Statistical optimization of L-asparaginase production by using *Fusarium solani*

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Background and objective
The enzyme L-asparaginase is important as a therapeutic agent in the treatment of acute lymphocytic leukemia. It has been observed that microorganisms such as yeast and filamentous fungi such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. are commonly produced asparaginases with fewer adverse effects. *Fusarium solani* was selected to be the most potent microbial isolate for L-asparaginase production. The factors controlling L-asparaginase production, such as containing media, were implemented to increase the yield of L-asparaginase. Statistical experimental design such as the Plackett–Burman enables finding out the most effective factors that increase the yield of L-asparaginase.
Materials and methods
Aspergillus rubber, *Aspergillus terreus*, *Epico niger*, *Penicillium cyclopium*, and *F. solani* were chosen for screening the L-asparaginase activity using phenol red – a pH indicator. L-Asparagine and the modified Czapek Dox media were tested for the production of L-asparaginase from *F. solani* under different incubation times. To optimize L-asparaginase production by using *F. solani*, the combined effect of seven variables (L-asparagine, sucrose, $NaNo_3$, K_2HPO_4, $MgSO_4$, KCl, and time) were assessed using the central composite design.
Results and conclusion
All the four fungal strains screened for L-asparaginase activity showed positive results in the rapid plate assay method. The medium employed contained asparagine with phenol red, and after incubation, pink zones around the colonies were observed. *F. solani* was a potential source for a high yield of L-asparaginase enzyme and high substrate specificity. Modified Czapek medium II was the most suitable for L-asparaginase, showing an activity of 121 U/ml. The result on L-asparaginase activity according to the screening Plackett–Burman experiments gives a medium composed of the following (g/l): sucrose (1); L-asparagine (0.5); KH_2PO_4. (0.75), $MgSO_4$ (0.7); H_2O (0.72); KCl (0.72); $(NH_4)_2SO_4$ (11.7) at an incubation time of 3 days. For further investigation, x_1 K_2HPO_4, x_2 sucrose, and x_3 time were found to be the most significant variables affecting L-asparaginase activity. The second optimization step was to identify optimal values of the three factors that bring about maximum L-asparaginase activity, using the central composite designed experiment. The results showed that K_2HPO_4 (2.5 g/l), sucrose (4 g/l), and the time (8 days) were critical in the production of L-asparaginase.

Keywords:
Box–Behnken design, *Fusarium solani*, L-asparaginase, Plackett–Burman design, submerged media

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Introduction

L-Asparaginase (E.C.3.5.1.1) is one of the most important biomedical and biotechnological group of enzymes as it constitutes nearly 40% of the total worldwide enzyme sales [1]. It is present in many organisms including animals, plants, microorganisms, and in the serum of certain rodents but not in humans [2].

Microbial enzymes are preferred because they are stable, consistent, less economically produced, and are well known for their ease of process modification and optimization [3].

L-Asparaginase is used in the treatment of acute lymphoblastic leukemia, pancreatic carcinoma, and bovine lymphomosarcoma [4]. It is known as ELSAPAR, ONCASPAR, ERWINASE, and KIDROLASE. The enzyme is produced by several microorganisms, for example, *Eschericia coli* [5], *Erwinia carotovor* [6], *Enterobacter aerogenes* [7],

Candida utilis [8], *Staphylococcus aureus* [9], and *Thermus thermophiles* [10].

Tumor cells depend on the presence of L-aspragine as an essential amino acid in their growth, whereas normal cells are not dependent as they can be synthesized in sufficient amounts for the metabolic needs using their own enzyme (L-asparaginase synthetase) [11].

Submerged fermentation is widely used for the production of L-asparaginase throughout the world. This method shares a number of disadvantages such as low concentration of the product, consequent handling reduction, and disposal of large volumes of water during the downstream processing [12,13].

