima, S.J., Kobayashi, T. Enhanced b-Galactosidase production by high cell-density culture of recombinant *Bacillus subtilis* with glucose concentration control. Biotechnol Bioeng. 1992; 40:686-696.

(10) Huang, H., Ridgway, D., Gu, T., Moo-Young, M. Enhanced amylase production by *Bacillus subtilis* using a dual exponential feeding strategy. Bioproc Biosyst Eng. 2004; 27:63-69.

(11) Skolpap, W., Scharer, J.M., Douglas, P.L., Moo-Young, M. Fed-batch optimization of [alpha]-amylase and proteaseproducing *Bacillus subtilis* using Markov chain methods. Biotechnol Bioeng. 2004; 86:706-717.

(12) Liu, J., Xing, J., Chang, T., Ma, Z., Liu, H. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Proc Biochem. 2005; 40:2757-2762.

(13) Anson M.L. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen Physiol. 1938; 22(1):79-89.

(14) Choi, J.H., Lee, S.J., Lee, S.J., Lee, S.Y. Enhanced production of insulinlike growth factor I fusion protein in *Escherichia coli* by coexpression of the down-regulated genes identified by transcriptome profiling. Appl Environ Microb. 2003; 69: 4737-4742.

(15) Yee, L., Blanch, H.W. Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*. Biotechnol Bioeng. 1993; 41:781-790.

(16) Nakano, M.N., Dailly, Y.P., Zuber, P., Clark, D.P. Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J Bacteriol. 1997; 179:6749-6755.

(17) Lee, S.Y. High cell-density culture of *Escherichia coli*. Trends Biotechnol. 1996; 14:98-105.

(18) Koo, T.Y., Park, T.H. Increased production of recombinant protein by *Escherichia coli* deficient in acetic acid formation. J Microbiol Biotechnol. 1999; 9:789-793.

(19) Yoon, S.K., Kang, W.K., Park, T.H. Fed-batch operation of recombinant *Escherichia coli* containing trp promoter with controlled specific growth rate. Biotechnol Bioeng. 1994; 43:995-999.

(20) Emanuilova, E.I., Toda, K. a-Amylase production in batch and continuous culture by *Bacillus caldolyticus*. Appl Microbiol Biotechnol. 1984; 19:301-305.

(21) Schwab, K., Bader, J., Brokamp, C., Popović, M.K., Bajpai, R., Berovic, M. Dual feeding strategy for the production of alpha-amylase by *Bacillus caldolyticus* using complex media. N Biotechnol. 2009; 26(1-2):68-74.

(22) Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S.V., Senthilkumar, S.R., Sangiliyandi, G. Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. Bioresource Technol. 2008; 99:8170-8174.

(23) Wang, D.S., Torng, C.C., Lin, I.P., Cheng, B.W., Liu, H.R., Chou, C.Y. Optimization of nattokinase production conduction using response surface methodology. J Food Process Eng. 2006; 29:22-35.

(24) Ku, T.W., Tsai, R.L., Pan, T.M. A simple and cost-saving approach to optimize the production of subtilisin NAT by submerged cultivation of *Bacillus natto*. J Agric Food Chem. 2009; 57:292-296.