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Optimization of phospholipase production condition in submerged medium for *Trichoderma atroviride* sp. ZB-ZH292

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ABSTRACT

Phospholipases are important groups of enzyme with wide applications in various industries. In this study, two-step optimization including Plackett-Burman screening design and response surface methodology (RSM) optimization were done with the goal of higher production level of phospholipase by selected mutant strains of *Trichoderma atroviride* sp.ZB-ZH292. First step was done by screening and evaluation of seven factors affecting the enzyme activity and biomass production by selected mutant, using Plackett-Burman design at two levels each namely temperature, time, amount of soybean phospholipids (as a carbon source), peptone level (as nitrogen source), equal ratio of mono and di-potassium hydrogen phosphate (as phosphorus source), seed size, and seed age. According to the result of screening design, incubation time (P<0.01), incubation temperature (P<0.01), and soybean phospholipids as a carbon source (P<0.05), had significant effect on the enzyme activity, so they were selected and used as independent variables in central composite design (CCD) under response surface methodology (RSM). In the analysis of 20 experimental runs, the effects of three independent variables including incubation temperature (20-30 °C), incubation time (3-5 day), and phospholipid concentration (3-9%) were evaluated on phospholipase activity. Analysis of variance (ANOVA) showed that the optimum values of soybean phospholipid, incubation temperature and incubation time were 4.32%, 29.73°C, and 101.76 h, respectively. At this optimum point, the phospholipase activity was found to be 3.57 (U/ml) which is in good agreement with the predicted value (3.56 (U/ml)) by the model.

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1. Introduction

Phospholipases with the ability to affect phospholipids, able to separate fatty acids and phosphate groups in the phospholipid molecule, have potential applications in various industrial sectors such as food industries (degumming of oils, degumming of gums and production of emulsifiers with special properties), pharmaceuticals and production of vaccines, cosmetics , oily chemicals and production of detergents [1-3]. These enzymes are divided into A_1 ،A_2 B, C, and D are divided and they are obtained from different sources, including animal sources and microbial sources, and a wide range of their catalytic properties depends on the producer strain [4].

phospholipases generally by microorganisms[1] and using submerged fermentation culture[2] are produced Due to the possibility of easier control of parameters and production of products in high volume, this method has been noticed compared to other methods of fermentation cultures, and more than 90% of industrial biocatalysts (enzymes) are produced by genetic manipulation of micro-organisms, optimization of cultivation environment and environmental conditions by immersion culture [5]. Many studies have been conducted to determine the optimal conditions for the production of different biocatalysts, it shows that these conditions are influenced by the factors affecting the production of the desired product, such as the concentration and type of different sources of carbon, nitrogen, phosphorus, pH of the cultivation environment, the temperature required for growth, the age of inoculation, the duration Warming is the effect of air and stirring [6]. The general method of checking the maximum production by checking all the factors, including the cultivation environment and environmental conditions, in addition to spending time and high cost, is not able to express the mutual effect of the factors. Therefore, it seems very necessary to use statistical methods to reduce costs and time. Statistical methods, especially screening[3] and optimization[4] The growth conditions of micro-organisms or the production of special products have been taken into consideration. Platelet method- Berman is a strong and fast method. This statistical design is able to evaluate the variables and select the most effective ones on the response with a minimum number of tests in cases where there is a large number of variables. Considering the multiplicity of factors in biotechnology experiments, platelet design- Berman can have high efficiency in the early stages [7]. The factors selected in the screening stage are used in optimization methods. Response level optimization method[5] (RSM) has a high efficiency in evaluating biotechnology processes and has been used in the production of many biological products [8-10]. The main advantage of this method is to reduce the number of tests required to evaluate multiple parameters, check their mutual effects and check the relationship between independent variables and responses [11].

