Statistical optimization of L-asparaginase production by *Cladosporium tenuissimum*

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Background
L-asparaginase produced by plant and bacteria can be used in the pharmaceutical and food industry. Unlike the bacterial counterparts, fungal L-asparaginase has more stability, more activity, and less adverse effects. Central composite design (CCD) was used to optimize temperature, pH, incubation time, and carbon-to-nitrogen ratio for L-asparaginase production by *Cladosporium tenuissimum* via submerged fermentation. CCD reduces the number of tests and time for optimization.
Objective
To optimize the culture conditions, such as temperature, pH, production time, and the ratio between concentration of carbon and nitrogen sources, for the production of L-asparaginase by isolated *C. tenuissimum* via submerged fermentation.
Materials and methods
Primarily, four significant parameters (temperature, pH, incubation period, and carbon-to-nitrogen ratio) were identified that affect the production process of L-asparaginase via submerged fermentation using the modified Czapek Dox medium. CCD was used to optimize the selected parameters concurrently, and their results were compared.
Results and conclusions
The highest L-asparaginase enzyme activity obtained was 2.6471 U/ml at 37°C, pH 6.2, incubation time 72 h, and 2 : 1 carbon-to-nitrogen ratio. The *P* value of interaction between every two factors was only significant for the interaction between temperature and incubation period ($P<0.0281$). The most significant factor was temperature followed by pH ($P<0.0154$) and carbon-to-nitrogen ratio ($P<0.0346$). Incubation period has no major effect on the production of L-asparaginase, but it has a quadratic effect ($P<0.0001$). Our results showed the significant role of culture conditions (temperature, pH, incubation period, and carbon-to-nitrogen ratio) in L-asparaginase production and confirmed the need for optimization.

Keywords:
enzyme, fungi, L-asparaginase, optimization, production

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Introduction

L-asparaginase (E.C.3.5.1.1) is an enzyme that catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia [1,2]. L-asparaginase is used in pharmaceutical and food industries [3]. It is produced by plant, animal tissues, and microbes [4,5]. Pharmaceutical L-asparaginase is obtained primarily from bacteria such as *Escherichia coli* and *Erwinia carotovora*. However, L-asparaginase enzymes obtained from bacteria have less stability, less enzyme activity, and are associated with undesired adverse effects, especially in the long-term use, causing some immunological reactions [4,5]. Fungal L-asparaginase is nontoxic and presumed to have an immune-suppressive activity [6].

Optimizing production medium is the most important step in L-asparaginase production [3,7] which is performed before scaling up production [8,9]. Design of experiment techniques offer a reliable result in a lesser time with reduced number of experiments [10]. In this work, we described optimization of L-asparaginase production using response surface methodology (RSM) as one of the design of experiment approaches.

Materials and methods

Microorganisms

A total of 55 fungal isolates were obtained from the stocks of the Laboratory of Mycology at the Basic and Industrial Microbiology Department, Ege University, Adnan Mendres University, and Celal Bayar University. Fungal isolates were maintained by cultivation in a slant of potato dextrose agar, Oxoid,

Wesel, Germany. These isolates were tested for L-asparaginase production by plate assay using modified Czapek Dox medium.

Chemicals and reagents

All chemicals were purchased from Merck (New Jersey, USA), unless otherwise specified.

Production of L-asparaginase via submerged fermentation

A 250-ml Erlenmeyer flask containing 50 ml sterile modified Czapek Dox medium [components (g/l): glucose: 2.0; L-asparagine, Sigma, St Louis, Missouri, USA: 10; KH_2PO_4: 1.52; $MgSO_4.7H_2O$: 0.52; KCl: 0.52; $FeSO_4$: 0.03; $CuNO_3.3H_2O$ 0.03; and $ZnSO_4.7H_2O$ 0.05] was used for production. Cultivation was done by adding 1 ml of the inoculum to the medium. The bottles were incubated at 30°C and 150 rpm. After 96 h, the fungal cell mass was separated by centrifugation at 5000 rpm for 15 min at 4°C. The supernatant was used as a raw enzyme to determine enzyme activity [11,12].

Effect of different types of carbon and nitrogen sources on L-asparaginase production

The effect of the nitrogen sources (proline, urea, asparagine, ammonium chloride, and sodium nitrate) and carbon sources (glucose, starch, lactose, glycerol, and sucrose) on the enzyme production was assessed by using the best three L-asparaginase producers from the semi-quantitative screening.