It was reported previously that most of the microbial L-asparaginase is secreted inside the cells except few that are extracellular [14], which in turn are more advantageous. They could be produced abundantly in the culture broth under normal conditions and could also be purified economically. L-Asparaginase of bacterial origin could cause an allergic reaction like a skin rash, deceased blood pressure, sweating, or loss of consciousness. It may also interfere with blood clotting, raise liver enzyme blood tests, and hence lead to liver disease. Fungal L-asparaginase is nontoxic and appears to have an immune suppressive activity [11].

Statistical experimental design is better acknowledged for the optimization of nutritional and environmental requirements for the L-asparaginase production compared with the traditional one variable at a time method [11]. It tackles the problem involving the specific design of experiments, which in turn minimizes the error in determining the effect of the parameters, and the results are achieved in an economical manner to get clear results with least expense. The response surface method quantifies the relation between the responses and the vital input factors. The most well-known response surface methods are central composite and Box–Behnken designs [3].

Our objective was to choose the best microorganism that produced significant yield of L-asparaginase. Statistical experimental design enabled finding out the optimal conditions using the solid state fermentation.

Materials and methods

Microorganisms

The fungi used throughout this study, *Aspergillus terreus*, *Aspergillus rubber*, *E. niger*, and *Fusarium solani*, were provided by the National Research Center, Cairo, Egypt. All strains were grown on potato dextrose agar slants at 30°C and preserved at 80°C in 50% glycerol.

Cultivation conditions and crude enzyme extraction

Fungal strains (*A. terreus*, *A. rubber*, *E. niger*, and *F. solani*) were grown for 3 days at 30°C in the modified Czapek medium, which had the following composition accurate optimized values (g/l): sucrose (30); $NaNo_3$ (3.0); KH_2PO_4 (0.1); $MgSO_4$ (0.7); H_2O (0.5); KCl (0.5); $FeSO_4$ (0.7); and H_2O (0.01). The medium was supplemented with L-asparaginase 1.5%. The pH of the medium was initially adjusted to 7.0. The flasks were autoclaved for 15 min at a pressure of 1.5 lb/inch^2. Cultivation was achieved by adding 2 ml of the fungal suspension previously prepared as a standard inoculum in 100 ml of the fermentation medium placed in 250 ml Erlenmeyer flasks, and then incubated at 30°C in a reciprocal shaker (200 rpm) for 3 days. The extracellular enzyme was prepared by centrifugation at 5000 rpm for 20 min. The supernatant obtained from the culture broth was used for the determination of L-asparaginase activity.

Enzyme assay

Qualitative method (rapid plate assay)

L-Asparaginase activity of six fungi was screened by using the Czapek Dox agar medium supplied with L-asparagine and three drops of phenol red indicator dye (pH 7). L-Asparaginase-producing microorganisms were selected on the basis of formation of pink zones around the colonies of the medium [15].

Quantitative method

L-Asparaginase was assayed colorimetrically [16]. A standard curve was prepared with ammonium sulfate. One L-asparaginase unit (1 U) is defined as the amount of enzyme that liberates 1 μmol of ammonia per minute under the optimal assay conditions.

Statistical design

Plackett–Burman design

Both the nutritional and environmental factors were evaluated. The different factors were prepared in two levels, one for low level and the other for high level, according to the Plackett–Burman statistical design. The design is practical especially when there are large numbers of factors are implemented in settings that produce optimal or near optimum responses [17]. Table 1 shows the factors under investigation as well as levels of each factor used in the experimental design. The Plackett–Burman experimental design is based on the first-order model:

$$Y = \beta_0 + \sum \beta_i X_i,$$

where Y is the response; β_0 is the model intercept; and β_1 is the variables estimates. This model describes no interaction among the factors and it is used to evaluate the important factors that affect the production of L-asparaginase. The effect of each variable was determined by the following equation:

$$E(x) = \frac{2(\sum M^{+} - M^{-})}{N},$$

where $E(x)$ represent L-asparaginase activity at the tested variable, M^{+} and M^{-} are the effect of this variable, at the high and low concentrations, respectively, and N is the number of trials. The SE of the concentration effect was the square root of the variance of an effect, and the significant level (P-value) of each concentration effect was determined using Student's t-test:

$$T(x) = \frac{E(x)}{SE},$$

where $E(x)$ is the effect of the variable x.

