

Enhancement of L-Asparaginase Production by Isolated *Bacillus circulans* (MTCC 8574) Using Response Surface Methodology

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Abstract L-asparaginase production was optimized using isolated *Bacillus circulans* (MTCC 8574) under solid-state fermentation (SSF) using locally available agricultural waste materials. Among different agricultural materials (red gram husk, bengal gram husk, coconut, and groundnut cake), red gram husk gave the maximum enzyme production. A wide range of SSF parameters were optimized for maximize the production of L-asparaginase. Preliminary studies revealed that incubation temperature, moisture content, inoculum level, glucose, and L-asparagine play a vital role in enzyme yield. The interactive behavior of each of these parameters along with their significance on enzyme yield was analyzed using fractional factorial central composite design (FFCCD). The observed correlation coefficient (R^2) was 0.9714. Only L-asparagine and incubation temperature, were significant in linear and quadratic terms. L-asparaginase yield improved from 780 to 2,322 U/gds which is more than 300% using FFCCD as a means of optimizing conditions.

Keywords L-Asparaginase · *Bacillus circulans* ·
Fractional factorial central composite design · Optimization ·
Red gram husk · Solid-state fermentation

Introduction

L-Asparaginase (EC.3.5.1.1; asparagine amidohydrolase) has been widely used for the chemotherapy of acute lymphoblastic leukemia and is gaining commercial importance in the pharmaceutical sector. This enzyme is produced by a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings [1]. L-asparaginase production pattern was studied in *Escherichia coli*, *Erwinia cartovora*, *Corynebacterium glutamicum*, *Cylindrocarpon obtusisporum*, *Pseudomonas stutzeri*,

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Rhodospiridium toruloids, *Tetrahymena pyriformis*, *Pseudomonas aeuginosa* 50071, *Aspergillus tamari* and *Aspergillus terreus* [2–11].

Most of the microbial enzymes are produced by submerged fermentation. In the recent years, solid-state fermentation (SSF) is increasingly used as an alternative economic process due to enhanced yield at low capital and energy cost using agricultural waste as medium [12, 13]. However, only a few publications are available on L-asparaginase production under SSF [1, 2, 14]. Literature reports suggested that each agricultural material is specific as source of energy and nutrients and influences the microbial metabolism and metabolite/enzyme production [1, 2, 14, 15]. Hence, selection of the most appropriate substrate and optimum fermentation conditions is crucial to develop the selected microbial strain at the commercial level. Therefore, an attempt was made to understand the significance of different agricultural waste materials and other medium components for L-asparaginase production using isolated *Bacillus circulans* (MTCC 8574) by response surface methodology and report the interaction of different medium components and their role on overall improvement of L-asparaginase yield.

Materials and Methods

Microorganism and Growth Conditions

Isolated *B. circulans* (MTCC 8574) was used for this study. The culture was maintained on M9 medium consisting of (g/L): Na₂HPO₄, 6.0; KH₂PO₄, 3; NaCl, 0.5; CaCl₂, 0.011; MgSO₄·7H₂O, 0.12, L-asparagine, 5.0; and pH 7.0). The culture slants prepared with the above agar-based medium was sub-cultured at every 7-day interval and stored at 4 °C.

Preparation of Solid Substrates

Five grams of the selected substrate was taken separately for each 250-ml Erlenmeyer flasks and moistened with 2.5 ml of distilled water, unless otherwise specified. The flasks were sterilized and inoculated with 2 ml of 24-h-old cell suspension (having absorbance of 0.8 at 600 nm) of *B. circulans*. The flasks were mixed thoroughly and incubated at 37 °C in an incubator for 24 h. All experiments were performed in triplicate and average values were reported.

Enzyme Extraction

The crude enzyme was extracted by using 50 ml of 0.1 M phosphate buffer (pH 8.3) with simple contact method for 30 min at 150 rpm. The enzyme solution was filtered through the muslin cloth. This extraction process was carried out for two times and the filtrates were pooled together and centrifuged at 6,000 rpm for 15 min. The resultant supernatant was collected and used for enzyme assay.