In the previous research, the strain*Trichoderma atroviride* As a producer of phospholipase from the effluent of oil industry, isolation, identification and title *T.atroviride* sp.ZB-ZH192 In the NCBI database[6] It was registered with the number KP233811, and in order to increase the production of phospholipase, it was randomly mutated by gamma radiation [12]. In this research, for the first time, the screening and optimization of the conditions for the production of phospholipase in the submerged culture medium for the strain*T.atroviride* sp.ZB-ZH292 (mutated by cobalt 60 gamma rays at a rate of 4.52 Gy/s, with an amount of radiation of Gy400) was investigated.

2- Materials and methods

2-1- Materials

Soybean phospholipid was obtained from Behpak (Behshahr, Iran) and other materials used in the chemical and microbial section with high purity were obtained from Merck and Sigma. This research was conducted during the years 1392-1394 in the Food Industry Department of Tarbiat Madras University.

2-2- Microorganism maintenance culture medium and primary culture preparation

For propagation and short-term storage of the strain*T.atroviride* sp.ZB-ZH292 at a

[1]. Microorganisms

[2]. Submerged Fermentation

[3]. Screening

[4]. Optimization

[5]. Response Surface Methodology

[6]. National Center for Biotechnology Information

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temperature of 4˚C, from slant culture medium[7]PDA was used. In order to prevent the reduction of the activity of the strain and possible contaminations, new slants were prepared from it every month, then the spores of the desired colonies were produced and mixed with glycerol-sterone (30%) until the time of use (long-term) and kept in a freezer at -20 [13]. After preparing the spores on the slant, the spores are suspended in sterile distilled water containing 0.1% Tween 80 (in order to better separate the spores from each other and count them more accurately) and with the help of a slide hemocytometer, the concentration of spores is 10 spores/ml.[6] was set

2-3- Investigating the ability to produce phospholipase by microorganisms

Investigating phospholipase production using spore transfer of strain colonies*T.atroviride* sp.ZB-ZH292 (spores/ml10^6) to immersion fermentation culture medium containing (g/L): soybean phospholipid, 4; Peptone, 5/7; starch, 10; meat extract, 7/5; sodium chloride, 5; Haftabe magnesium sulfate, 1; Done. The pH of the culture medium and the stirring speed were set to 7 and 150 rpm, respectively [14].

2-4- Determining enzyme activity

First, the mycelium was removed from the culture medium in a centrifuge (Sigma 3-30ks, USA) at a speed of 10000 g for 10 minutes at a temperature of 4˚C. Determination of phospholipase activity from the supernatant was done according to Jiang et al.'s method [15]. One unit of phospholipase enzyme is the amount of enzyme that is able to release one micromole of titratable fatty acid per unit of time (minute) under the test conditions. To prepare the substrate suspension, a solution of 25% soybean phospholipid and 4% polyenyl alcohol was combined in a ratio of 4:1. 4 milliliters of suspension solution, 5 milliliters of citric acid buffer (0.5M, pH: 0.01) and one milliliter of crude enzyme extract were placed in the Shikardar greenhouse for 10 minutes at 37˚C. The reaction was terminated by adding 15 ml of 95% alcohol. The titration of the released fatty acids was done by 0.05 M sodium hydroxide. Inactivation of the control sample (without enzyme extract) was done by placing it at 95˚C for 10 minutes. Experiments were performed in three replicates to calculate the average. The accuracy and precision of the enzyme activity determination method using standard phospholipase A enzyme$_1$ (PLA_1, Lecitase™ Ultra from*Thermomyces lanuginosus* , Novozyme) was investigated.

5-2- Determination of biomass

Biomass dry weight was determined by filtering the culture medium on Whatman 42 filter paper and washing the material on the funnel with distilled water until clear water came out of the funnel. The separated cell mass was dried in an oven at a temperature of 60 °C until reaching a constant weight [6].

