High cell density cultivation of a recombinant Bacillus subtilis for nattokinase production

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Abstract

Several hundred U mL-1 of Nattokinase (NK), a fibrinolytic enzyme, can be produced by culturing recombinant Bacillus subtilis in Luria-Bertani broth in a shaking flask. For use as a nutraceutical, large-scale preparation and a simple purification process is required. The present study utilized a fed-batch process with a pH-stat and low-glycerol-level-maintain feeding strategy to cultivate a B. subtilis strain carrying a pHT01 plasmid with an NK-encoding gene (B. subtilis/pHT01-aprN1). Finally, a NK activity of 7778 ±17.28 U mL-1 was obtained, which represented a 26-fold increase of NK activity by high cell density cultivation compared to the flask culture. Furthermore, fermentation supernatant was successively purified by ammonium sulfate precipitation and nickel column affinity chromatography with a total NK recovery rate of 65.2%.

Keywords

Nattokinase; *Bacillus subtilis* ; recombinant protein expression; high cell density fermentation; induction and feeding strategy

Introduction

Thrombosis, which is responsible for high morbidity and mortality in humans [1], can be effectively treated by thrombolytic drugs, but these are associated with adverse effects [2, 3]. Therefore, it is necessary to develop new biological substances, especially prophylactic food-source thrombolytic agents with low immunogenicity and preventative, long-term effects that are convenient for oral administration and stable in the gastrointestinal tract.

Nattokinase (NK), which decreases the ability of blood to clot, is traditionally taken from natto, a Japanese solid-state fermented soybean food [5, 6]. Currently, NK is used as a dietary supplement as well as a prophylactic or a curative thrombosis medicine [4, 5]; however, the process used to purify it from natto is complicated and accompanied by significant loss of bioactivity. Accordingly, fed-batch fermentation by adopting genetically modified bacteria may improve enzyme yield [7-9].

Several reports have shown that fed-batch culture led to increases in NK activity of 2.1–25-fold relative to batch culture and that the addition of glycerol during the cell growth phase increased NK production significantly [11–15]. Moreover, various protein purification methods have been utilized for NK purification [4, 16]. Taken together, these findings suggest that the expression of NK by genetically modified bacteria may reach a much higher level if efficient protocols for high cell density fermentation and subsequent purification are obtained.

We previously constructed a *B. subtilis* 168 strain containing a pHT01-*aprN* plasmid [10]. In this study, we further investigated the expression of NK by culturing this strain under different induction and feeding strategies. In addition, we investigated the efficiency of purification with ammonium sulfate precipitation and Ni–NTA affinity chromatography.

Materials and Methods

Microorganism and inoculum preparation

A gene-modified strain (*B. subtilis*168/pHT01-*aprN1*) was used to express NK. The medium for flask and fed-batch culture was as follows: yeast extract, 60 g L^{-1}; glucose, 40 g L^{-1}; glycerol, 20 mL L^{-1}; NH_4Cl, 3 g L^{-1}; $K_2HPO_4{\cdot}3H_2O$, 1 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 2 g L^{-1}; $MnSO_4{\cdot}H_2O$, 0.02 g L^{-1}; $FeSO_4{\cdot}7H_2O$, 0.05 g L^{-1}; $CoCl_2{\cdot}6H_2O$, 0.01 g L^{-1}; $ZnCl_2$, 0.01 g L^{-1}; 2,6-pyridinedicarboxylic acid, 0.167 g L^{-1}. Glucose and $MgSO_4{\cdot}7H_2O$ were autoclaved separately and aseptically added to the medium. Nutrient solution contained 100 g L^{-1} yeast extract and 250 mL L^{-1}glycerol was used as feed medium. To avoid plasmid loss and prevent microbial contamination, chloramphenicol (5 μg mL^{-1}) was added to all media after being dissolved in anhydrous ethanol and filtered through a 0.22 μm membrane.

Shake flask experiments

To prepare a stock culture, one colony of *B. subtilis*168/pHT01-*aprN1* was inoculated in 1 mL Luria-Bertani (LB) medium and then incubated overnight at 37°C in a shaking incubator at 200 revolutions per minute (rpm). A flask with 25 mL fed-batch culture media was then inoculated with 7.5% (v/v) of stock culture and incubated at 37°C until reaching mid-log phase (OD_{600} of 0.8–1.0). Next, the temperature was adjusted to 28°C and held for 20 min, after which a 25-μL aliquot of 0.5 mmol L^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the culture was further incubated for 45 h at 28°C in a shaking incubator (200 rpm) for NK expression.

