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Utilization of Red Lentil Flour Substrate for the Production of Nattokinase Enzyme by *Bacillus subtilis* Natto

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ABSTRACT

In this research, the optimal production conditions of nattokinase using *Bacillus subtilis* Natto on red lentil flour substrate were studied. To optimize the fermentation process, the effects of three variables of fermentation time (24, 48 and 72 h), molasses concentration (3, 5 and 7%) and water content (50, 75 and 100 ml) on fibrinolytic and protease activities were investigated. The central composite design (CCD) was employed, and the results were modelled and analyzed using response surface methodology (RSM). Coefficients of determination, R2, of fitted regression models for fibrinolytic (quadratic model) and protease (linear model) activities were 97.15 and 90.12%, respectively, and their lack-of-fit was not significant at 95%. Hence, the models for all the responses were highly adequate. Fibrinolytic activity increased significantly ($p\leq0.05$) with increasing water content and fermentation time, while increasing the amount of molasses decreased fibrinolytic activity ($p\leq0.05$). With increasing the amount of water, protease activity also increased, but this increase was not significant, while increasing the amount of molasses and fermentation time led to a significant decrease and increase in protease activity ($p\leq0.05$), respectively. Concerning optimization, the optimal fermentation conditions were determined as 92.38 ml of water, 3.66% molasses, and 70.90 h of fermentation. Under these conditions, the activities of fibrinolytic enzymes and proteases were predicted to be 2476.03 and 1.68 U/g, respectively. For validation of the model, the optimal sample was produced, and the experimental responses were compared with the responses predicted by the model. The experimental values obtained were quite close to those predicted by the model, indicating the validity of the model. The results of this study showed that red lentil could be used as a substrate for fermentation by *B. subtilis* to produce the enzyme nattokinase.

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1- Introduction

Fermentation is a process in which microorganisms are used to produce enzymes, food products, and nutraceuticals. Shelf life, safety, functionality, and sensory and nutritional properties of food are improved during fermentation. In addition, fermentation has beneficial effects on health by reducing the risk of various diseases such as type 2 diabetes and cardiovascular diseases [1].

Nattokinase (EC 3.4.21.62), also known as subtilisin NAT, is one of the most important extracellular enzymes produced by *B. subtilis* Natto. This enzyme, which belongs to the subtilisin family, is a serine protease consisting of 275 amino acids. The direct fibrinolytic activity of nattokinase is the main reason for interest in this enzyme [2]. Fibrinolytic enzymes have a significant effect on preventing the accumulation of fibrin platelets and preventing thrombosis. Not only does nattokinase break down blood clots, but it also breaks down accumulated fibrin, which is associated with heart disease [3].

B. subtilis is one of the gram-positive bacteria active in the production and secretion of various enzymes, and hence it is used in food-pharmaceutical biotechnology on an industrial scale. In fermented foods, this bacterium hydrolyzes the substrate and produces enzymes such as nattokinase, phytase, amylase, protease and lipase [4, 5].

Lentil, with the scientific name *Lens culinaris* Medik, is an edible pulse that is an important source of dietary protein in developing countries. Lentil is a highly nutritious legume with a sufficient amount of carbohydrates and a good amount of protein, minerals, vitamins and dietary fibres. The appropriate amounts of carbohydrate and protein make lentil a suitable substrate for the growth of *B. subtilis* and the production of nattokinase [6].

B. subtilis needs carbon and nitrogen sources to grow and produce nattokinase. Lentil is a good source of carbon and nitrogen, and as far as we know, there has been no study on the use of lentil flour substrate for the production of nattokinase by *B. subtilis*. In the present study, red lentil flour was used as the base fermentation substrate for nattokinase production by *B. subtilis*. To optimize the fermentation process, the effects of three variables of fermentation time, molasses concentration and water content on the fibrinolytic and protease activities of nattokinase were investigated using the response surface methodology (RSM).

2- Materials and methods

2. 1. Preparation of bacterial strain

B. subtilis subsp. Natto (ATCC23857.strain 168, DSM402, LMG 19457) was purchased from the Iranian Biological Resource Center (IBRC), Tehran, Iran, as IBRC-M:115.

2. 2. Reactivation of bacterial strain

At first, *B. subtilis* was cultured on nutrient agar (Oxoid, Altrincham, UK) medium by streaking technique, and then the cultured medium was incubated at 37°C for 24 hours. Next, one loop of cells was transferred from the plate to the nutrient broth medium (Oxoid, Altrincham, UK) and again placed in a 37°C incubator for 24 hours. Finally, bacteria grown in a nutrient broth medium were used to inoculate the substrate [7].