6-2- Screening and optimization of cultivation conditions and combinations

In order to screen the effective factors on the production of phospholipase and biomass, and to select the effective factors, 12 tests were performed at two levels, the lowest (-1) and the highest (1), with the Platelet-Berman method and with the help of JMP 7.0 software (SAS Institute Inc., 2007). was designed Temperature (20 ˚C and 30 ˚C), time (3 and 7 days), inoculation percentage (2 and 6%), age of inoculation (9 and 15 hours), amount of soybean phospholipid as a carbon source (10 and 2 g/L)), the amount of nitrogen source (10 and 5 g/L) and the amount of phosphorus source, equal ratio of mono and diptasymhydrogen phosphate (1 and 0 g/L) were the factors investigated in this research. All experiments were performed in 3 repetitions and their average was used as the response [16]. After determining the effective factors, optimization using RSM method and central composite design (CCD)[8] with three variables at three levels and 6 repetitions at the central point, with response variable (phospholipase activity level) and using Design Expert software (DX 7)[9], was designed. The tests were performed in three repetitions. The model validity test was also conducted in order to confirm the agreement between the predicted result and the actual measured value in 3 repetitions and their average was reported.

7. Potato Dextrose Agar

8. Central Composite Design

9. Design-Expert® version 7.0.0

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3. Results and Discussion

3-1- Screening of factors affecting phospholipase production and biomass growth

Screening of factors affecting phospholipase activity and biomass growth of mutant strain *T.atroviride* sp.ZB-ZH292 was performed using Platelet-Berman method with seven variables in two levels (Table 1). The results showed that based on (<2.447) t-Value and (>0.05)*P*-Value, three variables of temperature, time and the amount of soybean phospholipid as a carbon source with a confidence level of 95% (>0.05*P*) had a significant effect on the amount of phospholipase production, but no significance was observed in the mutual effect of these factors (Table 2).

Table 1 The Effect of Different Factors on of Phospholipase and Biomass production from *T. atroviride* sp.ZB-ZH292 by Plackett Burman method

	C (g/L)	N(g/L)	P(g/L)	S. S. (%)	S. A. (%)	Temp. (ºC)	Time (Day)	PL (U/ml)	Biomass (g/L)
1	-1	1	-1	-1	1	-1	1	3.02	11.64
2	1	-1	-1	1	-1	1	1	3.75	12.34
3	-1	-1	-1	-1	-1	1	1	3.32	11.14
4	-1	1	-1	1	1	1	-1	3.28	10.15
5	1	1	1	-1	-1	-1	1	3.37	11.36
6	1	-1	-1	-1	1	-1	-1	2.63	7.86
7	1	-1	1	-1	1	1	-1	3.23	8.81
8	-1	-1	1	1	1	-1	1	3.02	10.52
9	-1	-1	1	1	-1	-1	-1	2.50	7.71
10	1	1	-1	1	-1	-1	-1	2.90	10.21
11	1	1	1	1	1	1	1	2.92	13.86
12	-1	1	1	-1	-1	1	-1	3.08	9.06

C: Carbon Source (Soy Phospholipid); N: Nitrogen Source; P: Phosphate Source; S.S: Seed Source; S.A: Seed Age; Temp: Temperature; PL: Phospholipase

Table 2 The Effect of Different Factors on Phospholipase Activity from *T. atroviride* sp.ZB-ZH292 by Plackett Burman Method

Factors	Effect	t-value	p-value
Temp.	0.278	6.19*	0.0024**
Time	0.248	5.52*	0.0042**
C	0.148	3.30*	0.0208*
N	0.076	1.70	0.0996
S. S.	0.076	1.70	0.0996
S. A.	-0.001	-0.04	0.9736
P	0.001	0.04	0.9736
Temp.*Time	-0.046	-1.04	0.2661
Temp.*C	0.008	0.19	0.8707
Time*C	0.030	0.67	0.4846
Temp.*N	0.015	0.11	0.9226

C: Carbon Source (Soy Phospholipid); N: Nitrogen Source; P: Phosphate Source; S.S: Seed Source; S.A: Seed Age; Temp: Temperature; PL: Phospholipase; t-value=2.447; *: *P*<0.05; **: *P*<0.01