Fed-batch experiments

An in-situ sterilizable 15 L bioreactor (BIOSTAT®B, Germany) equipped with pH and pO_2 probes was used for fed-batch experiments. One milliliter of glycerol stock bacteria was inoculated into 100 mL LB medium and cultured for 12 h at 37°C in a shaking incubator (200 rpm), after which the pre-culture solution was aseptically inoculated into 1 L LB medium and incubated under the same conditions for 12 h. Next, 700 ml of seed cultures were inoculated for 7-L fed-batch fermentation in the bioreactor. The value of oxygen dissolved was set at 20%, which was cascade controlled by agitation speed (300–1000 rpm) and air flow rate (3–20 L min^{-1}). The pH was set as 7.0 and automatically controlled by adding 2 mol L^{-1} HCl or 2 mol L^{-1} NaOH.

In the first fed-batch experiment (FB A), the temperature was adjusted to 28°C at 3.5 h, while IPTG was added 30 min later as the OD_{600} value reached about 18. A total of 600 mL feed media was supplemented at 8 h with a flow rate of 30 mL min^{-1}. In the second fed-batch experiment (FB B), the expression was induced at 5 h when the OD_{600} value had reached 27, and the feed media was constantly fed after 14 h of cultivation with a flow rate of 6 mL min^{-1} for 1 h, then 3.6 mL min^{-1} for 2 h, and finally 2.4 mL min^{-1} for another hour. In the third experiment (FB C), the expression was induced at 3 h when the OD_{600} reached about 9 and the feed media was fed at 10 h. The feeding rate depended on the content of glycerol, which was controlled at a concentration of 50 mmol L^{-1}. Cell growth was monitored at various times by measuring the OD_{600} values.

NK activity assay

Quantitative analysis of NK activity was conducted by the fibrin plate method, with slight modification [17]. Briefly, bovine fibrinogen (Sigma, St. Louis, MO, USA) and thrombin (Sigma, St. Louis, MO, USA) were dissolved in 0.1 mol L^{-1} sodium phosphate buffer (PBS) at pH 7.4. An equi-voluminal mixture of 5 g L^{-1} bovine fibrinogen solution and 12 g L^{-1} agarose solution was then warmed in a 45°C bath, after which 10 μL of 500 U thrombin was added to 15 mL of the mixture solution in a 90 mm petri dish and kept at room temperature for 1 h to form fibrin. Holes with a 2 mm diameter were made on the fibrin plate and 10 μL of each diluted supernatant of fermentation broth was then added. The plates were subsequently incubated at 25°C for 16 h, after which the areas of the lysis zones on the fibrin plates were measured and the fibrinolytic activities were determined according to the standard curve of urokinase.

Glycerol concentration measurement

The concentration of glycerol was determined enzymatically using a free glycerol assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's procedures.

Purification and

lyophilization of NK

The final fermentation broth was collected and centrifuged (GL-25M, Luxiangyi, Shanghai, China) immediately at 18,300 × g for 10 min. Solid $(NH_4)_2SO_4$ was then gradually added until the supernatant was 20% saturated, after which it was stored at 4°C overnight. Next, the broth was centrifuged at 18,300 × g for 10 min and the precipitate was discarded. Additional $(NH_4)_2SO_4$ was subsequently added to make the supernatant 60% saturated, after which the mixture was stored at 4°C overnight again. Following centrifugation at 18,300 × g for 30 min, the supernatant was removed and the precipitate was dissolved in 10 mmol L^{-1} PBS (pH 7.4) as a crude enzyme. The crude enzyme was further purified by Ni–NTA affinity chromatography according to the manufacturer's (Novagen, San Diego, CA, USA) protocols. After purification, 10 μl of eluted fractions of different dilution times were used to assay their fibrinolytic activities. Residual solution was desalted by dialysis and lyophilized using a GLZY-0.5B vacuum freeze drier (Pudong Freeze Drying Equipment, Shanghai, China) to obtain NK Lyophilized powder.

SDS-PAGE and Western blotting analysis

SDS-PAGE protein analysis was performed in a Mini-Protean Tetra system (BioRad, Hercules, CA, USA) by loading 30 μL of 1: 1 (v: v) boiled supernatant and dye buffer onto a 4–20% precast Mini protean TGX gel (BioRad) and then running the samples in 1 × Tris-glycine-SDS running buffer (BioRad, Shanghai, China) at 100 V for 15 min followed by 200 V for 25 min. A protein standard (10-250 kDa, Precision Plus Protein Kaleidoscope, Bio-Rad) was used as a ladder.