Seven independent variables were screened in combinations organization. All trials were performed in duplicates and the average of L-asparaginase yield observations were treated as responses.

Table 1 Actual values of the process variables

Variables	Symbol	–	0	+
L-Asparagine	x_1	0.5	1	1.5
Sucrose	x_2	1	2	3
$NaNO_3$	x_3	0.1	0.2	0.3
K_2HPO_4	x_4	0.5	1	1.5
$MgSO_4$	x_5	0.25	0.5	0.75
KCl	x_6	0.25	0.5	0.75
Time (days)	x_7	4	5	6

Box–Behnken design

This design was used for the experimental plan to estimate the nature of the response surface in the experimental region [18]. Table 2 presents the design matrix of 13 trial experiments. Using this design, factors of highest confidence level percentage were prescribed into three coded levels, coded –1, 0, and +1 for low, middle, and high concentration, respectively. For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between variables and responses (asparaginase activity). For the three factors the equation is as follows:

$$Y = \beta_0 + \beta_1 \times_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2,$$

where Y is the predicted response; β_0 is the model constant; x_1, x_2, and x_3 are independent variables; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are cross product coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Microsoft excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of polynomial model equation was expressed by the coefficient of determination R^2. All experimental designs were randomized to exclude any bias. Experiments were carried out in duplicate and mean values were given.

Validation of the experimental model

The statistical model was validated with respect to L-asparaginase production under the conditions predicted by the model under submerged conditions. Samples were withdrawn at the desired intervals and L-asparaginase assay was carried out as described above.

Table 2 Box–Behnken factorial design for three independent variables for optimization for L-asparaginase production using the central composite design

Trials	Independent variables			Asparagine activity	Dry weight (g/10 ml)
	K_2HPO_4 x_1	Time x_2	Sucrose x_3		
1	1.5 (–)	6 (–)	4 (0)	124.3	0.196
2	2.5 (+)	6 (–)	4 (0)	128.8	0.255
3	1.5 (–)	8 (+)	4 (0)	133.6	0.209
4	2.5 (+)	8 (+)	4 (0)	144.4	0.233
5	1.5 (–)	7 (0)	3 (–)	103.5	0.202
6	2.5 (+)	7 (0)	3 (–)	118.1	0.217
7	1.5 (–)	7 (0)	5 (+)	92.2	0.265
8	2.5 (+)	7 (0)	5 (+)	83.7	0.267
9	2 (0)	6 (–)	3 (–)	115.9	0.222
10	2 (0)	8 (+)	3 (–)	73.7	0.219
11	2 (0)	6 (–)	5 (+)	71.4	0.223
12	2 (0)	8 (+)	5 (+)	63.7	0.245
13	2 (0)	7 (0)	4 (0)	115	0.251

Results and discussion

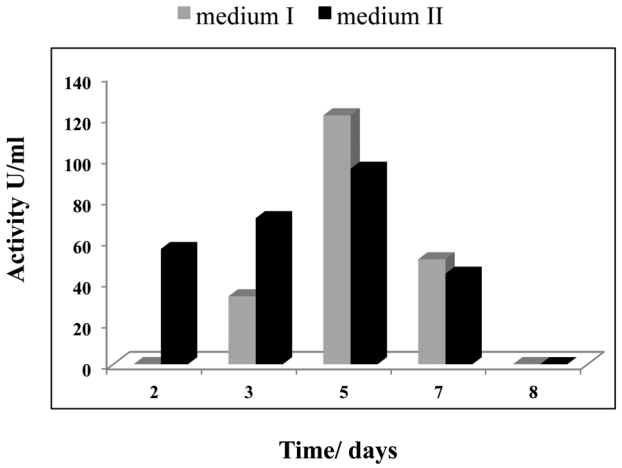
Screening of different fungi for L-asparaginase production by using the rapid plate assay