The screening of the factors affecting the growth of biomass also shows significance (<0.05*P*) variables were time and amount of peptone as nitrogen source. Although the temperature factor was not reported as significant in this section, its effect on biomass production was visible. In the study of biomass growth, as in the study of enzyme activity, the interaction of factors in screening was not significant (Table 3). Fermentation temperature and time are effective factors in the production of biotechnology products. The amount of phospholipase production also has a maximum in relation to temperature and time, when the maximum point is passed, the growth and sporulation of the microorganism will stop, and as a result, the production of the enzyme will decrease [16-17]. Many metabolic activities take place during fermentation to produce enzymes, and in all these activities, spore life

span and inoculation rate are also effective. Increasing the amount of inoculation first increases the amount of production, but then, the increase in the number of micro-organisms leads to the accumulation of cells, decreasing the process.

The amount of nutrients and in many cases the consumption of the product leads to the benefit of biomass production and as a result the production of the product decreases.. Adding a nitrogen source to the substrate increases the amount of enzyme production, but removing and increasing it too much has the opposite effect and causes the production of by-products more than the desired product or increases the growth of microorganism and decreases enzyme production [18].

Table 3 The Effect of Different Factors on Biomass from *T. atroviride* sp.ZB-ZH292 by Plackett Burman method

Factors	Effect	t-value	p-value
Time	1.437	6.22*	0.0033*
N	0.674	2.92*	0.0287*
Temperature	0.520	2.25	0.0504
S.S.	0.424	1.83	0.0908
C	0.337	1.46	0.1541
P	-0.154	-0.67	0.5627
S.A.	0.071	0.31	0.7921
Time*N	0.050	0.21	0.8505
Time*Temp.	0.074	0.32	0.7844
N*Temp.	-.0173	-0.75	0.4315
Time*S.S.	0.046	0.20	0.8598

C: Carbon Source (Soy Phospholipid); N: Nitrogen Source; P: Phosphate Source; S.S: Seed Source; S.A: Seed Age; Temp: Temperature; PL: Phospholipase; t-value=2.447; *: $P<0.05$

Based on the obtained results, the nitrogen source was completely significant on the microorganism growth (Table 3), but no significant effect was observed on the amount of enzyme production (Table 2). In the research conducted to investigate the production of phospholipase enzyme by strain 292.*T. atroviride* Phospholipase production takes place in the logarithmic phase and this enzyme is dependent on growth. The amount of nitrogen source, like the temperature and fermentation time, has a maximum for the production of phospholipase, after passing the maximum point, the growth of the biomass occurs more than the production of the enzyme [12].

2-3- Optimization of phospholipase production conditions

Levels of the effective factors of soybean phospholipid, temperature and time

greenhouse and the results of experiment design to optimize the conditions of phospholipase production along with the amount of phospholipase activity produced by*T.atroviride* sp.ZB-ZH292 are observed in Tables 4 and 5, respectively. By examining the data in different models, the quadratic model (quadratic[10]), with a lack of fit[11] equivalent to 0.54 and R values^2، R^2 Asla Hashda And R^2 The predicted values are 0.96, 0.93 and 0.83 respectively (Table 6) and these values can indicate the appropriateness of the proposed

[10]. Quadratic

[11]. lack of fit

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model.

Table 4 Variable Levels for Optimization of Phospholipase Production *T.atroviride* ZB-ZH292 by RSM

Name	Unit	Maximum level	Intermediate level	Minimum level
Soy Phospholipid	%	(9) +1	(6) 0	(3) -1
Temperature	°C	(30) +1	(26) 0	(22) -1
Time	day	(5) +1	(4) 0	(3) -1

Table 5 Results of Optimization of Phospholipase Production from *T.atroviride* ZB-ZH292 by RSM

