CI&CEQ
Chemical Industry & Chemical Engineering Quarterly

Available on line at
Association of the Chemical Engineers of Serbia *AChE*
www.ache.org.rs/CICEQ

Chem. Ind. Chem. Eng. Q. 20 (4) 541–547 (2014) CI&CEQ

AYDIN BERENJIAN[1]
RAJA MAHANAMA[1]
JOHN KAVANAGH[1]
FARIBA DEHGHANI[1]
YOUNES GHASEMI[1,2]

[1]School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney, Australia
[2]Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

SCIENTIFIC PAPER

UDC 615:577.15:633.34

DOI 10.2298/CICEQ130928037B

NATTOKINASE PRODUCTION: MEDIUM COMPONENTS AND FEEDING STRATEGY STUDIES

Article Highlights

- The highest nattokinase activity of 587 U/mL was obtained in the fermenter
- Maximum nattokinase activity was resulted when 3% fed-batch glycerol was added
- Results demonstrate a 25-fold increase of nattokinase activity

Abstract

In the present study, the effect of nutrients on nattokinase activity during the fermentation of Bacillus subtilis natto was investigated. The highest nattokinase activity of 587 U/mL was obtained in fermenter for a media consisting of yeast extract (6%), soy peptone (1.2%) and glycerol (6%). The second order polynomial equation was fitted to the results by using central composite face design. The polynomial model fitted the experimental data well with R^2 = 0.939 and R^2(Adj) = 0.861. In addition, contribution of fed-batch glycerol addition on the nattokinase production pathway was further investigated. Maximum nattokinase activity was resulted when 3% glycerol was added to the fermentation media during the cell growth phase. The results demonstrate a 25-fold increase of nattokinase activity compared to the batch and not optimized culture. This study provides valuable data on the key nutrients and feeding strategy for further investigations and scale up of nattokinase production process.

Keywords: nattokinase, Bacillus subtilis natto, fermentation, fed-batch glycerol.

Nattokinase is an enzyme considered to be a promising remedy for thrombosis healing due to its potent fibrinolytic activity [1]. Due to its presence in food and relatively robust fibrinolytic activity, nattokinase has benefits over other available commercially used drugs in prophylactic and extended effects, particularly due to stability in the GI tract and comfortable oral administration [1]. Oral administration of nattokinase could diminish plasma levels of fibrinogen, factor VIII, and factor VII which may be useful as a nutraceutical for cardiovascular disease [2-4]. In adults suffering hypercholesterolemia, nattokinase was well endured and had decreasing effects on the serum cholesterol level together with low cholesterol diet. Intravenous administration of fibrinolytic drugs such as tissue plasminogen activator (tPA) and urokinase has been extensively used in clinical platform for thrombolytic treatment [5]. In this regard, due to the costly prices and the detrimental side effects, microbial fibrinolytic enzymes such as nattokinase have now pulled toward much more consideration than current thrombolytic drugs [4].

Nattokinase is traditionally produced by fermentation of various microorganisms, among which the genus *Bacillus subtilis* natto is the preeminent nattokinase producer [1,6-8]. In biotechnology-based processes, the formulation of culture media (mainly carbon and nitrogen sources) is of critical importance; its composition affects the product concentration, yield, and volumetric productivity. To date, several medium optimization studies have been carried out to improve nattokinase production [9-12]. However, lack of knowledge about the sophisticated interactions among various factors and fermentation operating conditions usually results in the complexities and uncertainties in

Correspondence: Y. Ghasemi, Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

E-mail: Ghasemiy@sums.ac.ir; Younes.ghasemi@sydney.edu.au
Paper received: 28 September, 2013
Paper revised: 6 November, 2013
Paper accepted:21 November, 2013

locating the optimum condition. Moreover, feeding limiting nutrients to the culture media may also offer the possibility to enhance the target product concentration [13]. Fed-batch nutrient supplementation protocols can also significantly contribute to enhance the target product concentration and developing the scale up strategies. The aims of this study, therefore, were to investigate and develop an efficient medium and feeding strategy to increase nattokinase production while gaining a better understanding on the process behavior.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Bacillus subtilis natto [9] was cultivated in the media composed of 0.5% peptone, 0.05% yeast extract and 0.5% glucose. Cells were scrapped from tryptic soy agar plates after 5 days and harvested cells were suspended in a 0.9% sodium chloride solution. The spore suspension was kept in water bath at 80 °C for 30 min to inactivate the residues of vegetative cells, centrifuged at 3000 rpm for 10 min to remove the cell debris, diluted with 0.9% sodium chloride solution to obtain the standard solution of $(5.2\pm0.5)\times10^{10}$ spores/mL.