L-asparaginase activity assay

L-asparaginase activity test was performed using the Nesslerization method [9]. In the enzyme assay mix, 1 ml Tris-HCl buffer (pH 8.6), 0.9 ml distilled water, and 0.1 ml (40 mM) freshly prepared L-asparagine were used. The enzyme assay mixture was incubated with 0.1 ml culture supernatant for 30 min at 37°C, and the reaction was stopped by adding 0.1 ml of 1.5 M TCA. The reaction mixture was centrifuged at 10 000 rpm for 5 min at 4°C. Ammonia released in the supernatant was determined using a colorimetric technique at 425 nm by adding 0.5 ml of Nessler reagent (45.5 g HgI_2 and 35.0 g KI dissolved in 1 l distilled water containing 112 g KOH) to the sample containing 0.2 ml of supernatant and 4.3 ml of distilled water. Blank samples were prepared by adding TCA before the enzyme was added. Ammonia released in the reaction was calculated based on the standard curve obtained using ammonium sulfate. One unit of L-asparaginase activity was defined as the amount of enzyme that releases 1 μM ammonia at 37°C per minute, using asparagine as the substrate.

Experimental design and central composite design-based optimization

A RSM-based central composite rotatable design with four variables was used to study the response pattern and to define the interaction between parameters. The variables with five levels are temperature (27–47°C), pH (4.2–8.2), carbon-to-nitrogen ratio (2 : 10, 2 : 15, 2 : 20, 4 : 10, 6 : 10), and incubation time (24–120 h), which were optimized. The experimental design for central composite rotatable design was carried out as shown in Table 1. For detection of pure error sum square, six replicates (run order: 12, 19, 26, 3, 14, 22) at the center of the model were performed. The influence of the variables on the production of L-asparaginase (response) was expressed by second-order polynomial presented in the following equation:

$$Y = \beta 0 + \sum \beta iXi + \sum \beta iiX2i + \sum \beta ijXiXj.$$

Where Y is the predicted enzyme yield (response), β_0 is the intercept term, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interaction effect, and Xi and Xj are independent factors.

Statistical analysis

The statistical analysis was performed using the Design-Expert 7.0 software. Statistical analysis of the observations was evaluated through analysis of variance, R^2, and three-dimensional graphs. The observations resulted from RSM were recorded and interpreted with their respective possible surface interaction plots.

Validation of experimental design

The model was validated by selecting one of the experiments recommended by the program to perform

Table 1 Ranges of the independent variables used for the production of L-asparaginase by *Cladosporium tenuissimum* using response surface methodology

		Level			
Codes	Variables	−1	+1	−Alpha	+Alpha
A	Temperature (°C)	32	42	27	47
B	pH	5.2	7.2	4.2	8.2
C	Incubation time (h)	48	96	24	120
D	C vs. N	−1	1	−2	2

validation. After performing the experiment, the result was compared with the result that had been predicted by the model.

Results and discussion

Experimental design and central composite design-based optimization

The present study reveals that 2.6471 U/ml is the highest L-asparaginase level obtained by *Cladosporium tenuissimum* at 37°C, pH 6.2, incubation time 72 h, and 2 : 1 carbon : nitrogen ratio (Table 2) (trial 19), whereas the lowest L-asparaginase level (0.0037 U/ml) was obtained from trial 9 experiment (42°C, pH 5.2, incubation time 48 h, and carbon/nitrogen ratio 0.4 : 1), indicating that L-asparaginase production by *C. tenuissimum* is influenced by levels of the selected factors. Almost at a similar condition (incubation time 72 h, pH 6.3, and temperature 33°C), Vimal and Kumar [3] achieved the highest level of L-asparaginase production using *Aspergillus terreus*.

Statistical analysis

Regression analysis was conducted to calculate the effect of each factor and interaction between factors. The model is expressed by the following formula:

$$\begin{aligned} Y = {} & 1.44 - 0.30 \times A + 0.13 \times B + 0.096 \times C + 0.11 \\ & \times D - 0.082 \times A \times B - 0.15 \times A \times C \\ & - 0.077 \times A \times D - 0.084 \times B \times C \\ & + 0.066 \times B \times D + 0.041 \times C \times D - 0.17 \\ & * A^2 - 0.24 \times B^2 - 0.27 \times C^2 - 0.22 \times D^{2.} \end{aligned}$$