Run	Temperature	Time	SPL	PL.A (U/ml)	
				Actual Values	Predicted values
1	1	-1	1	3.26	3.25
2	-1	0	0	2.37	2.21
3	0	0	0	3.01	3.35
4	0	0	0	3.34	3.35
5	1	1	-1	3.56	3.47
6	-1	1	1	3.26	3.25
7	-1	-1	-1	0.86	0.94
8	0	-1	0	3.02	2.87
9	1	0	0	3.47	3.56
10	-1	1	-1	2.39	2.42
11	0	1	0	3.48	3.65
12	0	0	0	3.45	3.35
13	1	1	1	3.58	3.52
14	0	0	0	3.49	3.35
15	-1	-1	1	1.51	1.61
16	0	0	-1	3.21	3.11
17	0	0	1	3.47	3.44
18	0	0	0	3.23	3.35
19	0	0	0	3.41	3.35
20	1	-1	-1	3.11	3.18

PLA: Phospholipase Activity; SPL: Soy Phospholipid

Table 6 Analysis of Model Quadratic for optimization of Phospholipase Production from *T.atroviride* ZB-ZH292 by RSM

Source	Sum Square	Mean Square	Df	F Value	Wow P
Model	1.83	0.61	3	17.61	0.0003^{**}
Lack of Fit	0.17	0.035	5	1.00	0.5014^{n}
Net Error	0.17	0.035	5		
Total Error	192.05	0.60	20		
	R^2 = 0.96	**R^2 adjusted= 0.91**		**R^2 predicted = 0.83**	

(P<0.01) n: non-significant ; **: significant in 1% level

According to the analysis of variance (ANOVA) of the factors affecting the level of phospholipase enzyme activity (Table 7), and their mutual effects, and according to the coefficients determined for the coded factors, as well as the non-significance of the effect of all variables on the response and the elimination of the coefficients of the variables without significant effect The following final equation was determined to show the relationship between independent variables and phospholipase activity:

Equation (1)

$Y= +0.35 + 0.67\ b_1 + 0.44\ b_2 + 0.18\ b_3 – 0.30\ b_1 * b_2 – 0.15\ b_1 * b_3 – 0.47\ b_1^2$

where Y: phospholipase enzyme activity; β_1: greenhouse temperature; β_2: greenhouse time and β_3: The percentage of phospholipid is soy.

In examining the expressions of a model, higher F factor and lower P factor will indicate the greater influence of those expressions on the answer and the significance of the final model [11]. According to the analysis of variance presented in Table 7 and the equation above, all three variables of temperature, time and percentage of phospholipid as a carbon source, as well as the interaction effect of temperature-time and temperature-phospholipid percentage

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were significant. Although the factor of temperature and then time had a greater effect on the activity of phospholipase.

Table 7 Analysis of Variance to Optimization Condition for Phospholipase Production from *T.atroviride* ZB-ZH292

Sources	Sum of Square	Df	Mean Square	F Value	P Value
Model	9.48	9	1.05	30.42	0.0001^{**} >
b_1	4.52	1	4.52	130.43	0.0001^{**} >
b_2	1.92	1	1.92	55.41	0.0001^{**} >
b_3	0.32	1	0.32	9.25	0.0124^{*} >
$b_1 * b_2$	0.71	1	0.71	20.45	0.0011^{*} >
$b_1 * b_3$	0.19	1	0.19	5.37	0.0429^{*} >
$b_2 * b_3$	0.0002	1	0.0002	0.5776	0.9409^{n}
b_1^2	0.60	1	0.60	17.41	0.0019^{*} >
b_2^2	0.053	1	0.053	1.52	0.2463^{n}
b_3^2	0.011	1	0.011	0.32	0.5858^{n}
Remaining	0.35	10	0.035	-	-
Lack of fit	0.17	5	0.035	1.00	0.5014^{n}
Net Error	0.17	5	0.035	-	-
Total Error	9.82	19	-	-	-

*: significant in 5% level (P<0.05); **: significant in 1% level (P<0.01)

Table 8 The values of different parameters on optimal point to phospholipase activity from *T.atroviride* sp.ZB-ZH292

(U/ml)Phospholipase Activity		Soy Phospholipid (%)	Time (Day)	Temperature (°C)
Predicted values	Actual Value			
3.56	3.57	4.32	4.24	29.73