Materials

Chromogenic substrate *N*-succnyl-Ala-Ala-Pro--Phep-nitroanilide (S-7388) and *p*-nitroaniline were procured from Sigma-Aldich. Yeast extract was purchased from BD (USA). Glycerol was purchased from Chem-Supply (Australia). Acetic acid was obtained from Sigma-Aldrich (USA) and soy peptone was purchased from Oxoid (UK).

Fermentation

Each media was inoculated with the *B. subtilis* natto spore solution ($5.2\pm0.5\times10^{10}$ spores/ml) using an inoculum size of 2 vol.%. Fermentation was conducted aerobically at 40 °C in an incubator (Thermoline Scientific, Australia) in round bottles (25 ml) at 180 rpm. In each experiment the whole fermentation culturing media was extracted to avoid any sampling errors. The concentrations of 1 to 5% glycerol were used to study the most favourable concentration on nattokinase production. Additionally, a 3-L fermenter (BioFlo/CelliGen 115, New Brunswick Scientific Co., USA) was used to validate the optimized conditions acquired from the small scale studies on nattokinase activity. Temperature, agitation and aeration rates were maintained constant at 40 °C, 600 rpm, and 2 vvm, respectively.

Cell lysis

The *B. subtilis* natto cells were harvested from the fermentation media via centrifugation (6,000*g*) for 15 min at 4 °C and then resuspended in phosphate buffered saline (PBS). *B. subtilis* natto lysis was carried out by sonication on ice (6 times with 1-min intervals, at the 50 W). The sonicated cell suspension was centrifuged at 10,000*g* for 15 min at 4 °C to remove cellular debris. The supernatant (enzyme solution) was used for activity assay [14].

Nattokinase activity measurement

The enzyme activity was quantified by cleavage of the synthetic substrate *N*-succinyl-Ala-Ala-Pro--Phe-p-nitroanilide, as determined by absorbance of product (*p*-nitroaniline) at 405 nm. The reaction mixture containing 0.5 mL of 1 mM synthetic substrate, 0.5 mL of PBS buffter, and 0.5 mL of the enzyme solution was incubated at 37 °C for 10 min. The reaction was then stopped by adding 0.5 mL of 0.2 M acetic acid. The absorbance of released *p*-nitroaniline was measured at 405 nm. One unit of amidolytic activity was expressed as nmol of *p*-nitroaniline released due to substrate hydrolysis/min/mL by the enzyme [14-16].

Glycerol concentration measurement

The concentration of glycerol was determined enzymatically using a free glycerol determination kit (Sigma-Aldrich Co., USA) following its own procedure. Briefly, 800 µL of the glycerol free reagent was inoculated with 10 µL of fermentation media and then incubated for 5 min at 37 °C. The UV-absorbance of resulting solution was measured at 540 nm using the absorbance of water as the reference.

Experimental design

Experimental methods are finding increasing use in manufacturing to optimize the production process. Specifically, the advantage of these methods is to identify the optimum condition for the different factors that can affect the production process. The major classes of designs that are used in industrial experimentation are consist of central composite face (or response surface) design [13]. It is also often adventageous to explore the experimental region of interest at particular points that are not represented by a factorial designs. The central composite design analysis options do not make any assumptions about the number of different factor values or their combinations across the experiment runs. Hence, these options can be used to analyze any type of design to fit to the data of the general model. In this study, Central Composite Face design strategy was employed to optimize the levels of the effective factors on nattokinase activity.