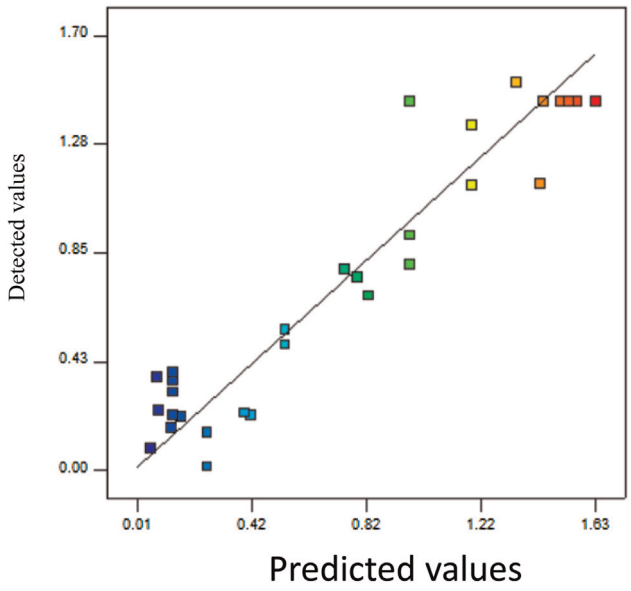
Where Y is the predicted enzyme yield, A is the temperature, B is the pH, C is the incubation time, and D is the C versus N.

The relationship between predicted and actual values are presented in Fig. 1, whereas Fig. 2 describes the approximate linear model for probability. According to the analysis of variance (Table 3), the most significant factor was temperature followed by pH ($P<0.0154$) and carbon-to-nitrogen ratio ($P<0.0346$). Incubation period has no major effect on the production of

Table 2 Experiments design for central composite rotatable design

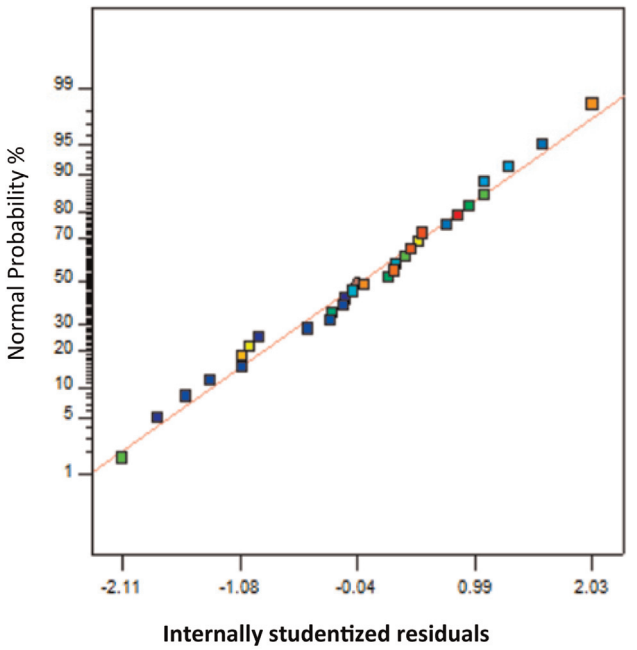
Run orders	Temp (°C)	pH	Incubation period (h)	Carbon-to-nitrogen ratio
1	32	7.2	48	4 : 10
2	37	6.2	120	2 : 10
3	37	6.2	72	2 : 10
4	32	5.2	48	4 : 10
5	32	5.2	48	2 : 15
6	42	7.2	96	4 : 10
7	32	7.2	96	2 : 15
8	42	7.2	48	4 : 10
9	42	5.2	48	4 : 10
10	42	7.2	48	2 : 15
11	32	5.2	96	2 : 15
12	37	6.2	72	2 : 10
13	47	6.2	72	2 : 10
14	37	6.2	72	2 : 10
15	42	5.2	96	4 : 10
16	37	4.2	72	2 : 10
17	32	7.2	48	2 : 15
18	37	6.2	72	6 : 10
19	37	6.2	72	2 : 10
20	32	5.2	96	4 : 10
21	42	5.2	96	2 : 15
22	37	6.2	72	2 : 10
23	27	6.2	72	2 : 10
24	32	7.2	96	4 : 10
25	37	6.2	24	2 : 10
26	37	6.2	72	2 : 10
27	37	6.2	72	2 : 20
28	42	5.2	48	2 : 15
29	37	8.2	72	2 : 10
30	42	7.2	96	2 : 15

Figure 1



Regression plot for L-asparaginase production detected and predicted values.