After determining the validity of the model statistically, it can be used to analyze and estimate the effect of different levels of variables on the amount of phospholipase production. All three factors of temperature, time and percentage of phospholipid have a significant effect on the phospholipase activity of the strain.*T.atroviride* sp.ZB-ZH292 (Table 7) and the highest level of activity in the high levels of all three variables was obtained with 3.58 U/ml (Table 5). The contours of the response surface show the relationship between the independent variables and the dependent variable, while the other variable (the third variable in this research) is kept constant at the optimal value. Two-dimensional and three-dimensional graphs of the interaction effect of temperature and incubation time at a fixed amount of phospholipase percentage and the interaction effect of temperature and soybean phospholipid percentage at a constant temperature are shown in Figures 1 and 2, respectively. By studying these diagrams, the optimal areas of the diagram based on the answer

It has been identified and additional optimization can also be done. In general, in the investigation of the effect of temperature, time and percentage of phospholipid on phospholipase activity, it was observed that the changes of all three parameters were significant, and in terms of the importance of individual effects, in order of greenhouse time (<0.01)*P*), greenhouse temperature (<0.01*P*) and then the percentage of phospholipid (<0.05).*P*) are more effective on enzyme

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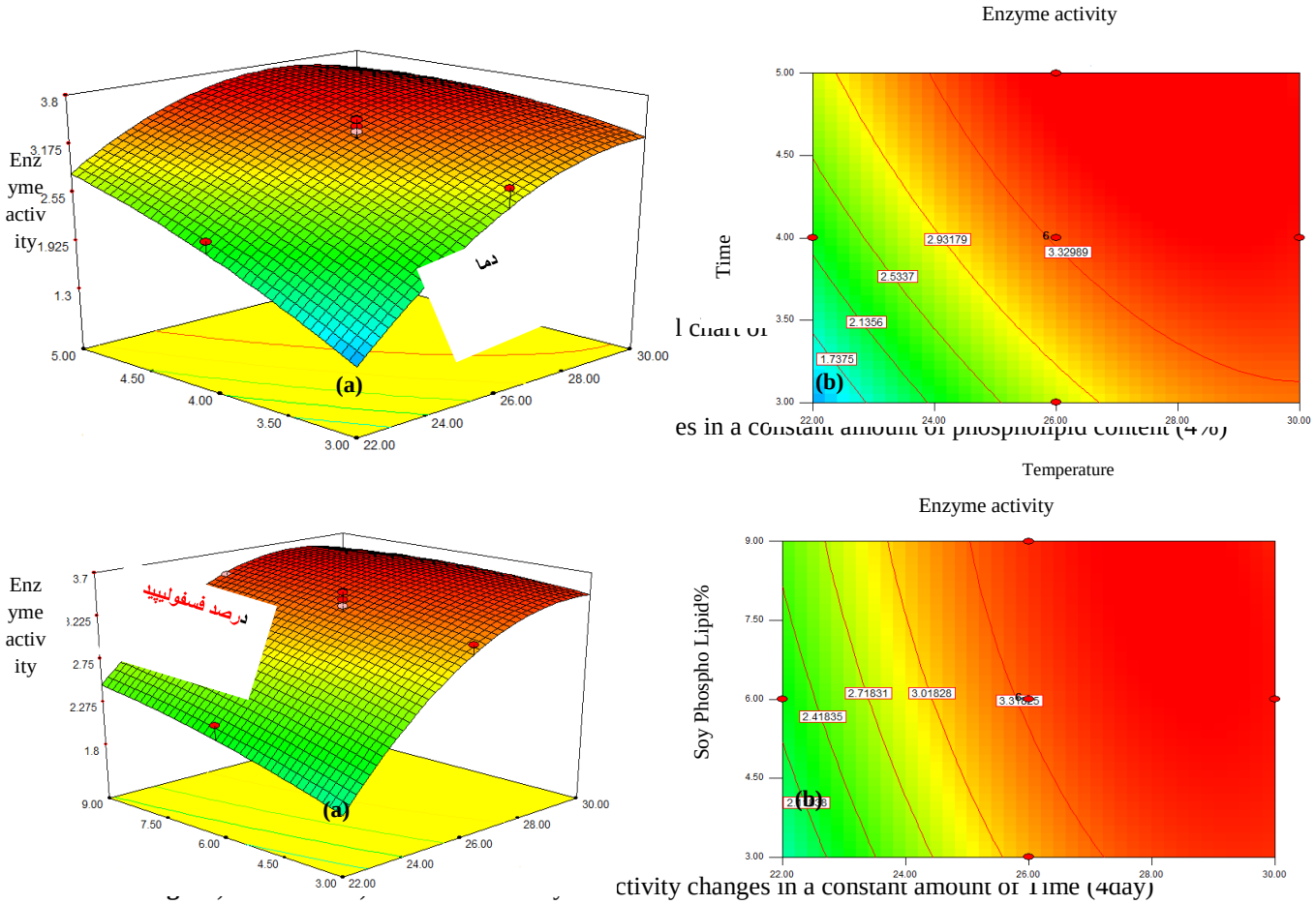
activity. The greenhouse temperature has a controlling and limiting role in enzyme activity changes (because it has quadratic effects and interaction effects with each variable with a negative coefficient), meaning that an increase in temperature (along with an increase in time or an increase in the percentage of phospholipid) up to limit (up to a temperature close to 27°C) has led to an increase in enzyme activity. The interaction of temperature-time has had a more significant effect on the process of enzyme activity compared to temperature-phospholipid percentage (Figures 1 and 2).