Response surface methodology was used for analyzing the results. The experimental values were scaled factors and the response was described by the quadratic equation. MODDE software version 9 (Umetrics, Sweden) was used to create design matrixes, develop a model and determine optimum concentration of each nutrient. The quality of fit for the regression model equation was expressed as R^2 and the statistical significant was determined by analysis of variance (ANOVA) test. Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Media optimization studies

Our preliminary studies have demonstrated that yeast extract, soy peptone and glycerol are good sources of amino acids and carbon for enhancing *Bacillus subtilis* natto growth rate [9]. In this study, therefore, we investigated the effects of these nutrients on nattokinase production. The CCF design matrix with the actual levels of the nutrient and the results of experimental studies are presented in Table 1. The polynomial model was regressed (Eq. (1)) for the prediction of nattokinase activity as a function of yeast extract, soy peptone and glycerol concentrations:

$$Y = 384 + 33.8X_1 + 65.47X_2 + 61.27X_3 - \\ -47.39X_1X_2 - 28.63X_1X_3 - 93.89X_2X_3 + \\ +30.32X_1^2 - 63.57X_2^2 - 26.19X_3^2 \quad (1)$$

where X_1: yeast extract, X_2: soy peptone X_3: glycerol and Y: nattokinase activity. The coefficients for the model are listed in Table 1, which includes all the linear, quadratic and interaction terms. According to the regression analysis of the experimental design, all single factors showed a significant effect ($p < 0.05$) on nattokinase activity. Interactive model terms X_1X_2 and X_2X_3 with p-values less than 0.05 and quadratic model term X_2^2 were significant combinations on nattokinase activity.

An R^2 value of 0.939 and an adjusted R^2 value of 0.861 confirmed the good model fit. The comparisons between predicted versus observed results demonstrate a good correlation of the model with the experimental data, as shown in Table 2.

The ANOVA test (Table 3) showed the goodness of the model fit as the standard deviation of the regression was much larger than standard deviation of the residuals. The F-value was very high compared to the upper critical value of the F-distribution at 5% significant level. Figure 1 shows the contour plots of nattokinase activity for each pair of variables by keeping the other constant at middle level. These data demonstrate that increasing the glycerol concentration up to 6% resulted in higher nattokinase activity.

At high levels of glycerol concentration, a further increase in soy peptone value (<4%) resulted in decrease in nattokinase activity. There was a high level of interaction between glycerol and yeast extract as shown in Figure 1b. The optimal levels for nattokinase activity were calculated by solving the regression equation inside the region of the experiments. Finally, the highest nattokinase activity of 477 U/mL was predicted by model for the conditions using 6% yeast extract, 1.2% soy peptone and 6% glycerol. The experimental result achieved at this condition was 488 U/mL, which resulted in higher activity with only 3% error as compared to the predicted value.

Nattokinase production was significantly enhanced by the addition of glycerol to the fermentation media. Glycerol also found to be the noteworthy carbon source on promoting cell density during the fermentation of *B. subtilis* natto. Soy peptone, yeast extract and their mixture were used as potent nitrogen

Table 1. Statistical analysis from the central composite face design experiments; X_1 = yeast extract; X_2 = soy peptone; X_3 = glycerol; R^2 = 0.939, R^2(Adj) = 0.861, R^2(Pred) = 0.792, Adeq Precision = 6.442; significance code: P < 0.05

Term	Coefficient	Standard Error	P-value
Constant	384.684	18.6881	1.6027e-007
X_1	33.8083	13.8109	0.0442403
X_2	65.4792	13.8109	0.00210512
X_3	61.2708	13.8109	0.00302077
X_1^2	30.3233	26.6818	0.293161
X_2^2	-63.5725	26.6818	0.0486947
X_3^2	-26.1975	26.6818	0.358885
X_1X_2	-47.3906	15.4411	0.0180896
X_1X_3	-28.6302	15.4411	0.106117
X_2X_3	-93.8906	15.4411	0.000500579

Table 2. Experimental conditions of the central composite face design and responses showing both original and scaled factors