Figure 2



Normal plot of residuals.

Table 3 Optimization of L-asparaginase production by *Cladosporium tenuissimum*

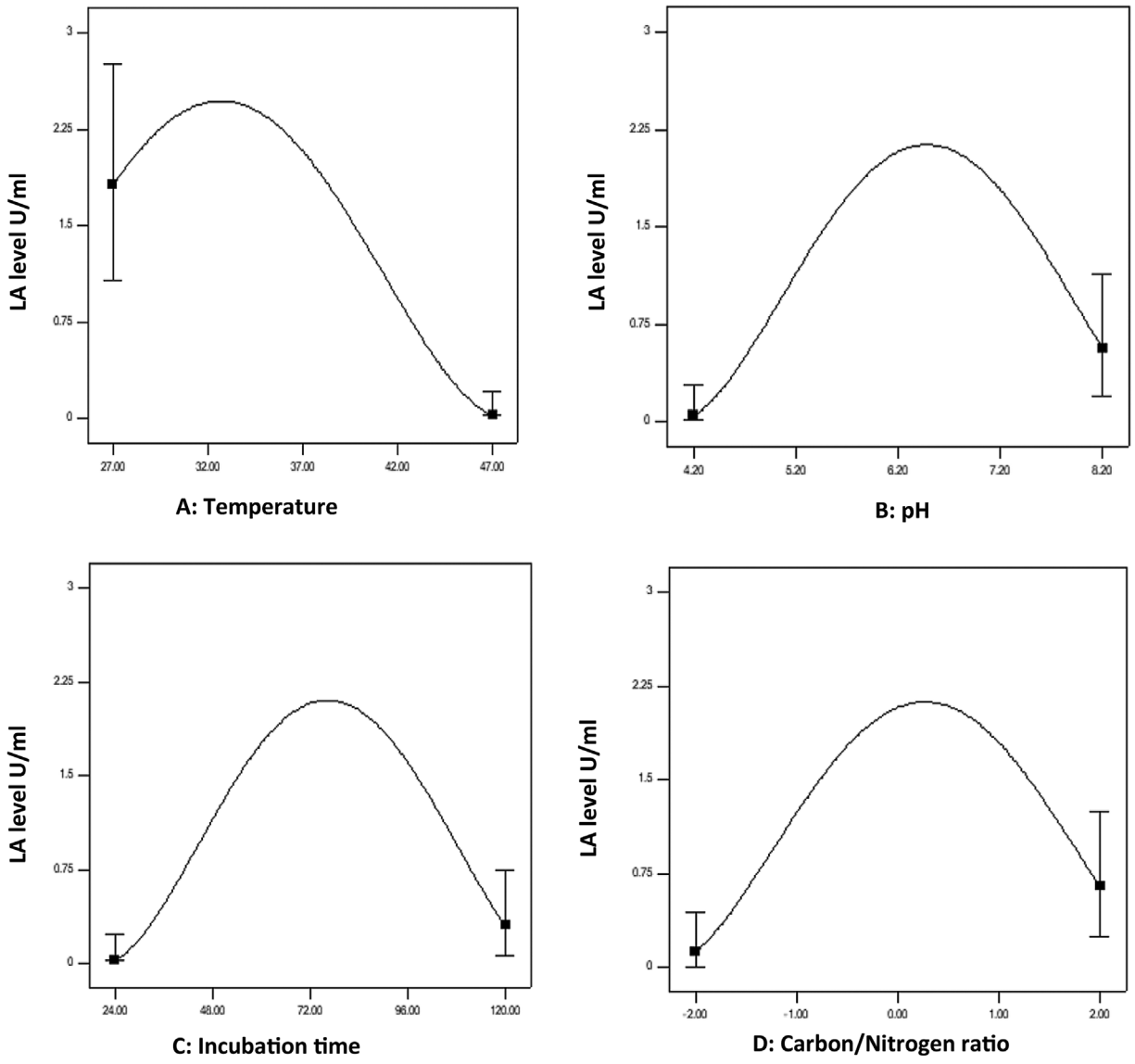
Runs	Temp (°C)	pH	Incubation period (h)	Carbon to nitrogen ratio	L-asparaginase activity (U/ml)
1	32	7.2	48	4 : 10	1.422
2	37	6.2	120	2 : 10	0.2867
s3	37	6.2	72	2 : 10	2.2643
4	32	5.2	48	4 : 10	0.0196
5	32	5.2	48	2 : 15	0.172
6	42	7.2	96	4 : 10	0.0196
7	32	7.2	96	2 : 15	0.9546
8	42	7.2	48	4 : 10	0.2867
9	42	5.2	48	4 : 10	0.0037
10	42	7.2	48	2 : 15	0.0073
11	32	5.2	96	2 : 15	0.5539
12	37	6.2	72	2 : 10	0.9546
13	47	6.2	72	2 : 10	0.0682
14	37	6.2	72	2 : 10	2.0889
15	42	5.2	96	4 : 10	0.0086
16	37	4.2	72	2 : 10	0.0196
17	32	7.2	48	2 : 15	0.6874
18	37	6.2	72	6 : 10	0.9456
19	37	6.2	72	2 : 10	2.6471
20	32	5.2	96	4 : 10	2.0532
21	42	5.2	96	2 : 15	0.0278
22	37	6.2	72	2 : 10	2.357
23	27	6.2	72	2 : 10	1.422
24	32	7.2	96	4 : 10	1.8228
25	37	6.2	24	2 : 10	0.0186
26	37	6.2	72	2 : 10	2.431
27	37	6.2	72	2 : 20	0.0196
28	42	5.2	48	2 : 15	0.1532
29	37	8.2	72	2 : 10	0.6206
30	42	7.2	96	2 : 15	0.0682