NK was identified by Western blotting analysis as described by Towbin, with slight modification [18]. Briefly, 10 μL aliquots of different supernatants were separated by 15% SDS–PAGE. After electrophoresis, the protein bands were transferred from the gel to an Amersham nitrocellulose (NC) membrane (GE Healthcare, Piscataway, NJ, USA) using a Mini Trans Blot electrophoretic transfer cell (BioRad). NK on the membrane was reacted with a mouse monoclonal antibody (Yisheng Biological Technology, Shanghai, China) against His-tag (1:1000 v/v), then incubated with anti-mouse *Ig* G alkaline phosphatase (Sigma) conjugate (1:2000 v/v). After washing, the NC membrane was stained using Super ECL Detection Reagent (Yisheng Biology Technology, Shanghai, China) according to the manufacturer's instructions, then covered by an X-ray film and exposed for 1 min. A Tanon v.3500 Gel Imaging System (Tanon Co., Shanghai, China) was used for the analysis of proteins.

Results and discussion

NK expression by flask culture and fed-batch experiment

At least five different NK activity assay methods have been reported to date [4, 11, 12, 17, 19,]. Among these, the fibrin plate method and chromogenic method are the most widely used, but the casein-degradation and JBSL (Japan Bio Science Laboratory Co., Ltd) methods may show much higher values of enzyme activity [11, 12, 14].

The NK activity obtained at 24 h in flask culture was 380.14 ± 5.71 U mL^{-1}, while the OD_{600} reached 13.45 ± 0.45.

Many studies have demonstrated the significant contribution of media ingredients [20, 21] and nutrient feeding strategy to NK production [4, 15]. Berenjian confirmed that glycerol was a noteworthy carbon source influencing cell density during the fermentation of *B. subtilis* natto, and that the highest activity of NK was obtained by adding 3% glycerol as a carbon source [15]. It has also been reported that 2,6-pyridine dicarboxylic acid (PDCA) and metal ions such as Ca^{2+} and Mg^{2+} could improve osmotic pressure and help to maintain enzyme conformation, thereby improving the NK activity [10, 22]. Moreover, Wang demonstrated

that glucose, $K_2HPO_4.3H_2O$ and $MgSO_4{\cdot}7H_2O$ played key roles in the production of NK, and they obtained an activity of 12.34 FU mL^{-1} [23]. Taken together, the results of these studies showed that NK activity could be improved dozens of times by media optimization.

In addition to media, feeding solutions are critical factors that influence NK activity, which should support cell growth and recombinant protein production while avoiding substrate inhibition and other related problems [12, 15].

Based on this information, we selected a mixture of glycerol, yeast extract, PDCA and a concentrated inorganic mixture solution as the fermentation broth, and a mixture of glycerol and yeast extract as the feed broth.

The strategy of induction, including cell density at the time of induction, inducer concentration, pre-induction growth and post-induction incubation time, can also affect the efficiency of protein expression. The aim of this study was to investigate the effects of using pH-stat and low-glycerol-level-maintaining strategies on NK expression by *B. subtilis* 168/pHT01-*apr* N1.

Three experiments were performed to examine the effects of induction time, feeding time and feeding rate on the NK activity of fermentation broth. The results are presented in Figure 1 and Table 1.

For FB A, the OD_{600} increased rapidly from the second hour. Although we fed 600 mL of media into the fermenter at the eighth hour, the nutrition was not sufficient for cell growth as indicated by the glycerol concentration decreasing rapidly to about 70 mmol L^{-1} from 4 to 12 h and the OD_{600}not varying markedly after 13 h. However, the NK activity still increased significantly until 17 h. The final NK activity was 2910.5 $\pm$ 21.6 U mL^{-1} and the specific activity was 30.32 U ml^{-1} $OD_{600}{}^{-1}$.