Run	Yeast extract, %	Glycerol, %	Soy peptone, %	Nattokinase activity observed, U/mL	Nattokinase activity predicted, U/mL
1	0.1 (-1)	0.1 (-1)	0.1 (-1)	12.1667	-5.23206
2	6 (+1)	0.1 (-1)	0.1 (-1)	179.5	214.426
3	0.1 (-1)	6 (+1)	0.1 (-1)	410.333	408.289
4	6 (+1)	6 (+1)	0.1 (-1)	462.5	438.385
5	0.1 (-1)	0.1 (-1)	6 (+1)	339.667	362.351
6	6 (+1)	0.1 (-1)	6 (+1)	466.875	467.489
7	0.1 (-1)	6 (+1)	6 (+1)	436.667	400.31
8	6 (+1)	6 (+1)	6 (+1)	299.917	315.885
9	0.1 (-1)	3 (0)	3 (0)	348.083	381.199
10	6 (+1)	3 (0)	3 (0)	476.208	448.816
11	3 (0)	0.1 (-1)	3 (0)	296.458	255.633
12	3 (0)	6 (+1)	3 (0)	340.042	386.591
13	3 (0)	3 (0)	0.1 (-1)	288.583	297.216
14	3 (0)	3 (0)	6 (+1)	422.667	419.758
15	3 (0)	3 (0)	3 (0)	424.667	384.684
16	3 (0)	3 (0)	3 (0)	344.125	384.684
17	3 (0)	3 (0)	3 (0)	396.708	384.684

Table 3. Analysis of variance for quadratic model; DF: degree of freedom; SS: sum of squares; MS: mean sum of squares; SD: standard deviation

Source of variation	*DF*	*SS*	*MS*	*SD*	*F*-value	*P*-value
Total Corrected	16	219025	13689	117	-	-
Regression	9	205673	22852.5	151.171	11.9809	0.002
Residual	7	13351.9	1907.41	43.6739	-	-

sources. The presence of soy peptone and yeast extract mixture significantly improved the nattokinase biosynthesis (Figure 1). This synergistic effect is mainly due to providing a broad range of required amino acids that are crucial for cell metabolism and enzyme production. The results show that a stronger correlation existed between nattokinase biosynthesis and cell density.

Monitoring nattokinase activity in 3-L fermenter

The bacterial growth, pH, nattokinase activity, glycerol and dissolved oxygen levels were measured in a 3-liter fermenter to acquire a better understanding on production pattern and governing factors on nattokinase activity. These results show that there is a correlation between nattokinase production and growth rate of bacteria (Figure 2a). Nattokinase activity was significantly increased within the exponential cell growth and steadily approached to a maximum of 587 U/mL after 24 h. In addition, as it was anticipated while bacteria growth rate was increased steadily, the concentration of glycerol and dissolved oxygen in the media were decreased during the fermentation. Additionally, over the first 10 h of fermentation the glycerol concentration in the media was decreased significantly and it was almost completely consumed by the end of fermentation. These data underlined that glycerol may play a key role in the production of nattokinase. Fed-batch supplementation of glycerol can be further investigated for possible increase on the nattokinase production.

Fed-batch glycerol addition studies

The aim of this study was to investigate the effect of glycerol feeding strategies to optimize the nattokinase production. The Fed-batch glycerol (1 and 3%, of the total fermentation volume) were added to the fermentor media namely at 2, 4, 6, 8, 10 and 12 h after the fermentation startup. Different timings were used to cover the whole *B. subtilis* growth stages during the nattokinase production. Based on the results, addition of glycerol after 10 h of fermentation showed a positive effect on the cell growth and nattokinase production. Addition of glycerol at different cell growth stages can significantly influence the phospholipids composition of cell membrane [12], consequently, cell growth and nattokinase production. The fed-batch addition of glycerol after 10 h of fer-

