Optimization of medium composition for the production of nattokinase by *Bacillus natto* NLSSE

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Abstract:

Nattokinase, primarily isolated from a traditional fermented food in Japan---" Natto" , is a novel fibrinolytic enzyme. It is considered to be a promising agent for thrombosis therapy. The composition of

the medium for the production of nattokinase by *Bacillus natto* NLSSE was optimized with a stepwise strategy. Effects of various nitrogen and carbon sources were investigated. Among the tested nitrogen sources, soy peptone was found to be most promising. As to carbon sources, maltose was found to be most effective for nattokinase production. Fractional factorial design (FFD) was applied to elucidate the key ingredients in the media and the results indicated that the soy peptone and yeast extract gave significant negative influence on nattokinase production , while the calcium chloride gave strong positive effect. Central composite design (CCD) was employed to search for the optimal concentration of the three key components, and the experimental results were fitted with a second-order polynomial model at 98% level ($p<0.02$). Canonical analysis results showed that the response surface had a maximal point. According to the model, the predicted maximal nattokinase volumetric concentration was about 1270 u/ml and the corresponding concentration of soy peptone, calcium chloride and yeast extract were around 8.28 g/ml, 0.64 g/ml, 0.74 g/ml, respectively. The trial checking the developed medium showed a high nattokinase activity of 1300±30 u/ml (average of three repeats).

Introduction

Nattokinase (subtilisin NAT, NK), a potent fibrinolytic enzyme, was primarily isolated from a traditional fermented food " Natto" in Japan. (Sumi et al. 1987). Sumi further demonstrated that oral administration of nattokinase capsules enhanced fibrinolysis in canine plasma in an experimental thrombosis model (Sumi et al. 1990). Moreover, fibrinolytic activity was retained in the blood for more than 3h (Fujita et al. 1995). As to the fibrinolytic mechanism of nattokinase, the enzyme was reported not only to possess plasminogen activator activity (Fujita et al. 1995), but also to directly digest fibrin by limited protelysis (Chang et al. 2000). And nattokinase could cleave plasminogen activator inhibitor-1 into low molecular weight fragments (Urano 2001). All the results suggest that nattokinase could be used as an agent for thrombosis therapy.

So far, many researchers have focused their efforts on the isolating and screening of microorganisms for enzyme production with high fibrinolytic activity(Chang et al. 2000), as well as purifying and characterizing newly found enzyme (Kim et al. 1996). In contrast, there were few reports concerning culture medium optimization. While a novel fermentation medium is of critical importance because medium composition can significantly affect product concentration. In this paper, we studied the nutritional fermentation condition with Bacillus natto NLSSE in order to maximize nattokinase yield.

The traditional one-at-a-time optimization strategy is simple and easy, and the individual effects of medium components can be seen on a graph without the need to apply statistical analysis. But the technique has some major flaws: interactions between components are ignored, the optimum can be missed completely, and it is time-consuming and laborious.

Statistical experiment design provides an efficient approach to optimization (Kennedy et al.1999). Factorial design (full or fractional) is especially suitable to identify the most significant components in the medium formula. While central composite design, provides as more information on the interaction of factors as on main factors, and is usually optimized with response surface methodology to find the optimal

point (Dean & Voss 1999).

The aim of this work is to optimize the medium to maximize the nattokinase productivity. A stepwise optimization was performed including: (1) employment of one-at-a-time method to screen the optimal carbon and nitrogen source, (2) elucidation of the medium components that affect nattokinase production significantly using a 2n-k fractional factorial design, (3) RSM optimization of these significant ingredients by CCD experiment design.

Materials and Methods

Microorganism and its maintenance

Bacillus natto NLSSE was obtained from State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China. Bacteria were maintained as spores suspended in 50% (v/v) glycerol, stored at – 25℃.

Cultivation and production of nattokinase by Bacillus natto NLSSE

A 5%(v/v) spore suspension was added to seed medium composed of (g/l): glucose, 10; yeast extract, 10; K2HPO4.3H2O, 1; MgSO4.7H2O, 0.5 and pH 7.0~7.2. The subsequent cultivation lasted for 12 h at 37℃ and 170 rpm in an orbital shaker to obtain seed culture with a OD600nm value of 7~8. Then A 5%(v/v) seed culture was added to production medium consisting of the ingredients resulted from experimental design. Throughout the work, liquid cultures were incubated at 37℃ and 170 rpm in an orbital shaker. After 48 h of fermentation, cells were removed by centrifugation, and the supernatant were used for nattokinase activity assay.