2. 3. McFarland solution quality control

The standard absorbance of 0.5 McFarland at 625 nm should be between 0.08 and 0.1. The density of McFarland solutions was checked by a spectrophotometer (CT-8200 Double beam, ChromTech) and the correctness of the standard was confirmed [8].

2. 4. Fermentation process

Red lentil was purchased from a local store and then ground using a home grinder. For discontinuous fermentation, 10 grams of lentil flour was poured into a 250 ml Erlenmeyer flask and mixed with some distilled water. After shaking the flask to mix

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the contents, its lid was closed and autoclaved. After autoclaving and cooling the flask, under sterile conditions, 2 ml of cultured bacteria from the previous day were added to it using the McFarland method. After that, the flask was placed in a 37°C incubator shaker for 48 hours. Finally, the produced nattokinase was extracted and its fibrinolytic and protease activities were measured.

2. 5. Extraction of nattokinase

To extract nattokinase, normal saline was added to lentil flour inoculated with *B. subtilis*, which was incubated at 37°C for 24, 48 and 72 hours. Then the obtained mixture was shaken for 1 hour at 37°C using a shaker at 150 rpm. After that, the supernatant was removed and poured into 2 ml microtubes and centrifuged at 14000 rpm for 15 min at 4°C. Finally, the supernatant, the liquid containing nattokinase, was used to measure fibrinolytic and protease activities.

2. 6. Determination of fibrinolytic activity

The fibrinolytic activity of nattokinase was performed according to the method described by Gowthami and Madhuri [9]. Human plasma was used to extract fibrin for the fibrinolytic test. The reaction mixture containing 2.5 ml of fibrin solution, 2.5 ml of 0.1 M Tris buffer (pH = 7.8) and 1 ml of purified enzyme solution was incubated for 15 min at 37°C before adding 5 ml of 0.1 M trichloroacetic acid (TCA). The mixture was then kept at room temperature for 20 min. After centrifugation for 10 min at 12000 rpm, the supernatant was collected and its absorbance was determined at 275 nm.

2. 7. Determination of protease activity

Briefly, 1 ml of enzyme solution was added to 5 ml of casein (1% w/v in 50 mM potassium phosphate buffer, pH 7.5) and incubated at 37 °C for 10 min. After 10 min, 5 ml of 0.4 M TCA was added and incubated again for 30 min. Then, the obtained mixture was filtered with a 0.45-micron syringe filter. After filtration, 5 ml of 500 mM sodium carbonate was added to 2 ml of the filtrate, and then 1 ml of phenol reagent (Folin–Ciocalteu phenol solution: D–H2O=1:2) was added and incubated at 37°C for an additional 10 min. Finally, the absorbance of the filtered sample was measured at 660 nm [10].

2. 8. Experimental design and statistical analysis

The statistical Design Expert software version 11.0.3.0 (Stat-Ease Inc., Minneapolis, MN, USA) was used to design the experimental plan and data analysis. Twenty experiments (including 6 center points, 6 axial points and 8 factorial points) were carried out according to a quadratic central composite design (CCD) with three numerical variables (water content (X_1), molasses concentration (X_2) and fermentation time (X_3)) at three levels. The variables had 3 levels, coded as – 1, 0 and +1. The levels of independent variables are shown in Table 1. Twenty runs of experiments at different combinations of variables along with the actual and predicted responses (fibrinolytic activity (Y_1) and protease activity (Y_2)) were given in Table 2. Statistical analysis was performed using analysis of variance (ANOVA) test to estimate the statistical parameters and determine the significance of the model.

Table 1- Independent variables and their coded and uncoded levels according to central composite design

Independent Variables	Symbol	Coded levels		
		-1	0	+1
Water content (ml)	X_1	50	75	100
Molasses concentration (%)	X_2	3	5	7
Fermentation time (h)	X_3	24	48	72

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Table 2- Experimental design matrix and responses (actual and predicted) by central composite design CCD)

Run	Variable			Response			
				Y_1 (U/g)		Y_2 (U/g)	
	X_1 (ml)	X_2 (%)	X_3 (h)	Actual	Predicted	Actual	Predicted
1	75	5	48	1876.47	1990.69	1.04	1.20
2	100	7	24	2228.83	2249.91	0.79	0.7632
3	32.96	5	48	570.43	656.93	1.22	1.11
4	75	1.64	48	2165.20	2366.70	1.50	1.41
5	75	5	48	1998.07	1990.69	1.07	1.20
6	75	8.36	48	2179.66	2165.96	0.99	0.9978
7	50	7	72	1106.65	1121.05	1.44	1.40
8	75	5	88.36	2344.21	2251.98	1.24	1.71
9	75	5	48	1894.79	1990.69	1.11	1.20
10	75	5	48	2120.65	1990.69	1.21	1.20
11	75	5	48	1937.87	1990.69	1.33	1.20
12	75	5	7.64	1696.14	1774.69	0.75	0.5756
13	50	3	24	1776.72	1713.57	0.43	0.8147
14	50	7	24	1271.10	1193.65	0.55	0.6521
15	100	3	24	1925.24	1920.51	0.83	1.01
16	100	3	72	2473.63	2560.75	1.78	1.76
17	100	7	72	2398.82	2471.64	1.52	1.51
18	50	3	72	2070.88	2059.47	1.61	1.64
19	70	5	48	2113.98	1990.69	1.19	1.20
20	117.05	5	48	2066.47	1966.34	1.42	1.30