L-asparaginase ($P<0.0686$) but it has a quadratic effect ($P<0.0001$). These results were in agreement with the previous results of Gurunathan and Sahadevan [2], Kumar *et al.* [9], and Prakasham *et al.* [10], in which the authors reported a significant main effect of temperature on L-asparaginase production.

Contrary to the findings of Vimal and Kumar [3] and Prakasham *et al.* [10], in our study, pH showed significant effect on L-asparaginase production, supporting the previous results of Gurunathan and Sahadevan [2], Dias and Sato [7], Kumar *et al.* [9], and Talluri *et al.* [13]. The R^2 was 0.9679, which is very close to 1.0, and the adjusted R^2 (0.8671) and projected R^2 (0.8591) were close to each other, reflecting that the model is suitable for prediction.

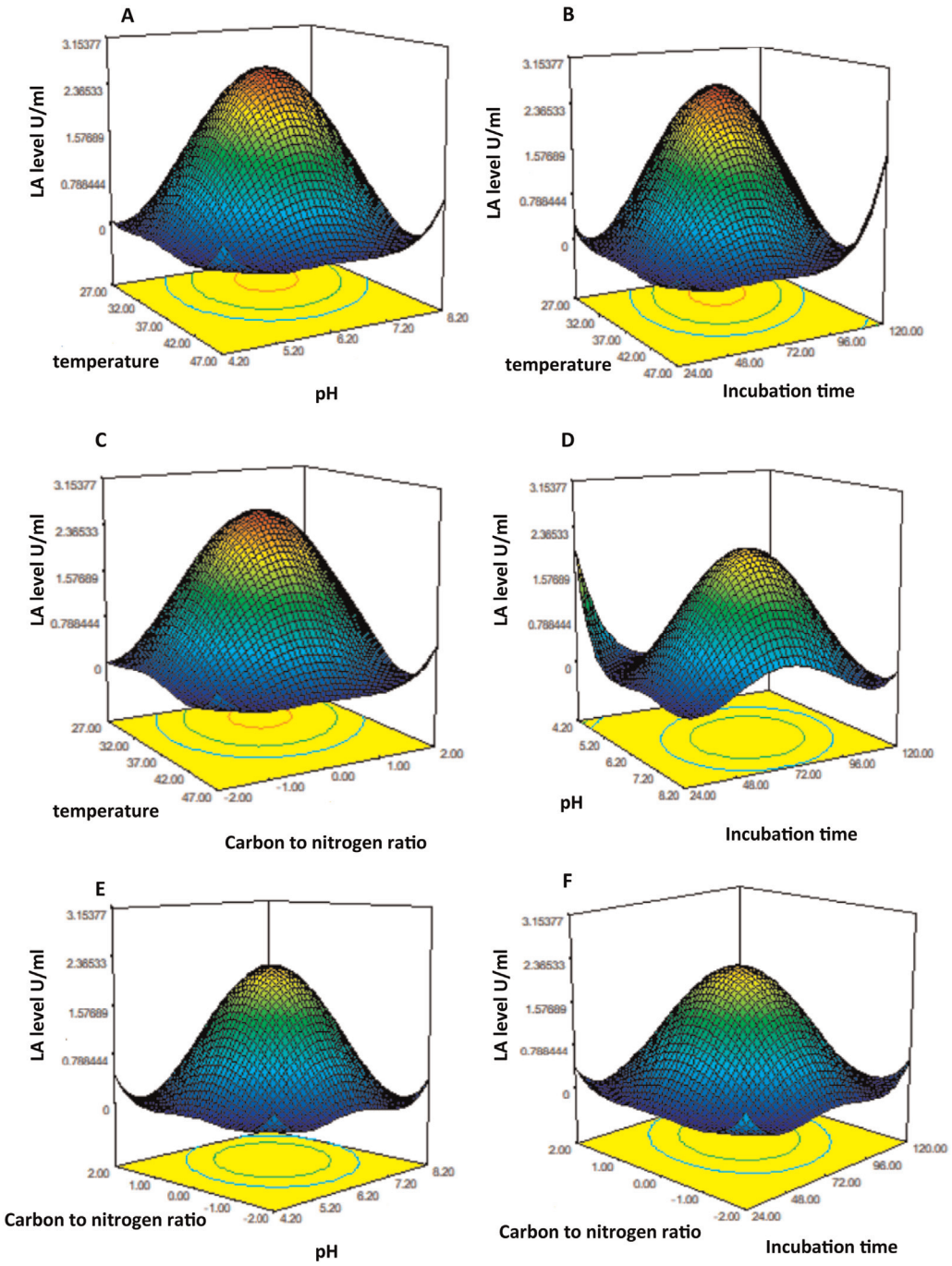
Figure 3 shows the main effect of each factor separately on the production of L-asparaginase by *C. tenuissimum*. Figure 3b, c, and d shows a curve with a top in the middle of the curve, indicating that the range of factors used in optimization is well maintained. Moreover, it