Four fungi, namely *A. terreus*, *A. rubber*, *E. niger* and *F. solani*, and *P. cyclopium*, were screened for L-asparaginase activity. They all showed positive results by using the rapid plate assay method (Fig. 1). L-Asparagine was hydrolyzed by L-asparaginase into ammonia, thus increasing the pH of the media, which in turn was detected by using phenol red – a pH indicator. This method is quick and L-asparaginase production can be seen directly [19]. It was proposed that the strain exhibited zone diameter between 30 and 40 cm was referred to as good L-asparaginase producer. *F. solani* exhibited the higher zone of diameter 5 cm and was considered as a potential strain for L-asparaginase production (Fig. 1). This result is in agreement with that obtained by Siddalingeshwara *et al.* [20], who found that eukaryotic microorganisms such as yeast and filamentous fungi such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. have the ability to produce L-asparaginase without side effects.

Submerged fermentation using two different media

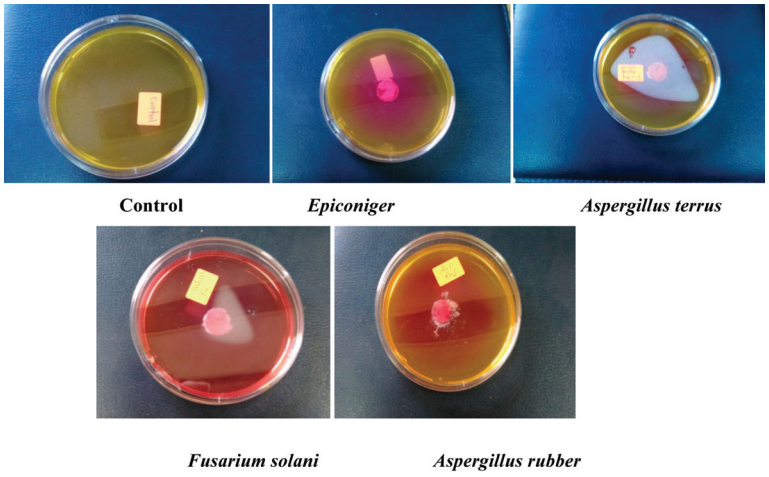
In two different submerged fermentation media (L-asparagin and modified Czapek), the production of L-asparaginase by using *F. solani* was tested under different incubation periods (Fig. 2). Maximum enzyme activity was exhibited after 5 days with both media. Similar results were obtained by Niharika and Supriya [21], who reported that maximum enzyme activity (182.54 ml) was reported on using *Fusarium oxysporum*. Further increase in the incubation period showed a decrease. This may be due to depletion of nutrients, accumulation of toxic products, or change of pH of the medium. Modified Czapek was the most suitable for L-asparaginase, showing an activity of 121 U/ml. Medium 2 showed better enzyme production; this might be attributed to the fact that sucrose has an inductive effect as an inexhaustible source of carbon as compared with other carbon sources and it helps in stabilizing the enzyme [22]. Similar results were obtained by Niharika and Supriya [21], who found that the presence of sucrose as a carbon source in the fermentation medium was an effective inducer for L-asparaginase production using *F. oxysporum*. Fungi previously showed great specificity toward different nitrogen sources, which in turn influenced the metabolism of the microorganism. Nitrogen source is a limiting nutrient and plays an important role in L-asparaginase production. The higher production of the enzyme using medium 2 may be attributed to the presence of sodium nitrate,

Figure 2



Effect of the two submerged fermentation media (I and II) at different time intervals. Medium I: Czapek medium; medium II: L-asparagine medium.