3- Results and discussion

3. 1. Effects of independent variables on fibrinolytic activity

The results of the ANOVA showed that the quadratic model was the best model for predicting fibrinolytic activity, and the corresponding F value (34.10) also indicated the significance of the model ($p \leq 0.05$) (Table 3). According to the table, the effects of independent variables (X_1, X_2 and X_3) on fibrinolytic activity were significant ($p \leq 0.05$). The interaction effects of variables (except X_1X_3) on fibrinolytic activity were also significant ($p \leq 0.05$) in the mentioned model. The square of variables of water and molasses contents also had significant effects on the model ($p \leq 0.05$). The square of variables of water and molasses contents also had significant effects on the model ($p \leq 0.05$). It should be noted that the effects of X_1X_3 and x_3^2 on the presented model were not significant ($p > 0.05$).

After removing insignificant terms, the quadratic model obtained for fibrinolytic activity (Y1) in terms of coded factors was given as follows:

$$Y_1 = +1990.69 + 389.38\,X_1 - 152.26\,X_2 + 141.91\,X_3 + 212.33\,X_1X_2 - 104.63\,X_2X_3 - 240.11\,X_1^2 + 152.73\,X_2^2$$

(Eq. 1)

The positive and negative signs in front of each term indicate synergistic and antagonistic effects, respectively. Based on this, the water content and its square showed the most positive and negative impacts on fibrinolytic activity, respectively. The value of R2 (0.9715) for the above equation indicates a good correlation between the measured and predicted values by the model.

Table 3- Analysis of variance (ANOVA) for response surface quadratic model for fibrinolytic activity (Y_1)

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	F-value	*P* value (Probe > *F*)	
Model	3.861 E+06	9	4.290 E+05	34.10	<0.0001	significant
X_1	2.071 E+06	1	2.071 E+06	164.58	<0.0001	
X_2	2.073 E+05	1	2.073 E+05	16.48	0.0028	

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X_3	2.750 E+05	1	2.750 E+05	21.86	0.0012	
X_1X_2	3.607 E+05	1	3.607 E+05	28.86	0.0005	
X_1X_3	43316.55	1	43316.55	3.44	0.0965	
X_2X_3	87573.22	1	87573.22	6.96	0.0270	
X_1^2	7.905 E+05	1	7.905 E+05	62.83	<0.0001	
X_2^2	1.957 E+05	1	1.957 E+05	15.55	0.0034	
X_3^2	878.78	1	878.78	0.0698	0.7975	
Residual	1.132 E+05	9	12581.55	-	-	
Lack of Fit	56057.39	4	14014.35	1.23	0.4054	not significant
Pure Error	57176.57	5	11435.31	-	-	
Cor Total	3.974 E+06	18	-	-	-	

Fig. 1 shows the effects of each independent variable on fibrinolytic activity separately. According to the figure, increasing the water content led to an increase in fibrinolytic activity. Increasing the amount of water up to about 90 ml was associated with a steep slope of the graph, and with an increase of more than 90 ml, the enzyme activity remained almost constant. Also, increasing the concentration of molasses decreased fibrinolytic activity. The decrease in enzyme activity up to 6% molasses was associated with a steep slope of the graph, and after 6% no significant change in fibrinolytic activity was observed. Increasing the fermentation time also resulted in an increase in fibrinolytic activity, so the highest fibrinolytic activity was observed at the end of the process (72 hours of fermentation).

Microorganisms grow and reproduce the most in their optimal moisture content. The moisture level plays an important role in the biosynthesis and secretion of many types of enzymes [11]. The best moisture content for the production of enzymes in bacteria is the condition in which the bacterial growth rate is optimal and the presence of water molecules does not prevent the proper release of oxygen and the transfer of nutrients into the bacterial cells [12]. Ding, Yao [13], investigating the fermentation conditions of tea residue using mixed strains, reported that the growth rate of bacteria increased by increasing the amount of water to 55% and then decreased with a gentle slope. They stated that the lower moisture content reduces nutrient diffusion and enzyme stability and causes matrix swelling, which harms the growth of fermenting microorganisms inside the environment. High moisture content also weakens the effect of air circulation, which is likely to cause bacterial contamination.