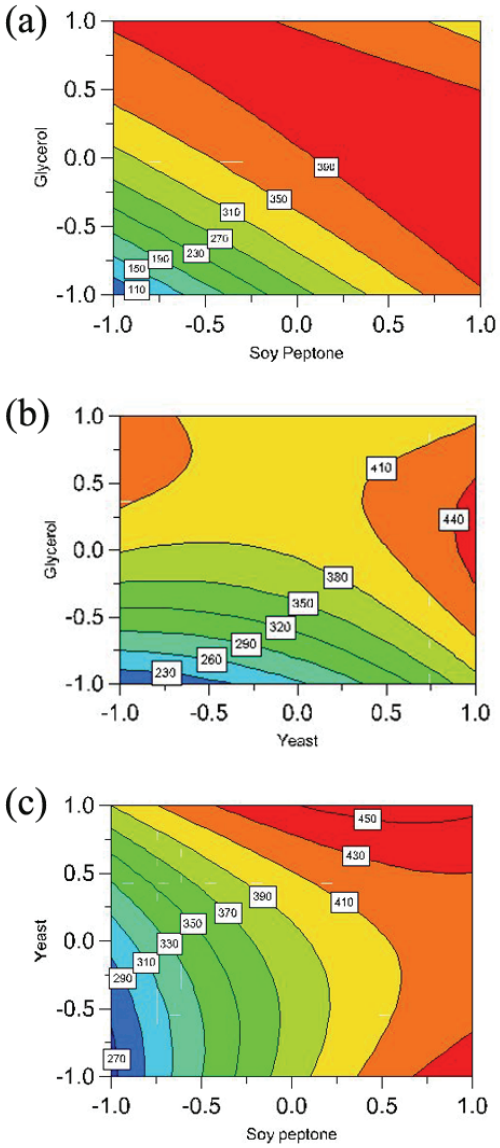

Figure 1. Response surface plot of nattokinase activity showing the effect of: a) glycerol and soy peptone; b) yeast extract and glycerol; c) yeast extract and soy peptone.

mentation was, therefore, selected as the optimum time for enhancing the nattokinase production in the fermentation process.

The effect of addition of various concentrations of glycerol was further investigated to evaluate the optimum concentration on nattokinase production. As depicted in Figure 3, the highest nattokinase activity was resulted when 3% fed-batch glycerol was added to the fermenter which was ~12% higher than the batch culture ($p < 0.05$). Glycerol consumption, pH, bacterial growth and nattokinase production at optimized fed-batch condition are summarized in Figure 2b.

Fed-batch glycerol addition resulted in increasing both nattokinase activity and cell growth as compared to the control media. Upon addition of 3% glycerol in a fed-batch mode, the cell growth was increased 1.6 fold as compared to control media; the nattokinase activity was increased from 587 to 654 U/mL. Fed-batch glycerol addition into the fermentation media may decrease the viscosity, enhances the diffusion of oxygen and consequently the growth rate of *B. subtilis* natto [17-19]. Unrean *et al*. reported the successful production of nattokinase using *B. subtilis* K-C3 while keeping a specific growth rate constant [20]. Authors have used different strategies, operating conditions and nutrients to investigate the role of fed-batch glycerol addition. However, the results of the present study are in-line with the previous report that shows the efficiency of the designed feed strategy used for fed-batch process. After addition of different glycerol concentrations the respective pH values of cultures were varied between 6.8 and 6.2. Fed-batch process resulted in high yield, high titer and high productivity of nattokinase by *B. subtilis* natto as summarized in Table 4.

These data demonstrates that glycerol has a key role on nattokinase production in *Bacillus subtilis*