Assay of nattokinase activity

Fibrinolytic activity of nattokinase was measured by the hydrolysis of fibrin. The incubation mixture contained 2.5ml of 12g/l fibrin solution (pH7.8), 6.5ml of 0.1M Tris-HCl buffer (containing 10mM CaCl2, pH7.8), and 1ml enzyme solution with suitable dilution. The incubation was carried out at 37℃ for 15 min and was stopped by adding 5ml of 0.11M trichloroacetic acid containing 0.22M sodium acetate and 0.33M acetic acid. The absorbency at 275nm of the supernatant obtained after centrifugation was determined. A fibrinolytic unit was defined as the amount of enzyme that gave an increase in absorbency at 275nm equivalent to 1ug of tyrosine per minute at 37℃.

Optimization procedure and Experimental design

All the statistical experimental designs and results analyses were carried out using STATISTICA 5.0 for Windows (Statsoft, Inc.).

Screening the optimal nitrogen and carbon sources

Seven kinds of nitrogen sources and five kinds of carbon sources were investigated with one-at-a-time strategy. In the investigation of nitrogen sources, growth was carried out in the minimal

synthetic medium (MSM) containing (g/l): K2HPO4.3H2O, 1, MgSO4.7H2O, 0.5, supplemented with 10 of glucose and the nitrogen source to be investigated. And in the process of screening carbon sources, fermentation was carried out in the same MSM (mentioned above) supplemented with the optimal nitrogen source found out above and the carbon source to be investigated.

Elucidation of significant components with fractional factorial design (FFD) (Dean & Voss 1999)

As a preliminary step for optimization, a 26-1 fractional factorial design was employed to find out the key ingredients that affect nattokinase production significantly. There are six nutrient factors in the medium, such as carbon source, nitrogen source, MgSO4.7H2O, K2HPO4.3H2O, yeast extract, CaCl2, and each factor had the chance to be examined at a high level (coded +1) and a low setting (coded -1) which are corresponding to the basal level ±50% respectively. And a 1/2 fraction of the full factorial design was adopted, consequently the experiment included 32 (26-1) combinations, as shown in Table 1.

Optimization of the key ingredients with central composite design (CCD) (Dean & Voss 1999)

Medium components that affect nattokinase production significantly were optimized with a CCD design. According to Statistica, a full CCD design of three factors consisted of 17 combinations, as was shown in Table 3. The experimental results were fitted with a second-order polynomial function. Meanwhile the significance level of each co-efficient was determined using student t-test and the analysis of variances were conducted to check if the model gave a satisfactory fit. In the end, canonical analysis was used to find the stationary point and determine its specification (maximal, minimal, or saddle) .

Results and Discussion

Effects of different nitrogen sources on production of nattokinase

Six kinds of nitrogen sources were examined: ammonium sulfate 3.5g/l, sodium nitrate 10g/l, sodium glutamate 21g/l, casein 10g/l, powder of soy cake 20g/l, and soy peptone 10g/l, all of which had equiva-

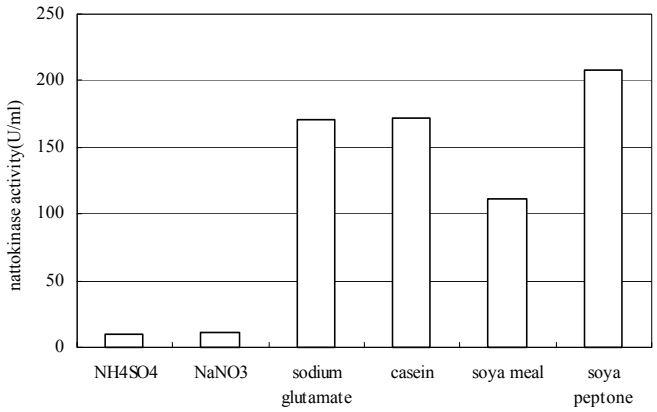

Figure 1. Effects of different nitrogen sources on production of nattokinase.

lent nitrogen element content. The results were shown in Fig. 1

Of all the nitrogen sources tested, soy peptone was found to be the most promising one, and the corresponding nattokinase activity is 208.3 u/ml. When inorganic nitrogen sources were used, very poor enzyme activities were obtained. While much higher activities were obtained by using the organic nitrogen sources. Powder of soy cake gave poor enzyme activity in compare with the other two organic nitrogen sources, which most likely resulted from its poor solubility. The optimal nitrogen source was soy peptone, which was used as nitrogen source in the following investigations.

Effect of different carbon sources on production of nattokinase

Five kinds of carbon sources were investigated: glucose, sucrose, maltose, xylose, and glycerol at

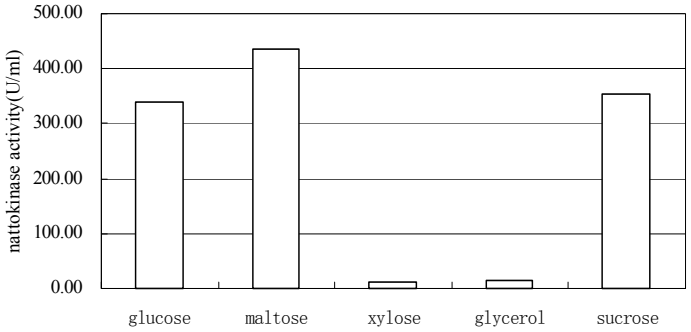

Figure2 Effect of different carbon sources on production of nattokinase

the concentration of 20g/l. The results were shown in Figure 2.