In the present study, the highest fibrinolytic activity was achieved at the concentration of 3% molasses. In a study, Zeng, Li [14] used a medium containing soybean residue and cane molasses to investigate the fibrinolytic activity and stated that the use of 2% cane molasses increased the production of enzymes. Li, Zhang [15] also reported that the use of more than 3% molasses led to the inhibition of the growth of *B. subtilis*, and the reason for this was an increase in the viscosity of the medium and a decrease in the diffusion of nutrients.

Wang, Chen [16] used shrimp skin substrate as a carbon source to investigate the production and activity of the nattokinase enzyme and stated that 48-hour fermentation had the highest nattokinase activity and after that, the enzyme activity gradually decreased. Wang, Torng [17] also optimized the fermentation conditions of *B. subtilis*, intending to achieve the highest activity of nattokinase, and stated that the optimal fermentation time was 37.07 hours. Pan, Chen [7] reported that if soybean meal and cassava starch are used as substrates, nattokinase activity reaches its maximum after 72 hours of fermentation, which is consistent with the results of this study.

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Guo, Jiang [18] optimized fibrinolytic activity by culturing *B. subtilis* natto on Ginkgo seeds substrate and obtained the highest fibrinolytic activity in conditions of relative humidity of 80%, the initial water content of 73%, fermentation temperature of 38°C, inoculum volume of 18% and fermentation time of 38 hours. They also found that long-term fermentation causes a lack of nutrients needed for bacterial growth, as well as the accumulation of toxic inhibitors, and finally suppresses the fermentation process.

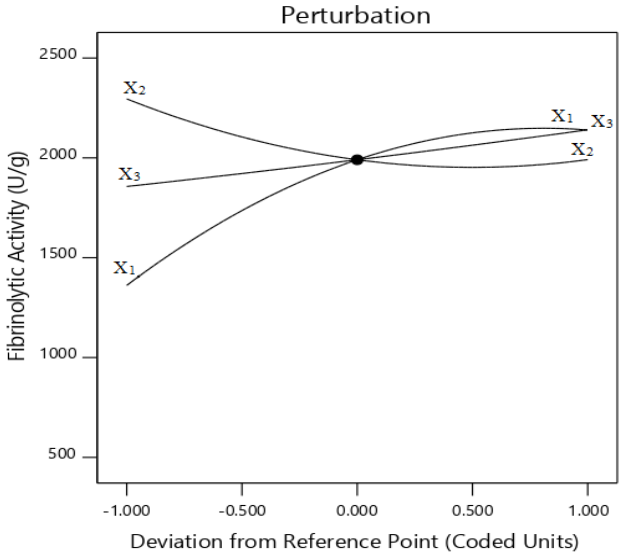
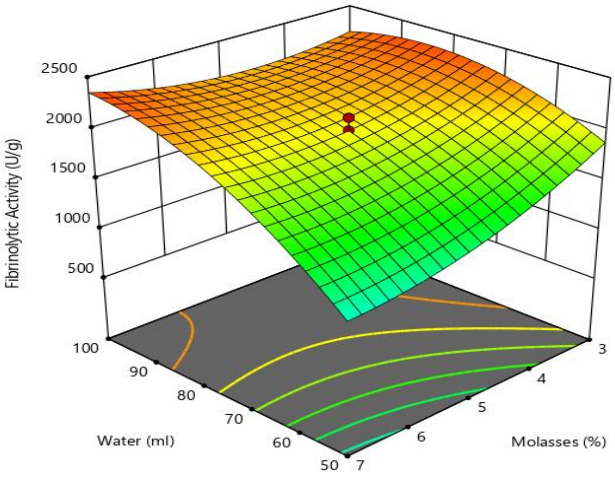


Fig. 1. The individual effects of independent variables of water content (X_1), molasses concentration (X_2) and fermentation time (X_3) on fibrinolytic activity in the center point.

Fig. 2 shows the combined effects of water content and molasses concentration on the fibrinolytic activity at constant fermentation time (48 hours). As it is known, the fibrinolytic activity increased in all molasses concentrations with increasing water content. In the amount of water below about 75%, increasing the concentration of molasses led to a decrease in fibrinolytic activity, although in higher amounts of water, with the increase in the concentration of molasses, the enzyme activity first decreased and then increased. Based on this, the role of water content in increasing fibrinolytic activity was greater than molasses concentration, and high fibrinolytic activity can be achieved in lower molasses concentrations by increasing water content.