l chart of

es in a constant amount of phospholipid content (4%)

ctivity changes in a constant amount of Time (4day)

Figure 1 shows that in lower periods of time (3-4 days) with increasing temperature (22-26°C), the activity of phospholipase increases with a high slope. Also, this figure shows that the highest activity of the enzyme is at high temperatures (26-30°C) and long times (4-5 days) and in the cultivation times of 3 to 4 days, with increasing temperature, the enzyme activity increases significantly, but the cultivation time 5 days, the temperature has no effect on the increase in activity, and these changes indicate the interaction effect of time and temperature of greenhouse. In fact, the simultaneous increase in temperature and time by creating a positive and upward slope led to an increase in enzyme activity, but with an increase in temperature to 30°C and a time of 5 days, the activity of phospholipase decreased to some extent. This trend can be attributed to the reduction of nutrients in the culture medium due to the increase in the number of microorganisms with increasing temperature and time, which leads to the creation of unsuitable conditions for the growth of microorganisms and less production of phospholipase. Singh et al., in the study of various conditions on the activity and survival of several Trichoderma strains, found that these microorganisms are very sensitive to the environmental conditions and the composition of the culture medium. They also stated that in the temperature conditions of 25°C to 30°C, with the increase of greenhouse time in Tricordema's special culture medium, the growth rate and subsequently the resulting enzymes increased, and this increase was

different at different temperatures. With the increase in temperature from 30 °C, a decrease in the growth and production of its biological products has been observed [19]. In an exclusive research on*Trichoderma Atriviridi*It was determined that the temperature of 25°C to 30°C and the pH range of 0.7-5.7 were determined as the best conditions for its growth [20], which is in line with the results obtained in this research.

It seems necessary to use lipid carbon sources to obtain high performance of phospholipase. In the process of producing lipolytic enzymes such as lipases and phospholipases, triglyceride carbon sources can act as stimulants or inhibitors [21]. In the graphs related to the effect of soybean phospholipid on phospholipase production (Figure 2), it can be seen that increasing the percentage of phospholipid from 3 to 9% increases the activity of phospholipase with a positive slope, which is due to the stimulation of enzyme production at lower temperatures (-26°C). 22) is The simultaneous increase in temperature (26-30°C) and soybean phospholipid content (6-9%) caused the slope of the curve to be somewhat negative. The reason for this can be attributed to the production of free fatty acids in high concentration of phospholipids, which act as inhibitors of enzyme production. These results are consistent with the results of other researchers due to the inhibition of lipid carbon sources in high concentrations in the production of lipolytic enzymes [22-23].