For FB B, we attempted to achieve higher enzyme production via higher cell density; hence, the expression was induced at the fifth hour, which was 1 h later than FB A, and the induced OD_{600} was up to 27.3 $\pm$ 1.0, which was higher than that of FB A (17.6 $\pm$ 0.4). The feeding time of FB B was 14 h, when the glycerol concentration was as low as 119.8 $\pm$ 1.3 mmol L^{-1}, which might have favored cell growth by reducing substrate inhibition. The glycerol content was sufficient to support the cell growth for 24 h, and the OD_{600} reached a high value of 208.8 $\pm$ 1.9 at 24 h, with the highest activity reaching 4521.8 $\pm$ 23.8 U mL^{-1}. Interestingly, the specific activity was 21.66 U $ml^{-1}OD_{600}{}^{-1}$ at 24 h, which was lower than that of FB A, and the NK activity did not synchronously increase following cell growth during the late fermentation stage, which implied that there should be a balance between the cell growth rate and enzyme expression. Consequently, we did not unilaterally pursue high cell density in FBC, and instead induced at an earlier time and kept the glycerol content low during the feeding period in FB C.

For FB C, continuous feeding was adapted, starting at 10 h when the OD_{600} had reached 105.3 $\pm$ 1.1 and almost two-thirds of the initial glycerol had been consumed. The glycerol concentration was controlled to around 50 mmol L^{-1} by adjusting the feeding rate.

As expected, an activity of 7778.0 $\pm$ 17.3 U mL^{-1} and a specific activity of 44.86 U $ml^{-1}OD_{600}{}^{-1}$ was achieved at 20 h, and these values were 1.7-, 2.6-, and 26-fold higher than those of batch B, batch A and the flask culture, respectively. These values were also higher than those of other studies in which the reported NK production levels were 587 U mL^{-1} [15], 7100 U mL^{-1} [12], and 3194.3 U mL^{-1}[4], respectively.

Recovery rate of NK by purification

The recovery rate of NK obtained by ammonium sulfate precipitation was 89.1% (Table 2), which was consistent with a study by Garg [16]. The high recovery rate and simple operation showed that this method could be used to purify NK on a large scale.

The purification was followed by Ni–NTA affinity chromatography, and the total recovery rate was 65.2% (Table 2). The high imidazole concentration in wash buffer B led to a high loss of NK when samples were purified using Ni–NTA affinity chromatography. Accordingly, the imidazole concentrations were adjusted to zero in wash buffer A and incubated supernatant, and to 10 mmol L^{-1} in wash buffer B; thus, a recovery rate

of 88% was obtained in this step. Different imidazole concentrations in elution buffer C were also investigated, but there were no significant differences in the range of 100 mmol L^{-1} to 500 mmol L^{-1}. According to the NK expression level and the recovery rate, the process developed here may be applied for large scale production of NK.

SDS-PAGE and Western blotting analysis of NK

SDS-PAGE analysis demonstrated that a 28 kDa protein was a crucial component in the supernatant from induced *B. subtilis*168/pHT01-*apr* N1, but that it was not present in the supernatant from *B. subtilis* 168/pHT01 and non-induced *B. subtilis* 168/pHT01-*apr* N1 fermentation broth (Fig. 2 A/B), suggesting that recombinant NK could be expressed in a soluble form. Western blotting with a His-tag-specific monoclonal antibody also showed a specific signal at 28 kDa, whereas no cross-reaction occurred in the total soluble proteins from non-induced *B. subtilis* 168/pHT01-*apr* N1 broth,which confirmed that the 28 kDa protein was the recombinant NK, as expected (Fig. 2 C).

In view of these reports, NK was produced by fed-batch cultures of recombinant *B. subtilis* , and its production was improved to 7,778 U/mL from 380 U/mL of flask culture using pH-stat and low-glycerol-level strategies. Future studies will design a process and set a kinetic model for fermentation optimization.

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Conflict of interest

The authors have no conflicts of interest to declare.

References

[1] Wei, X., Luo, M., Xie, Y., Yang, L., Li, H., Xu, L., Liu, H. (2012) Strain screening, fermentation, separation, and encapsulation for production of nattokinase functional food. *Appl. Biochem. Biotechnol.* 168:1753-1764.

[2] Aloni, P. D., Nayak, A. R., Chaurasia, S.R., Deopujari, J.Y., Chourasia, C., Purohit, H.J., Taori, G.M., Daginawala, H.F., Kashyap, R.S. (2015) Effect of Fagonia arabicaon thrombin induced release of t-PA and complex of PAI-1 tPA in cultured HUVE cells. *J. Tradit. Compl. Med.* 6:219-223.

[3] Peng, Y., Yang, X., Zhang, Y. (2015) Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo. *Appl. Microbiol. Biotechnol.* 69:126-132.