Figure 1



Screening of L-asparaginase production by some fungi.

which may be more suitable compared with peptone and yeast. These results were in agreement with those reported by El-Shafei and El-Ghonemy [23], who showed that sodium nitrate as an inorganic nitrogen source enhanced the production of L-asparaginase using *Trichoderma viride* F_2.

Analysis of the fermentation factors affecting L-asparaginase production

These experiments were designed upon the optimization of the fermentation conditions of L-asparaginase production using the Plackett–Burman design. The tested levels of the seven variables are presented in Table 1. The experiments were carried out with nine trials of the different cultural conditions (Table 3 and Fig. 6). All experiments were performed in duplicates and their averages were recorded. The data on L-asparaginase activity using Plackett–Burman (PB) experiments showed a wide variation from 24.06 to 123.6 μ/ml. This significant variation in the enzyme activity reflects the importance of optimization of the composition of the fermentation medium. The results obtained were used for the analysis of the variance (ANOVA) (Table 4). The main effects of the seven examined factors on L-asparaginase activity were calculated and represented graphically in Fig. 1. The results indicated that the activity of L-asparaginase was positively affected by sucrose, $NaNO_3$, K_2HPO_4, and the time. On the other hand, L-asparaginase, $MgSO_4$, $7H_2O$, and KCl showed negative effects. The data obtained from the experiments were used for the ANOVA (Table 4). The model *F*-value of 4.0701 implies the model is significant. Values of 'Prob>*F*' less than 0.0522 indicates that the model terms are significant. In this case, x_1, x_2, x_3, x_1x_2, x_1x_3, x_2x_3, x_1^2, x_2^2, and x_3^2 are significant models. Values greater than 0.1000 indicate the model terms are not significant.

Based on results shown in Table 4, the calculated *t*-test, with the *P*-value showed that x_1 K_2HPO_4, x_2 sucrose and x_3 time were the most significant variables affecting L-asparaginase activity.

Optimization of L-asparaginase activity by using the Box–Behnken design

To approach the optimum response region of the enzyme activity, each of the effective independent variables including K_2HPO_4 concentration (x_1),

Table 3 Plackett–Burman experimental design for the evaluation of factors (coded levels and real values) affecting L-asparaginase activity

Trial number	Independent variables							Asparagine activity	Biomass (g/100 ml)
	x_1	x_2	x_3	x_4	x_5	x_6	x_7		
1	−	−	−	+	+	+	−	42.6	0.198
2	+	−	−	−	−	+	+	38.7	0.261
3	−	+	−	−	+	−	+	75.4	0.288
4	+	+	−	+	−	−	−	104.4	0.225
5	−	−	+	+	−	−	+	120.4	0.236
6	+	−	+	−	+	−	−	24.06	0.226
7	−	+	+	−	−	+	−	63.4	0.245
8	+	+	+	+	+	+	+	123.6	0.266
9	0	0	0	0	0	0	0	111	0.263

Table 4 Analysis of variance for L-asparaginase production by *Fusarium solani*

Term	Coefficient estimate	DF	SE	SS	*t*-value	*F*-ratio	*P*-value
Corrected model		9		6873.1573		4.6701	0.0522
Intercept	105	1	7.383044		14.22		0.0001*
x_1 K_2HPO_4	0.175	1	4.521172	0.2450	0.04	0.0015	0.9706
x_2 time	−8.125	1	4.521172	528.1250	−1.80	3.2296	0.1323
x_3 sucrose	−15.025	1	4.521172	1806.0050	−3.32	11.0440	0.0209*
x_1x_2	−3.425	1	6.393903	46.9225	−0.54	0.2869	0.6151
x_1x_3	−5.775	1	6.393903	133.4025	−0.90	0.8158	0.4078
x_2x_3	13.625	1	6.393903	742.5625	2.13	4.5409	0.0863
x_1^2	17.9875	1	6.654986	1194.6467	2.70	7.3055	0.0426*
x_2^2	4.7875	1	6.654986	84.6283	0.72	0.5175	0.5041
x_3^2	−23.6125	1	6.654986	2058.6467	−3.55	12.5890	0.0164*