a



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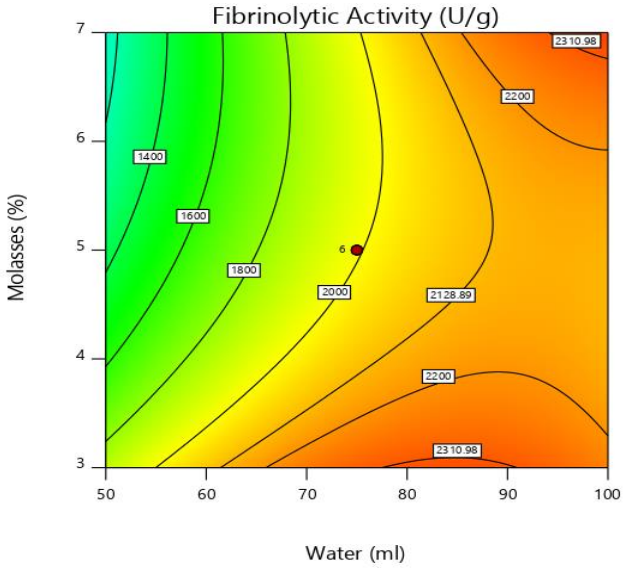

Fig. 2. The combined effects of water content and molasses concentration on fibrinolytic activity at constant fermentation time (48 hours); a: 3D; b: counter.

The combined effect of molasses concentration and fermentation time on the fibrinolytic activity at constant water content (75 ml) was shown in Fig. 3. According to the figure, with increasing fermentation time, fibrinolytic activity increased in all concentrations of molasses, especially in concentrations below 4%, so the highest enzyme activity was related to the concentration of 3% molasses and fermentation time of 72 hours. In lower amounts of molasses, the increase in fibrinolytic activity during fermentation was higher compared to higher concentrations of molasses. At all fermentation times, especially at lower times, increasing the amount of molasses first caused a decrease in enzyme activity, although, at high concentrations of molasses, fibrinolytic activity increased again to some extent. Anggraeni and Poernomo [19] evaluated the effect of different concentrations of molasses on the activity of fibrinolytic enzymes produced by *B. subtilis*. In this study, the highest fibrinolytic activity was observed at 0.5% concentration of molasses and at 30 hours of fermentation. According to the results, increasing the concentration of molasses up to 0.5% and increasing the fermentation time led to an increase in enzyme activity, and fibrinolytic activity decreased at molasses concentrations higher than 0.5%.

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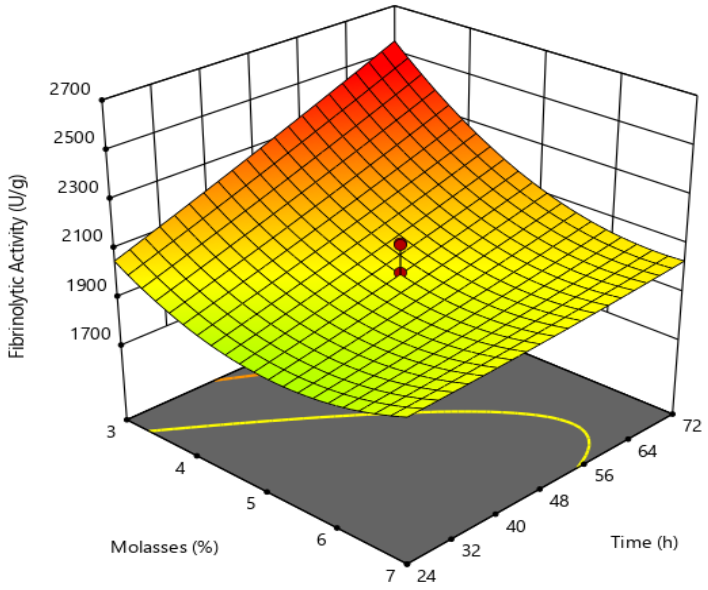
a



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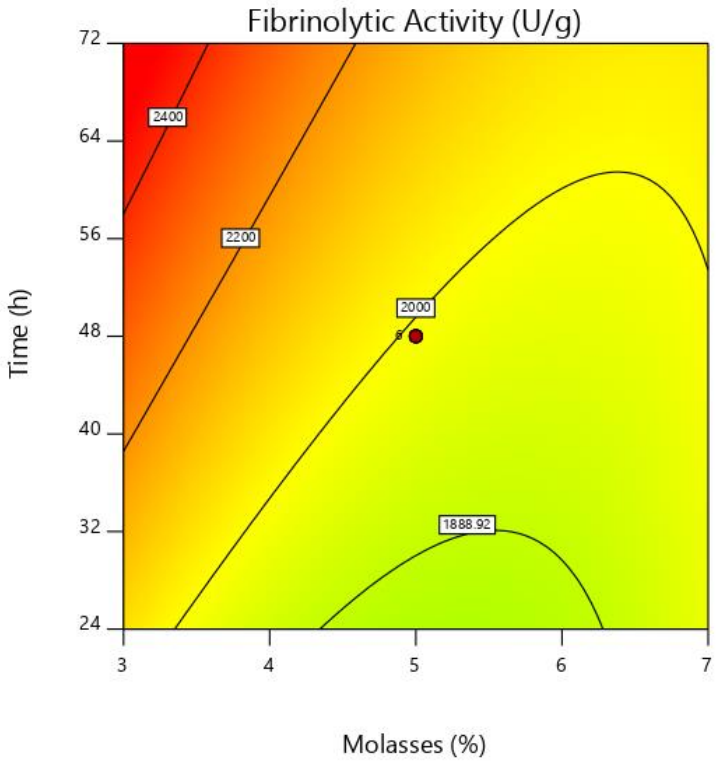