The optimal value of phospholipase activity along with the characteristics of the optimal point for each of the parameters are shown in Table 8. To determine the validity of the model, microorganism cultivation was carried out in the obtained optimal conditions. The maximum phospholipase activity (3.57 U/ml) obtained was almost equivalent to the values predicted by the model (3.56 U/ml).

4 - Conclusion

In this study, the Platelet-Berman design was used to screen effective factors in enzyme activity and the response surface method was used to optimize selected factors from the screening plan on the phospholipase activity of the mutant strain.*T.atroviride* sp.ZB-ZH292 was used in immersion culture and after that optimization of effective factors was done by response surface method. The results showed that the two-stage optimization with Platelet-Berman and response level statistical methods is a suitable method in evaluating the effective factors and introducing the optimal treatment to achieve the highest efficiency. In this research, the enzyme activity of 3570 U per liter was determined in the submersion culture medium in optimal conditions, including 29.73 °C, 101.76 hours and 4.32% soybean phospholipid.

5- Resources

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مقاله علمی_پژوهشی

بهینه‌سازی شرایط تولید آنزیم فسفولیپاز در محیط‌کشت غوطه‌وری توسط سویه *Trichoderma atroviride* sp. ZB-ZH292

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چکیده

فسفولیپازها گروه مهمی از آنزیم‌ها با کاربردهای گسترده در صنایع مختلف هستند. در این پژوهش بهینه‌سازی دو مرحله‌ای شامل استفاده از طرح غربالگری پلاکت- برمن و سپس بهینه‌سازی سطح پاسخ با هدف افزایش تولید فسفولیپاز سویه منتخب جهش‌یافته *Trichoderma atroviride* sp.ZB-ZH292 انجام شد. در مرحله اول جهت غربالگری و انتخاب مهمترین فاکتورها بر میزان توده زیستی و فعالیت فسفولیپاز، از طرح پلاکت - برمن با هفت متغیر دما، زمان، میزان فسفولیپید سویا (به عنوان منبع کربنی)، میزان پپتون (به عنوان منبع نیتروژنی)، نسبت مساوی از منو و دی‌پتاسیم‌هیدروژن فسفات (به عنوان منبع فسفر)، درصد تلقیح و سن تلقیح، در دو سطح استفاده گردید. بر اساس نتایج حاصل از غربالگری متغیرهای زمان گرمخانه‌گذاری ($P<۰/۰۱$)، دما گرمخانه‌گذاری ($P<۰/۰۱$) و فسفولیپید سویا ($P<۰/۰۵$) به عنوان منبع کربنی اثر معنی‌داری بر میزان فعالیت آنزیمی داشتند و در نتیجه جهت اجرای بهینه-سازی نهایی به عنوان متغیرهای مستقل در طرح مرکب‌مرکزی به روش آماری سطح پاسخ وارد شدند. بر این اساس طی ۲۰ آزمایش طراحی شده، اثر سه متغیر دمای گرمخانه‌گذاری (°C ۲۰-۳۰)، زمان گرمخانه‌گذاری (۵-۳ روز) و فسفولیپید سویا (٪ ۹-۳) بر میزان فعالیت آنزیم مورد بررسی قرار گرفت. بر اساس نتایج آنالیز واریانس (ANOVA) میزان ٪۴/۳۲ فسفولیپید، دما °C ۲۹/۷۳ و زمان ۱۰۱/۷۶ ساعت گرمخانه‌گذاری به عنوان شرایط بهینه در تولید فسفولیپاز پیش‌بینی شدند. در این شرایط، میزان فعالیت فسفولیپاز (U/ml) ۳/۵۷ تعیین گردید که با نتیجه پیش‌بینی‌شده توسط مدل (U/ml ۳/۵۶) مطابقت داشت.