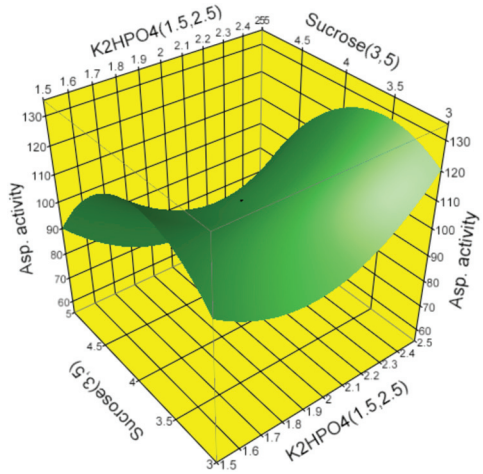
R^2=0.893686 (adjusted R^2=0.702321). SS, sum of squares. *Significant at 5% level.

sucrose (x_2), and time (x_3) were investigated at three levels according to the Box–Behnken design (Table 3). However, all other variables were kept at their low levels – that is, L-asparaginase, $NaNO_3$, $MgSO_4$, $7H_2O$, and KCl.

Analyzing the regression equation and examining the surface contour plots helped to reach the optimum values of the selected variables. Response surface plots as a function of two factors at a time keeping the other factors at a fixed level helped us to understand both the main and interaction effects of the two factors. The contour plots can be obtained by calculating the data from the model. The values were obtained by one factor, where the second varies with constant of a given *Y*-value. The values of the different concentrations of the variable can be also predicted from response surface plots. Figures 3–5 show the relative effect of the two variables with enzyme activity. The coordinates of the central point within the highest contour levels in each of these figures corresponded to the optimum concentration of the respective components.

Figure 3 shows the response surface plot obtained as a function of K_2HPO_4 versus sucrose, whereas all other variables were kept at zero level. This figure shows that, as the concentration of K_2HPO_4 and sucrose increases to 2.5 g/l, respectively, L-asparaginase is increased (Fig. 4) because of the effect of both K_2HPO_4 and time on the activity. As concentration of K_2HPO_4 reaches 2.5 g/l and the time is increased to 6 days, the activity increases (Fig. 5), whereas with time and concentration increased, while sucrose concentration was 2 g/l, the enzyme activity reached its maximum.

Figure 3



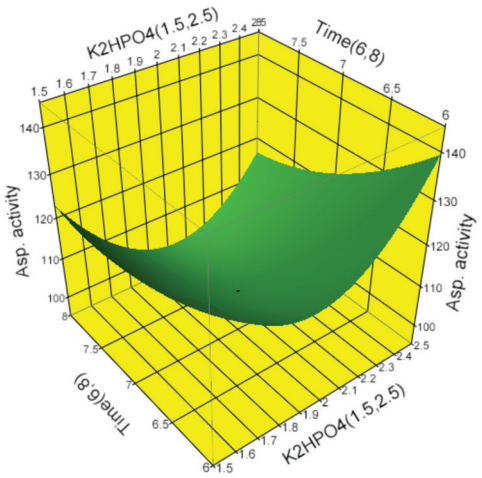
Response surface plot and its contour plot of L-asparaginase production by *Fusarium solani* showing the interactive effects of different concentrations of K_2HPO_4 and sucrose and asparagine at ×3=0.