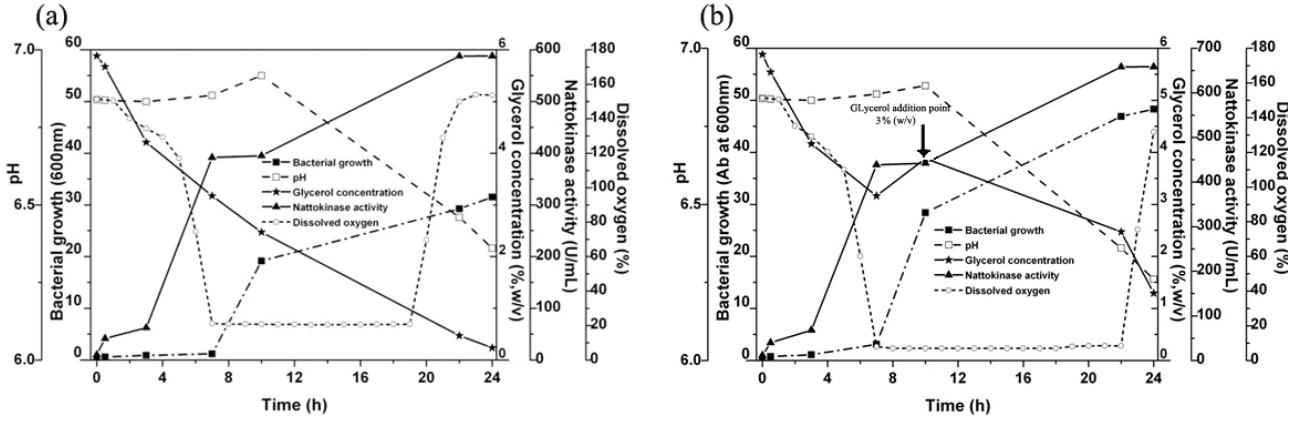

Figure 2. Changes in bacterial growth at 600 nm, glycerol concentration, nattokinase activity, pH and dissolved oxygen during the time course of fermentation for batch (a) and fed-batch (b) optimized conditions at 600 rpm, 2 vvm at 40 °C.

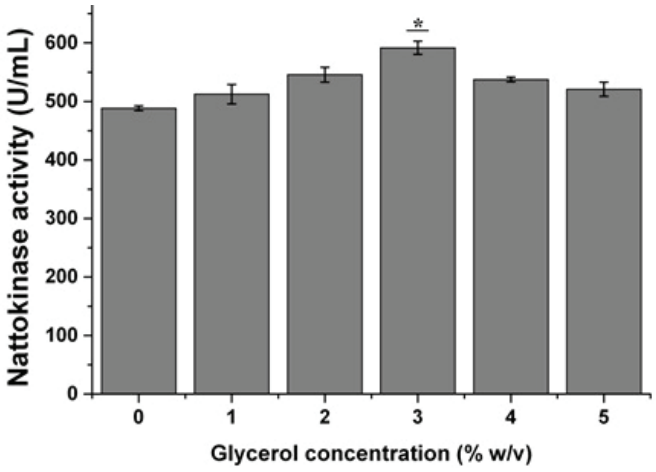

Figure 3. Effect of glycerol concentration on nattokinase activity.

natto metabolic pathway. Fed-batch supplementation of glycerol as the cheap and limiting nutrient, therefore, can be used for increasing the nattokinase production at scale up level.

Table 4. Summary of cell growth and production kinetics during batch and fed-batch process

Term	Batch culture	Fed-batch culture
Cell density (OD_{600})	32.12	48.33
Nattokinase activity, U/ml	587.14	654.84
Specific activity, U/mlOD_{600}	18.36	13.65
Volumetric production rate, U/(ml h)	24.58	27.53
Nattokinase yield, U/g-glycerol	63.24	78.61

CONCLUSIONS

In this study, nattokinase was successfully produced in batch and fed-batch fermentation of *B. subtilis* natto. Yeast extract, soy peptone and glycerol were found to be the most effective nutrients for enhancing nattokinase production. Moreover, the fed-batch addition of glycerol during cell growth stage resulted in significant increase on nattokinase production as compared to the batch fermentation. The results of this study demonstrate the significant contribution of adjusting the concentration and feeding strategy of essential nutrients on enhancing nattokinase production.

Acknowledgment

The authors acknowledge the financial support from Australian Research Council and also Agricure Pty Ltd.

REFERENCES

[1] H. Sumi, H. Hamada, H. Tsushima, H. Mihara, H. Muraki, Experientia **43** (1987) 1110-1111

[2] C.H. Hsia, M.C. Shen, J.S. Lin, Y.K. Wen, K.L. Hwang, T.M. Cham, N.C. Yang, Nutr. Res. **29** (2009) 190-196

[3] R.L. Hsu, K.T. Lee, J.H. Wang, L.Y. Lee, R.P. Chen, J. Agric. Food. Chem. **57** (2009) 503-508

[4] J.Y. Kim, S.N. Gum, J.K. Paik, H.H. Lim, K.C. Kim, K. Ogasawara, K. Inoue, S. Park, Y. Jang, J.H. Lee, Effects of nattokinase on blood pressure: a randomized, controlled trial, Hypertens. Res. **31** (2004) 1583-1588

[5] D.J. Wu, C.S. Lin, M.Y. Lee, Lipid-lowering effect of nattokinase in patients with primary hypercholesterolemia, Acta. Cardiol. Sin. **25** (2009) 26-30

[6] S.H. Kim, N. S. Choi, Purification and characterization of subtilisin DJ-4 secreted by Bacillus sp. strain DJ-4 screened from Doen-Jang, Biosci. Biotechnol. Biochem. **64** (2000) 1722-1725