Glucose is a generally preferred carbon source for growth of bacteria, and it was used as reference. The other four carbon sources had all been reported to be the best in respect of protease production by strains of Bacillus subtilis..

The results showed that, based on nattokinase production, maltose was the optimal carbon source (434 u/ml). Glucose and sucrose had similar positive effects, while xylose and glycerol performed poorly. So maltose was chosen as carbon source for the following investigations.

Elucidation of medium constituents controlling nattokinase production

The experimental design and results of the FFD observations were illustrated in Table 1. The nattokinase production varied greatly from 113.6 u/ml to 839.3 u/ml with different combinations of the components in the media. The analysis of variances and effect estimates for main effects were shown in Table 2. Three different conclusions could be obtained: (1) variations in the concentrations of maltose, K2HPO4.3H2O and MgSO4.7H2O didn’ t affect nattokinase production significantly, suggesting that their minus (-) settings are plentiful, (2) enzyme production was greatly affected by soy peptone ($P<0.05$), calcium chloride ($P<0.01$), and yeast extract ($P<0.01$). (3) The effect estimate of calcium chloride was

positive, which suggested that the increase of its concentration in the medium will result in promotion of nattokinase production. While the effect estimates of yeast extract and soy peptone were negative, which meant low level of the yeast extract content and soy peptone would benefit nattokinase production process. The three nutrient components will be further investigated with central composite design.

Table 1. Experimental design and results of the 2^{6-1} fractional factorial design

Trial	Block	x_1	x_2	x_3	x_4	x_5	x_6	NK(u/ml))
1	1	-1	-1	-1	1	1	-1	614.0
2	1	-1	1	1	-1	-1	-1	349.6
3	1	1	-1	1	-1	1	1	138.0
4	1	1	-1	1	-1	-1	-1	725.2
5	1	1	1	-1	-1	-1	-1	418.1
6	1	-1	-1	-1	-1	1	1	332.3
7	1	1	1	-1	-1	1	1	158.1
8	1	-1	-1	-1	1	-1	1	399.9
9	1	-1	1	1	-1	1	1	557.0
10	1	1	1	-1	1	1	-1	502.4
11	1	1	-1	1	1	-1	1	295.0
12	1	1	-1	1	1	1	-1	839.3
13	1	1	1	-1	1	-1	1	113.6
14	1	-1	1	1	1	-1	1	155.4
15	1	-1	-1	-1	-1	-1	-1	304.1
16	1	-1	1	1	1	1	-1	631.2
17	2	1	1	1	-1	-1	1	153.9
18	2	1	1	1	-1	1	-1	550.6
19	2	-1	-1	1	1	-1	-1	567.5
20	2	1	-1	-1	1	-1	-1	571.7
21	2	1	-1	-1	1	1	1	312.3
22	2	1	-1	-1	-1	1	-1	726.6
23	2	-1	-1	1	1	1	1	812.3
24	2	-1	1	-1	-1	1	-1	635.0
25	2	-1	1	-1	-1	-1	1	172.9
26	2	-1	-1	1	-1	1	-1	578.7
27	2	1	-1	-1	-1	-1	1	269.0
28	2	1	1	1	1	-1	-1	311.2
29	2	-1	1	-1	1	1	1	585.8
30	2	-1	1	-1	1	-1	-1	455.9
31	2	1	1	1	1	1	1	327.5
32	2	-1	-1	1	-1	-1	1	550.6

Note:$x_1=(X_1-40)/20$;$x_2=(X_2-30)/20$; $x_3=(X_3-2)/2$; $x_4=(X_4-1)/0.5$; $x_5=(X_5-0.3)/0.1$; $x_6=(X_6-3)/2$. X_1, X_2, X_3, X_4, X_5 and X_6 stood for natural variables (concentration, g/l) of maltose, soy peptone, $K_2HPO_4.3H_2O$, $MgSO_4.7H_2O$, $CaCl_2$, yeast extract, respectively. And x_1, x_2, x_3, x_4, x_5 and x_6 stood for the coded variables.