The multiple regression analysis of the experimental results was titled with the second-order polynomial function for the estimation of L-asparaginase production:

$$Y\ \text{activity} = 105 + 0.1075x_1 - 8.125x_2 - 15.025x_3 - 3.425x_1x_2 - 5.775x_1x_3 + 13.625x_2x_3 + 17.9875{x_1}^2 + 4.7875{x_2}^2 - 23.6125{x_3}^2,$$

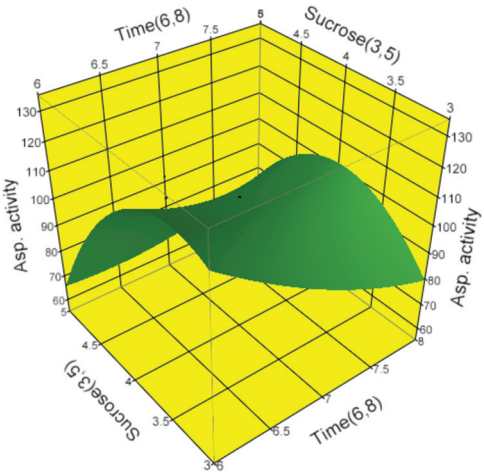
where Y is the response variable (L-asparaginase activity), and x_1, x_2, and x_3 are K_2HPO_4, time, and sucrose, respectively. The factorial design helped to show the importance of the medium composition and the suitable time for L-asparaginase production. The second-degree polynomial equation was optimized by using the software.

Figure 4



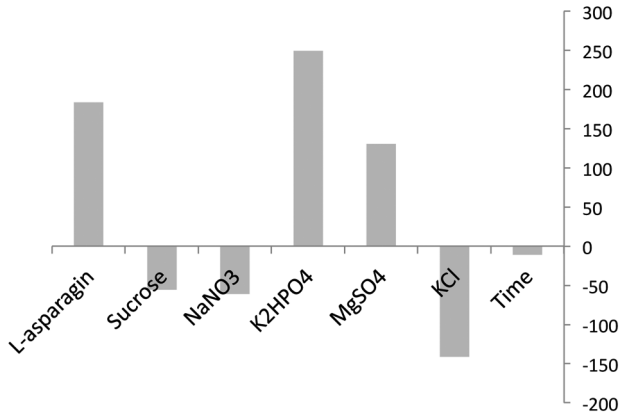
Response surface plot and its contour plot of L-asparaginase production by *Fusarium solani* showing the interactive effects of different concentrations of KH_2PO_4 and time (6 and 7 days) ×1=0.

Figure 5



Response surface plot and its contour plot of L-asparaginase production by *Fusarium solani* showing the interactive effects of different concentrations of sucrose and time (6 and 7 days).

Figure 6



Effect of environmental and nutritional factors on activity of L-asparaginase produced by *Fusarium solani* based on the Plackett–Burman design.

The ANOVA showed that the model was highly significant and fitted the second-order polynomial model to explain the observed yields. The degree of fitting the model was relatively high as the calculated coefficient of determination R^2 was 0.893. The closer the R^2-value to 1, the stronger the model is, and the better the response predicted [24].

Thus, the L-asparaginase production was greatly influenced by the amount of both sucrose and K_2HPO_4 together with time. The optimal levels of the three variables from the maximum point of the polynomial model were found to be K_2HPO_4 (2.5 g/l), sucrose (4 g/l), and the time (8 days).

Results obtained in this study were in agreement with those obtained by Radhika and Girisham (2012) [25], who reported that 2.09 g/l was optimum for K_2HPO_4. Other researchers reported the importance of sucrose as a carbon source.

Verification of model

A verification experiment was carried out to compare between the predicted optimal conditions and the basal fermentation medium. The estimated L-asparaginase activity was 123.6 μ/ml, whereas the predicted value from the polynomial model was 144.4 μ/ml. This revealed a high degree of accuracy of the model (85.59%), which was evidence for the validation of the model.

Conclusion

To study the factors affecting the production of L-asparaginase, it is important to test as many variables as possible and the effect of each one. The Plackett–Burman design offers a good and fast screening procedure and thus saves a lot of time. The effect of nutritional and environmental factors was studied using the PB design and it was concluded that K_2HPO_4, sucrose, and time of fermentation were the most important factors that influence L-asparaginase activity within their tested limits. In addition, the optimum values of these variables were estimated using the response surface methodology.

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Conflicts of interest

There are no conflicts of interest.

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