Estimation of L-asparaginase Activity

L-asparaginase enzyme assay was performed by a colorimetric method, according to Wriston and Yellin [16] at 37 °C, using UV-visible spectrophotometer (CECIL instruments, UK), by estimating the ammonia produced during L-asparagine catalysis using Nessler's reagent. Reaction mixture consisting of 0.5 ml of 0.08 mM L-asparagine, 1.0 ml of

0.05 mM borate buffer (pH 7.5) and 0.5 ml of enzyme solution. The reaction was terminated by the addition of 0.5 ml of 15% trichloroacetic acid solution after 30 min of incubation. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using a calibration curve from standard solutions of ammonia. One unit of the L-asparaginase (IU) is defined as that amount of enzyme capable of producing 1 μ mol of ammonia per minute at assay conditions.

Experimental Design and Optimization

Five variables (moisture content, incubation temperature, glucose concentration, inoculum level, and asparagines concentration) were selected for RSM study. These factors were coded at five levels starting from -2 , -1 , 0 , 1 , and 2 defined by Eq. 1.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \dots, k \quad (1)$$

Where x_i is the dimensionless coded value of the variable X_i , X_0 the value of the X_i at the center point and ΔX_i the step change.

For statistical calculations, the variables X_i were coded as x_i according to the following transformation according to Eq. 1. The range and levels of the variables in coded units for RSM studies were reported in Table 1. The behavior of the system was explained by the following quadratic model Eq. 2.

$$Y = \beta_0 + \sum \beta_i^* x_i + \sum \beta_{ii}^* x_i^2 + \sum \beta_{ij}^* x_{ij} \quad (2)$$

Where Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction effect.

In the present investigation, FFCCD design was employed to fit the second order polynomial model, which indicated 30 experimental tests (Table 1). For analysis of the data MATLAB 7.0 (Mathworks USA) software was used.

Results and Discussion

Preliminary studies on L-asparaginase production with *B. circulans* (MTCC 8574) in SSF was performed using various locally available agricultural materials. Among selected materials, red gram husk supported the maximum asparaginase production (780 U/gds) followed by bengal gram husk (600 U/gds). Ground nut (360 U/gds) and coconut oil cake (380 U/gds) indicating the importance of nature of agro-material in enzyme production by this isolate. This data suggested that the isolated bacterial strain of *B. circulans* (MTCC 8574) has higher potential in L-asparaginase production compared to the literature reported microbes such as 40 U/gds by *Aspergillus niger* [2] and 142.8 IU by *P. aeruginosa* [14] under solid-state fermentation and 24.5 IU/10 ml by *Serratia marcescens* [17] under submerged fermentation. Therefore, further optimization studies were performed using red gram husk as solid support/substrate medium for L-asparaginase production.

Conventional one-at-a-time experimental procedure revealed that five independent variables such as moisture content, incubation temperature, inoculum concentration, glucose (as carbon source) and asparagine (as nitrogen and as inducer of asparaginase enzyme) were the most influential factors for this enzyme production with red gram husk as solid medium (data not shown). These factors concentrations were optimized using a 2^{6-2} factorial design

Table 1 Experimental design along with observed and predicted asparaginase production.