Table 2. Analysis of variances (ANOVA) and effect estimates for main effects of FFD

Factors	SS	DF	MS	F	Prob-ability	Effect esti-mate
Blocks	34330.61	1	34330.61	1.63	0.21	
x_1	51994.32	1	51994.32	2.47	0.13	-80.62
x_2	119845.9	1	119845.9	5.69	0.03	-122.40
x_3	29485.78	1	29485.78	1.40	0.25	60.71
x_4	23945.98	1	23945.98	1.14	0.30	54.71
x_5	193386.1	1	193386.1	9.17	0.01	155.48
x_6	371459.1	1	371459.3	17.6	0.00	-215.48
Resid-ual	505882.3	24	21078.43			

Note: x_1, x_2, x_3, x_4, x_5, x_6 stood for the coded variables of maltose, soy peptone, $K_2HPO_4.3H_2O$, $MgSO_4.7H_2O$, $CaCl_2$, yeast extract, respectively.

Optimization of the key ingredients in the media with CCD

The key components including soy peptone, calcium chloride, and yeast extract were optimized with a CCD experimental design. The experimental design and results are shown in Table 3. According to the

RSM methodology, a full second-order polynomial model was used to fit the dependent variable into the following equation:

$$Y=b0+b2x2+b5x5+b6x6+b22x22+b55x52 +b55x52 +b25x2\ x5+b26x2\ x6+b56x5\ x6$$

where Y (volumetric nattokinase activity, u/ml) was the dependent variable to be modeled, and bi were coefficients of the model, and x2 (soy peptone), x5 (calcium chloride) and x6 (yeast extract) were independent variables. And the results of regression analysis were shown in Table 4 and ANOVA results were illustrated in Table 5.

Table 3. Experimental design and results of CCD

Trial	Block	x_2	x_5	x_6	Nattokinase (u/ml)
1	1	-1	-1	-1	850.48
2	1	1	-1	-1	933.55
3	1	-1	1	-1	958.89
4	1	1	1	-1	1079.98
5	1	-1	-1	1	630.76
6	1	1	-1	1	763.18
7	1	-1	1	1	820.88
8	1	1	1	1	982.83
9	1	0	0	0	1200.24
10	1	0	0	0	1278.51
11	2	-1.682	0	0	509.00
12	2	1.682	0	0	705.67
13	2	0	-1.682	0	946.22
14	2	0	1.682	0	920.88
15	2	0	0	-1.682	972.97
16	2	0	0	1.682	750.00
17	2	0	0	0	1309.55

Note: $x_2=(X_2-8)/2$; $x_5=(X_5-0.6)/0.2$; $x_6=(X_6-0.8)/0.2$. X_2, X_5, X_6 stood for natural variables (concentration, g/l) of soy peptone, $CaCl_2$, yeast extract, respectively. While x_2, x_5 and x_6 stood for coded variables.

Table 4 Coefficients and significance level of regression analysis

	Coefficients	t	P-level
C vs. S	-31.31	-1.67	0.15
b_0	1253.96		
b_2	60.72	2.53	0.04
b_5	45.54	1.90	0.10
b_6	-73.24	-3.05	0.02
b_{22}	-208.12	-7.89	0.00
b_{55}	-92.79	-3.52	0.01
b_{66}	-118.26	-4.48	0.00
b_{25}	8.44	0.27	0.80
b_{26}	11.28	0.36	0.73
b_{56}	19.36	0.62	0.56

Table 5 ANOVA of RSM regression analysis

Mode	Sum of squares	Degree of freedom	Mean of squares	F	Probability
Regression	899510.1	9	99945.57	10.13	0.02
Residual	69087.4	7	9869.63		
Total	968597.5	16			

Note: R^2 adj. 0.9287

Table 6. Canonical analysis

	Soya peptone (x_2)	$CaCl_2$ (x_5)	Yeast extract (x_6)
Critical value	0.1426	0.2220	-0.2847
eigenvalue	-89.05	-121.3	-208.59

Note: Predicted response at stationary point: 1273.8 u/ml, x_2, x_5 and $x_6$stood for coded variable of soy peptone, $CaCl_2$, yeast extract, respectively.

The ANOVA results showed that the F value was high enough to give a significance of 98% level and the adjacent R2 was 0.9287. It could be concluded that the applied model fitted the experimental values well. The regression analysis results told all coefficients of the model and their significant level. And all the three linear and three quadratic terms had significant effect ($P<0.10$) on nattokinase production, while the cross product of any two factors didn't. Canonical analysis was further conducted, and the results were illustrated in Table 6. All the eigenvalues were negative, so the stationary point is a maximum. The optimal concentration for production of nattokinse was 8.28 g/l for soy peptone, 0.64 g/l for calcium chloride, and 0.74 g/l for yeast extract, and the predicted maximum nattokinase activity was 1273.8 u/ml. In order to confirm the predicted results, experiments using the improved formula were performed, and a value of 1300±30 u/ml (n=3) was obtained.

Conclusions

Statistical experiment methods are efficient in improving fermentation media. In this paper, nattokinase activity rises up to about 1300 u/ml, almost two times higher than the original medium. And the batch fermentation technology of nattokinase production will be further investigated in the near future.

Acknowledgements

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