Fig. 3. The combined effects of molasses concentration and fermentation time on fibrinolytic activity at constant water content (75 ml); a: 3D; b: counter.

3. 2. Effects of independent variables on protease activity

In Table 4, the results of the linear model analysis of variance to determine the effect of independent variables and their interactions on protease activity are shown. The F value of the model was 42.58, which means that the model is significant ($p \leq 0.05$). According to

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the table, molasses concentration and fermentation time had significant effects on the model ($p \leq 0.05$), while the influence of water content was not significant. The non-significance of the lack-of-fit factor confirmed the correctness of the model for predicting protease activity.

The linear model of protease enzyme activity (Y2) in terms of the coded factors and after removing non-significant terms is as follows:

$$Y_2 = +1.20 - 0.1227\ X_2 + 0.3737\ X_3$$ (Eq. 2)

Among the variables, fermentation time showed the greatest effect on the increase of protease activity.

Table 4- Analysis of variance (ANOVA) for response surface linear model for protease activity (Y_2)

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	F-value	*P* value (Probe > *F*)	
Model	1.66	3	0.5532	42.58	<0.0001	significant
X_1	0.0383	1	0.0383	2.95	0.1080	
X_2	0.1865	1	0.1865	14.36	0.0020	
X_3	1.35	1	1.35	103.61	<0.0001	
Residual	0.1819	14	0.0130	-	-	
Lack of Fit	0.1246	9	0.0138	1.21	0.4393	not significant
Pure Error	0.0573	5	0.0115	-	-	
Cor Total	1.84	17	-	-	-	

Individual effects of independent variables on protease activity are shown in Fig. 4. As can be seen, the water content had no significant effect on the protease activity, while the high slope of the graph of the other two variables indicated their significant effect on the enzyme activity, and among them, the influence of the fermentation time was higher. Increasing fermentation time led to a significant increase in protease activity, while the relationship between protease activity and molasses concentration was the opposite and increasing the amount of molasses significantly decreased enzyme activity.

B. subtilis reached the stationary phase of the growth curve after 72 hours, and the maximum accumulation of enzymes was also observed in this phase. Also, the decrease in enzyme production after 72 hours may be due to the release of a high level of intracellular proteases at the same time as endospore formation [12].

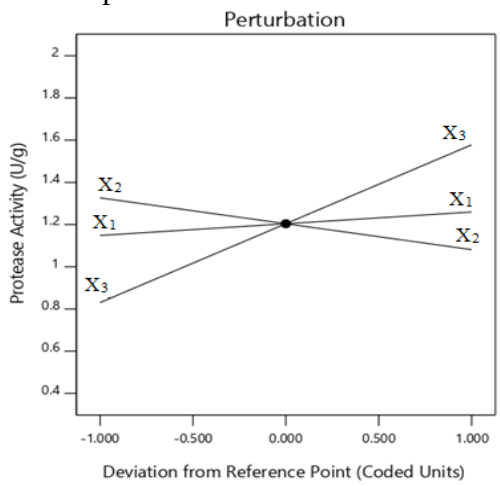

Fig. 4. The individual effects of independent variables of water content (X_1), molasses concentration (X_2) and fermentation time (X_3) on protease activity in the center point.