S.NO	Moisture content (% w/w)		Temperature (°C)		Glucose concentration (w/v)		Inoculum concentration (ml)		Asparagine concentration (w/v)		Asparaginase activity (U/gds)		
	Coded	Real	Coded	Real	Coded	Real	Coded	Real	Coded	Real	Experimental	Predicted	Error
1	-1	110	-1	36	-1	0.75	-1	3.5	1	1.25	1,774.8	1,790.24	-15.44
2	-1	100	-1	36	-1	0.75	1	3	-1	0.75	1,944.1	1,912.57	31.52
3	-1	110	-1	36	1	1.25	-1	3.5	-1	0.75	2,005.5	1,972.57	32.92
4	-1	100	-1	36	1	1.25	1	3	1	1.25	2,103.5	2,123.01	-19.51
5	-1	110	1	38	-1	0.75	-1	3.5	-1	0.75	2,167.7	2,135.71	31.99
6	-1	100	1	38	-1	0.75	1	3	1	1.25	2,016.1	2,036.54	-20.44
7	-1	110	1	38	1	1.25	-1	3.5	1	1.25	2,222.4	2,241.44	-19.04
8	-1	100	1	38	1	1.25	1	3	-1	0.75	2,162.9	2,134.97	27.92
9	1	130	-1	36	-1	0.75	-1	3.5	-1	0.75	2,191.2	2,159.44	31.75
10	1	120	-1	36	1	0.75	1	3	1	1.25	2,202.2	2,222.87	-20.67
11	1	130	-1	36	1	1.25	-1	3.5	1	1.25	1,953.8	1,973.07	-19.27
12	1	120	-1	36	1	1.25	1	3	-1	0.75	1,992.1	1,964.41	27.69
13	1	130	1	38	-1	0.75	-1	3.5	1	1.25	1,331.6	1,351.81	-20.21
14	1	120	1	38	-1	0.75	1	3	-1	0.75	1,851.7	1,824.94	26.75
15	1	130	1	38	1	1.25	-1	3.5	-1	0.75	1,731.8	1,703.64	28.15
16	1	120	1	38	1	1.25	1	3	1	1.25	1,890.2	1,914.47	-24.27
17	0	100	0	37	0	1	0	2.5	0	1	2,195.9	2,228.21	-32.31
18	-2	80	0	37	0	1	0	2.5	0	1	2,111.8	2,126.78	-14.98
19	2	120	0	37	0	1	0	2.5	0	1	1,813.7	1,818.68	-4.98
20	0	100	-2	35	0	1	0	2.5	0	1	2,222.7	2,237.21	-14.51
21	0	100	2	39	0	1	0	2.5	0	1	2,038.1	2,043.54	-5.44
22	0	100	0	37	-2	0.5	0	2.5	0	1	1,948.8	1,961.44	-12.64
23	0	100	0	37	2	1.5	0	2.5	0	1	2,102.5	2,109.81	-7.31
24	0	120	0	37	0	1	-2	3.5	0	1	1,792.0	1,807.44	-15.44
25	0	80	0	37	0	1	2	1.5	0	1	2,004.4	2,008.91	-4.51
26	0	100	0	37	0	1	0	2.5	-2	0.5	1,946.7	2,056.08	-109.38
27	0	100	0	37	0	1	0	2.5	2	1.5	2,106.8	2,017.38	89.42
28	0	100	0	37	0	1	0	2.5	0	1	2,259.6	2,228.21	31.39
29	0	100	0	37	0	1	0	2.5	0	1	2,231.2	2,228.21	2.99
30	0	100	0	37	0	1	0	2.5	0	1	2,246.1	2,228.21	17.89

(Table 1). The L-asparaginase production varied from 1,330 to 2,260 U/gds indicating the influence of selected parameters levels role on *B. circulans* metabolism and subsequent enzyme production as reported for *P. aeruginosa* [14]. Only $\pm 6\%$ variation in enzyme yields between experimental and software predicted amounts was noticed (Table 1) indicating the accuracy of experimentation. The calculated coefficient of determination (R^2) was 0.9714 (Fig. 1). The high value of R^2 (0.9081) suggested a higher significance of the model [18, 19]. The observed low difference (0.063) of the adjusted R^2 value (0.9081) and the R^2 value (0.9714) further confirm the data accuracy. At the same time, a relatively lower value of the coefficient of variation ($CV=3.03\%$) indicated a better precision and reliability of the performed experiments.

To understand an empirical relationship between the L-asparaginase yield (Y) and test variables (in coded units) as well as to perform the analysis of variance (ANOVA), the data was analyzed using second order surface model using following Eq. 3.

$$\begin{aligned}
 Y = & 2228.21 - 77.02 \times M - 48.41 \times T + 37.09 \times G + 50.36 \times I - 9.67 \times A - 63.87 \\
 & \times M^2 - 21.95 \times T^2 - 48.14 \times G^2 - 80 \times I^2 - 47.87 \times A^2 - 142.2 \times M \times T \\
 & - 37.52 \times M \times G + 41.97 \times M \times I - 14.1 \times M \times A + 43.6 \times T \times G + 9.42 \times T \times I \\
 & - 22.2 \times T \times A - 19.6 \times G \times I + 69.22 \times G \times A + 67.17 \times I \times A
 \end{aligned} \quad (3)$$

Where Y , is response asparaginase production in U/gds.