[7] W. Kim, K. Choi, Y. Kim, H. Park, J. Choi, Y. Lee, H. Oh, I. Kwon, S Lee, Appl. Environ. Microbiol. **62** (1996) 2482--2488

[8] Y. Peng, X. Yang, Y. Zhang, Appl. Microbiol. Biotechnol. **69** (2005) 126-132

[9] A. Berenjian, R. Mahanama, A. Talbot, R. Biffin, H. Regtop, P. Valtchev, J. Kavanagh, F. Dehghani, N. Biotechnol. **28** (2011) 665-672

[10] V. Deepak, K. Kalishwaralal, S. Ramkumarpandian, S. VenkateshBabu, S.R. Senthilkumar, G. Sangiliyandi, Bioresource. Technol. **99** (2008) 8170-8174

[11] J. Liu, J. Xinga, T. Chang, Z. Ma, H. Liu, Process. Biochem. **40** (2005) 2757-2762

[12] D.S. Wang, C.C. Torng, I.P. Lin, B.W. Cheng, H.R. Liu, C.Y. Chou, J. Food. Process. Eng. **29** (2006) 22-35

[13] Y. Ghasemi, M. Mohkam, A. Ghasemian, S.R. Amini. J. Food. Sci. Technol., in press

[14] P.M. Mahajan, S.V. Gokhale, S.S. Lele. Food. Sci. Biotechnol. **19** (2010) 1593-1603

[15] C.T Chang, M.H. Fan, F.C. Kuo, H.Y. J. Agric. Food. Chem. **48** (2000) 3210-3216

[16] J.H. Ko, J.P. Yan, L. Zhu, and Y.P. Qi, Comp. Biochem. Physiol., C **137** (2004) 65-74

[17] N.H.A.P. Unrean, Bioprocess. Biosystem. Eng. **36** (2013) 45-56

[18] S.Y. Lee. Trends. biotechnol. **14** (1996) 98-105

[19] A. Berenjian, R. Mahanama, A. Talbot, H. Regtop, J. Kavanagh, F. Dehghani, Am. J. Biochem. Biotechnol. **8** (2012) 105-110

[20] P. Unrean, N.H.A. Nguyen, W. Visessanguan, P. Kitsubun, KKU Res. J. **17** (2012) 769-777.

AYDIN BERENJIAN[1,2]
RAJA MAHANAMA[1]
JOHN KAVANAGH[1]
FARIBA DEHGHANI[1]
YOUNES GHASEMI[1,2]

[1]School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney, Australia
[2]Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

NAUČNI RAD

PRODUKCIJA NATOKINAZE: PROUČAVANJE SASTAVA HRANLJIVE PODLOGE I STRATEGIJE DOLIVANJA

U ovom radu je istraživan uticaj nutrienata na aktivnost natokinaze tokom fermentacije pomoću mikroorganizma Bacillus subtilis natto*. Najveća aktivnost natokinaze od 587 U/mL je dobijena u fermentoru sa hranljivom podlogom na bazi ekstrakta kvasca (6%), soja peptona (1,2%) i glicerola (6%). Fitovanje podataka pomoću polinomne jednačine drugog reda je izvršeno pomoću ka centru orijentisanog centralnog compositnog dizajna. Polinomni model fituje eksperimentalne podatake koeficijentom determinacije R^2 = 0,939 i podešenim koeficijentom determinacije R^2(Adj) = 0,861. Pored toga, uticaj dolivanja glicerola na put produkcije natokinaze je dodatno istraživan. Maksimalna aktivnost natokinaze je postignuta kada je dodato 3% glicerola fermentacionom medijumu tokom faze rasta ćelija. Rezultati pokazuju da se aktivnost natokinaze povećava 25 puta u odnosu na šaržnu i neoptimizovanu kulturu. Ovo istraživanje daje dragocenei podatke o ključnim komponentama hranljive podloge i strategiji dolivanjae za dalja istraživanja i povećanja razmere procesa produkcije natokinaze.*

Ključne reči: natokinaza, Bacillus subtilis natto*, fermentacija, polušaržno dodavanje glicerola.*