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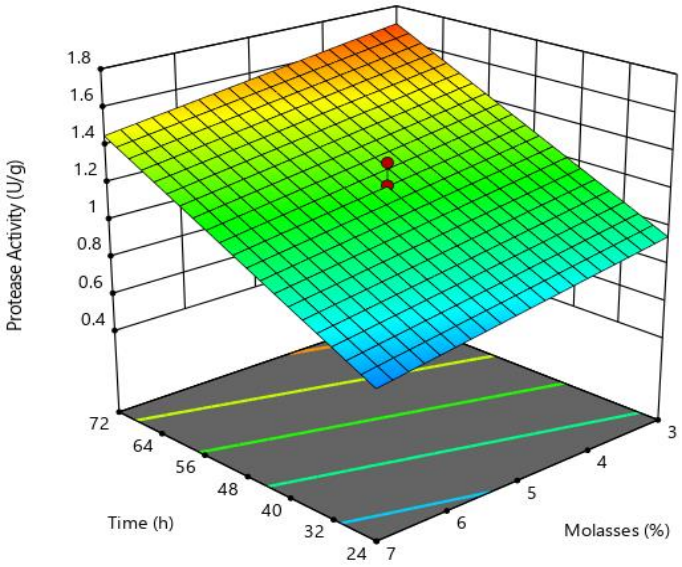
In Fig. 5, the effects of molasses concentration and fermentation time on protease activity in a constant amount of water (75 ml) are shown. According to the figure, with the increase in fermentation time, the protease activity increased significantly in all amounts of molasses, especially in its low concentrations, so the highest enzyme activity was related to the concentration of 3% molasses and fermentation time of 72 hours. Enzyme activity decreased with increasing amounts of molasses in all fermentation times, so the lowest enzyme activity was observed in a 7% concentration of molasses during 24 hours of fermentation. In a similar study, Sharan and Darmwal [20] investigated the activity of alkaline protease produced by *Bacillus pantotheneticus* on molasses substrate and showed that the use of molasses during 24 hours of fermentation increased the activity of the protease enzyme and further increase in fermentation time led to decrease enzyme activity.

a



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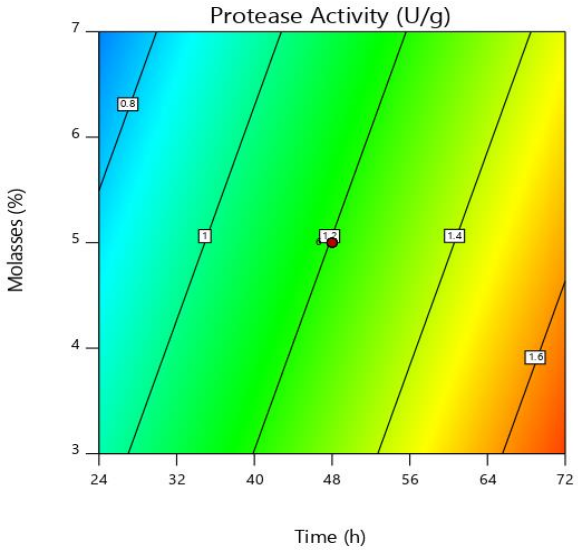

Fig. 5. The combined effects of molasses concentration and fermentation time on protease activity at constant water content (75 ml); a: 3D; b: counter.

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3. 3. Process optimization

The optimization parameters are presented in Table 5. The optimal fermentation conditions of lentil flour were obtained using the numerical optimization feature of the software. For optimization, the values of the independent variables were set in the "In range" feature. Due to the relationship between nattokinase production and fibrinolytic activity and the high importance of this enzyme activity, the fibrinolytic activity was considered to be the maximum with an importance factor of 5. The "In range" feature was also considered for protease activity.

The optimal treatment was selected based on the highest degree of desirability in order to achieve the desired responses. Based on this, the optimal fermentation conditions predicted by the model were: 92.38 ml of water, 3.66% molasses and 70.90 hours of fermentation, in which fibrinolytic and protease activities were predicted as 2476.03 and 1.68 U/g, respectively. To validate the accuracy of the model, fermentation was carried out under optimal conditions, and the responses obtained were compared with those predicted by the model (Table 6). As it is clear, the experimental values were quite close to the values predicted by the model, indicating the validity of the model.

Table 5- Parameters for numerical optimization

Name	Goal	Lower Limit	Upper Limit	Importance factor
Water content	is in range	50	100	3
Molasses concentration	is in range	3	7	3
Fermentation time	is in range	24	72	3
Fibrinolytic activity	maximize	570.43	2473.63	5
Protease activity	is in range	0.55	1.78	3

Table 6- Model validation

Water content, X_1 (ml)	Molasses concentration, X_2 (%)	Fermentation time, X_3 (h)	Fibrinolytic activity, Y_1 (U/g)		Protease activity, Y_2 (U/g)	
			Experimental	Predicted	Experimental	Predicted
92.38	3.66	70.90	2462.45	2476.03	1.69	1.68

In their research, Moharam, El-Bendary [21] studied the optimization of fibrinolytic enzyme production by *B. subtilis* and stated that the incubation period of 96 hours was the most optimal time to achieve maximum enzyme activity. Nguyen and Nguyen [5] optimized the content of protease produced from soybean fermentation by *B. subtilis* and obtained the highest amount of protease during 48 hours of fermentation at 32°C. Sahoo, Mahanty [22] also used cheese whey substrate to produce nattokinase enzyme by *B. subtilis* and declared the optimal fermentation time to be 51 hours. Thu, Khue [23] investigated the fibrinolytic activity of *B. subtilis* serine protease on shrimp skin substrate and found that the highest fibrinolytic activity was related to the 16th hour of fermentation.