reveals that the optimum level of the factor for the production of maximum L-asparaginase is the medium level in the range. Although the curve of temperature (Fig. 3a) shows a top near to the left of the curve, the optimum level for maximum production was not missed. The three-dimensional graphs (Fig. 4) were done by plotting the response of the regression model; it shows the interaction effect between parameter on the overall produced enzyme level. Moreover, it shows clearly the optimum level of the parameters required for the maximum yield of L-asparaginase enzyme.

Figure 3



Effect of different parameters on the production of l-asparaginase by *Cladosporium tenuissimum* (a) temperature, (b) pH, (c) incubation time, and (d) carbon versus nitrogen source.

Figure 4



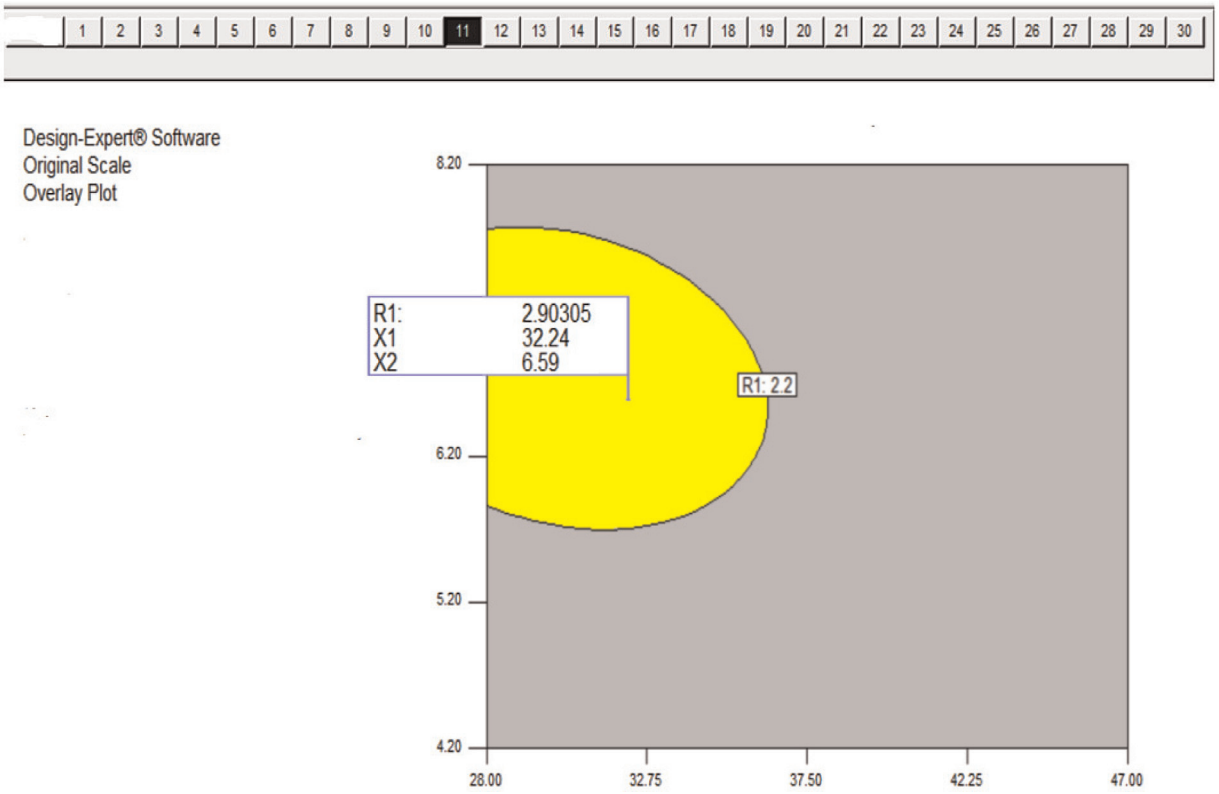
Three-dimensional response surface plot for the effect of temperature and pH (a), temperature and incubation time (b), temperature and carbon : nitrogen ratio (c), incubation time and pH (d), carbon : nitrogen ratio and pH (e), carbon : nitrogen and incubation time (f).