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

[5] Yanagisawa, Y., Chatake, T., Chibakamoshida, K., Naito, S., Ohsugi, T., Sumi, H., Yasuda, I., Morimoto, Y. (2010) Purification, crystallization and preliminary X-ray diffraction experiment of nattokinase from *Bacillus subtilis* natto. *Acta Crystallogr. Sect. F: Struct Biol Cryst Commun.* 66:1670-1673.

[6] Fujita, M. , Nomura, K., Hon,g K., Ito, Y. A. Asada, S. Nishimuro (1993) Purification and Characterization of a Strong Fibrinolytic Enzyme (Nattokinase) in the Vegetable Cheese Natto, a Popular Soybean Fermented Food in Japan. *Biochem. Biophys. Res. Commun.* 197:1340-1347.

[7] Chiang, C.J., Che, H.C., Chao, Y.P., Tzen, J.T. (2005) Efficient system of artificial oil bodies for functional expression and purification of recombinant nattokinase in *Escherichia coli .J. Agric. Food Chem.* 53:4799-4804.

[8] Liang, X., Jia, S., Sun, Y., Chen, M., Chen, X., Zhong, J., Huan, L. (2007) Secretory Expression of Nattokinase from *Bacillus subtilis* YF38 in *Escherichia coli* . *Molecul. Biotechnol.*37:187-194.

[9] Ni, H., Guo, P.C., Jiang, W.L., Fan, X.M., Luo, X.Y., Li, H.H. (2016) Expression of nattokinase in *Escherichia coli* and renaturation of its inclusion bod. *J. Biotechnol.* 231:65-71.

[10] Cui, Q., Qian, B.J., Yao, X.M., Ji, S.L., Zhang, J.H. (2017) Construction of nattokinase-producing genetic engineering strain and its activity analysis. *Food Sci. China* , 38:1-8.

[11] Chen, P.T, Chiang, C.J., Chao, Y.P.. (2007) Medium optimization for the production of recombinant nattokinase by *Bacillus subtilis* using response surface methodology. *Biotechnol. Prog.*23: 1327–1332.

[12] Cho, Y.H., Song, J.Y., Kim, K.M., Kim, M.K., Lee, I.Y., Kim, S.B., Kim, H.S., Han, N.S., Lee, B.H., Kim, B.S. (2010) Production of nattokinase by batch and fed-batch culture of *Bacillus subtilis* .*New Biotechnol.* 27:341-346.

[13] Dabbagh, F., Negahdaripour, M., Berenjian, A., Behfar, A., Mohammadi, F., Zamani, M., Irajie, C., Ghasemi, Y. (2014) Nattokinase: production and application, *Appl. Microbiol. Biotechnol.*98:9199-9206.

[14] Kwon, E.Y., Kim, K.M., Kim, M.K., Lee, I.Y., Kim, S.B. (2011) Production of nattokinase by high cell density fed-batch culture of*Bacillus subtilis* . *Bioprocess Biosyst. Eng.* 34:789-793.

[15] Berenjian, A., Mahanama, R., Kavanagh, J., Dehghani, F., Ghasemi, Y. (2014) Nattokinase production: Medium components and feeding strategy studies. *Chem. Ind. Chem. Eng.* 20:541-547.

[16] Garg, R., Thorat, B.N. (2014) Nattokinase purification by three phase partitioning and impact of t-butanol on freeze drying. *Sep. Purif. Technol.* 131:19-26.

[17] Kim, W., Choi, K., Kim, Y., Park, H., Choi, J., Lee, Y., Oh, H., Kwon, I., Lee, S. (1996) Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.*62:2482-2488.

[18] Towbin, H., Staehelin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Anal. Biochem.* 76:459-471.

[19] Friberge, P., Knos, M., Gustavsson, S., Aurell, L., Claeson, G. (1978) Methods for determination of plasmin, antiplasmin and plasminogen by means of substrate S-2251. *Haemostasis* 7:138-145.

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

[21] Chen, P.T., Chao, Y. (2006) Enhanced production of recombinant nattokinase in *Bacillus subtilis* by the elimination of limiting factors. *Biotechnol. Lett.* 28:1595–1600.

[22] Ikeda, S., Ohsugi, T.T., Sumi, H. (2006) Activation of fibrinolysis (Nattokinase) induced by dipicolinic acid and related compounds. *Food Sci. Technol. Res.* 12:152-155.

[23] Wang, J.K., Chiu, H.H., Hsieh, C.S. (2009) Optimization of the medium components by statistical experimental methods to enhance nattokinase activity. *Fooyin J. Health Sci.* 1:21-27.

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