Bajaj, Singh [24] studied different carbon sources to optimize the production of fibrinolytic protease enzyme using *B. subtilis*. The results showed that the enzyme activity was the highest in the presence of molasses. Al Mamun, Mian [25] reported that the addition of 0.92% molasses along with soybean flour increased protease activity. Gaddad [26] optimized the fermentation conditions to increase the alkaline protease activity. The study showed that the addition of 0.75% molasses resulted in a significant increase in protease activity.

4- Conclusions

This research investigated the optimal conditions for the production of nattokinase

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using *B. subtilis* on red lentil flour substrate in a fermentation method. The results of ANOVA showed that the best models for predicting fibrinolytic and protease activities were quadratic and linear models, respectively. In both models, with the increase in water content, molasses concentration and fermentation time, fibrinolytic and protease activities increased, decreased and increased, respectively. Also, the optimal fermentation conditions were 92.38 ml of water, 3.66% molasses and 70.90 hours, in which the fibrinolytic and protease activities were predicted as 2476.03 and 1.68 U/g, respectively, which confirmed the accuracy of the model due to its closeness to the experimental data. Overall, the findings of this study indicated that red lentil flour was a suitable substrate for the production of the nattokinase enzyme through fermentation by *B. subtilis*. Further investigations are recommended to optimize the conditions of fermentation in order to increase the yield of enzyme produced using red lentil flour substrate.

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مقاله علمی_پژوهشی

استفاده از سوبسترای آرد عدس قرمز برای تولید آنزیم ناتوکیناز توسط *باسیلوس سابتیلیس* ناتو

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چکیده

در این پژوهش شرایط تولید بهینه ناتوکیناز با استفاده از باکتری *باسیلوس سابتیلیس* بر روی سوبسترای عدس دال مورد بررسی قرار گرفت. برای بهینه‌سازی فرایند تخمیر، اثر سه متغیر زمان تخمیر (۲۴، ۴۸ و ۷۲ ساعت)، غلظت ملاس (۳، ۵ و ۷ درصد) و مقدار آب (۵۰، ۷۵ و ۱۰۰ میلی‌لیتر) بر میزان فعالیت فیبرینولیتیکی و پروتئازی مورد بررسی قرار گرفت. نتایج در قالب طرح مرکب مرکزی (CCD) بررسی و به روش سطح پاسخ (RSM) مدل‌سازی و تجزیه و تحلیل شد. ضریب تبیین مدل‌های رگرسیونی برازش شده برای فعالیت‌های فیبرینولیتیک (مدل درجه دوم) و پروتئازی (مدل خطی) به‌ترتیب ۹۷/۱۵ و ۹۰/۱۲ درصد بوده و فاکتور عدم برازش آنها در سطح اطمینان ۹۵ درصد معنی‌دار نبود، از این رو صحت مدل‌ها برای برازش اطلاعات تایید گردید. با افزایش میزان آب، ملاس و مدت زمان تخمیر، فعالیت فیبرینولیتیک به‌ترتیب افزایش، کاهش و افزایش یافت ($p\leq0.05$). با افزایش مقدار آب، فعالیت پروتئازی نیز افزایش یافت، اما این افزایش معنی‌دار نبود، در حالی‌که افزایش میزان ملاس و مدت زمان تخمیر به‌ترتیب منجر به کاهش و افزایش معنی‌دار فعالیت پروتئازی گردیدند ($p\leq0.05$). در ارتباط با بهینه‌سازی، شرایط بهینه تخمیر عبارت بود از: ۹۲/۳۸ میلی‌لیتر آب، ۳/۶۶ درصد ملاس و ۷۰/۹۰ ساعت تخمیر که در چنین شرایطی میزان فعالیت آنزیم‌های فیبرینولیتیک و پروتئاز به‌ترتیب ۲۴۷۶/۰۳ و ۱/۶۸ واحد بر گرم پیش‌بینی گردید. برای اعتبارسنجی مدل، تخمیر تحت شرایط بهینه انجام شد و پاسخ‌های به‌دست آمده با پاسخ‌های پیش‌بینی شده توسط مدل مقایسه گردید. نزدیکی داده‌های آزمایشگاهی به مقادیر پیش‌بینی شده توسط مدل صحت مدل را تایید نمود. نتایج این پژوهش نشان داد که از عدس دال می‌توان به‌عنوان سوبسترای تخمیر توسط باکتری *باسیلوس سابتیلیس* جهت تولید آنزیم ناتوکیناز استفاده نمود.

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