However, Gurunathan and Sahadevan [2,4] showed that there was no effect of interaction between any of these variables for l-asparaginase production by *A. terreus* in submerged fermentation.

Maximum activity and model validation

From the experiment proposed by the program, 3.25 U/ml L-asparaginase activity was obtained. The model predicted that we would obtain 2.903 U/ml, and for the validation of the model, the result should be between 2.2 and 3.8 U/ml. Our result of 3.25 U/ml is fitting the range, indicating that our model is valid. As shown in Fig. 5, the yellow region determines the levels of factors that produce maximum L-asparaginase level. Maximum L-asparaginase activity could be obtained from an experiment carried out under any selected levels in the yellow zone (Table 4).

Figure 5



The levels of factors that produce maximum L-LA level. R1: maximum L-LA level, X1: optimum temperature, X2: optimum pH.

Table 4 Analysis of variance for L-asparaginase production

Sources	Sum of squares	DF	Mean square	*F* value	*P* value >*F*	
Model	7.942155	14	0.567297	9.790152	< 0.0001	Significant
A-Temperature	2.165705	1	2.165705	37.37477	< 0.0001	
B-pH	0.432752	1	0.432752	7.468246	0.0154	
C-Incubation period	0.223034	1	0.223034	3.849021	0.0686	
D-Carbon : nitrogen ratio	0.312701	1	0.312701	5.396461	0.0346	
AB	0.106687	1	0.106687	1.84115	0.1949	
AC	0.342368	1	0.342368	5.90843	0.0281	
AD	0.093958	1	0.093958	1.621493	0.2223	
BC	0.112863	1	0.112863	1.947747	0.1831	
BD	0.069691	1	0.069691	1.202689	0.2901	
CD	0.027102	1	0.027102	0.467712	0.5045	
A^2	0.8268	1	0.8268	14.26854	0.0018	
B^2	1.571344	1	1.571344	27.11754	0.0001	
C^2	2.019511	1	2.019511	34.85181	< 0.0001	
D^2	1.282915	1	1.282915	22.13997	0.0003	
Residual	0.869185	15	0.057946			
Lack of fit	0.5924	10	0.05924	1.070147	0.5013	Not significant
Pure error	0.276784	5	0.055357			
Cor total	8.81134	29				

R^2=0.9679, predicted R^2=0.8591, adjusted R^2=0.867, and CV%=27.87.

Conclusion

The type of carbon and nitrogen sources present in the production environment can affect the level of L-asparaginase produced by the fungi. Glucose is the best carbon source, whereas urea was found to be the best nitrogen source for L-asparaginase production by *C. tenuissimum*. Optimization strategy used here can also be used to set up optimization for production of other enzymes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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