Table 2 denoted the significance of each coefficient in terms of Student's t test and p -values. Critical analysis of the data denoted that all other factors were significant in linear terms, except for asparagine and temperature which remained in quadratic terms. Similar results were reported by Abdel-Fattah and Olama [14] working on L-asparaginase production using *P. aeruginosa* under SSF using soya bean meal. The observed highest effect of inoculum, glucose, and asparagine concentrations in quadratic terms than their linear terms denoted that nutritional (carbon and nitrogen sources) and biological (initial biomass concentration) parameters are the most important factors and needs fine regulation for effective L-asparaginase production by *B. circulans* (MTCC 8574) (Table 2). However, at intercalative terms, the effect of temperature vs. moisture content interaction (-142.2) was higher than other factors interaction effects. Results identify that these two factors are critical in the production L-asparaginase by *B. circulans* (MTCC 8574) under SSF with red gram

Fig. 1 Regression plot for L-asparaginase production observed and predicted values

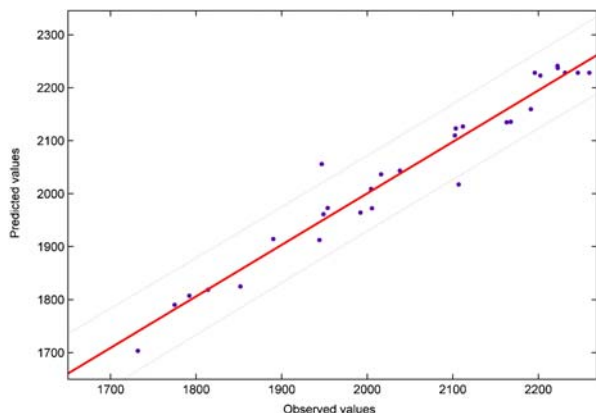


Table 2 Regression coefficients, effects and ANOVA.

	Coefficient	Effect	SS	df	MS	F-value	p-value
Mean/intercept	2,228.21	2,228.210	–	–	–	–	0.000000
M	–77.02	–154.050	142,388	1	142,388.4	38.0928	0.000164
T	–48.41	–96.833	56,260	1	56,260.2	15.0511	0.003734
G	37.09	74.183	33,019	1	33,019.0	8.8334	0.015648
I	50.36	100.733	60,883	1	60,883.2	16.2879	0.002947
A	–9.67	–19.350	2,247	1	2,246.5	0.6010	0.458077
M*M	–63.87	–127.740	108,783	1	108,783.4	29.1025	0.000436
T*T	–21.95	–43.915	12,857	1	12,856.8	3.4395	0.096640
G*G	–48.14	–96.290	61,812	1	61,811.8	16.5363	0.002814
I*I	–80.00	–160.015	170,699	1	170,698.7	45.6666	0.000083
A*A	–47.87	–95.740	61,108	1	61,107.7	16.3479	0.002914
M*T	–142.20	–284.400	323,533	1	323,533.4	86.5541	0.000007
M*G	–37.52	–75.050	22,530	1	22,530.0	6.0274	0.036452
M*I	41.97	83.950	28,190	1	28,190.4	7.5417	0.022616
M*A	–14.10	–28.200	3,181	1	3,181.0	0.8509	0.380337
T*G	43.60	87.200	30,415	1	30,415.4	8.1369	0.019010
T*I	9.42	18.850	1,421	1	1,421.3	0.3802	0.552754
T*A	–22.20	–44.400	7,885	1	7,885.4	2.1095	0.180335
G*I	–19.60	–39.200	6,147	1	6,146.6	1.6443	0.231769
G*A	69.22	138.450	76,674	1	76,673.6	20.5123	0.001428
I*A	67.17	134.350	72,200	1	72,199.7	19.3154	0.001733
Error			33,641	9	3,737.9		
Total SS			1,179,516	29			

SS sum of squares, MS mean square, df degree of freedom

husk. The data also identified that the highest interaction was between glucose and asparagine (69.225) with their individual linear (37.09, –9.67) and quadratic terms (–48.14, –47.87) indicating their interactive importance in enzyme production (Table 2). Similar interaction influence was reported in other fermentation optimization studies [15, 20–23].

The regression model data presented in the form of contours revealed that L-asparaginase production was highly and interactively influenced by all selected fermentation parameters. L-asparaginase production predicted to be increased with increase in moisture content when incubation temperature is maintained in the selected lower level (Fig. 2a). However, higher selected level of moisture content is detrimental for enzyme production at lower glucose concentrations. Optimum L-asparaginase yield could be possible with 80–90% moisture content and in presence of 1–1.5 g of glucose supplementation (Fig. 2b). Similar trend could be predicted with respect to the interaction of moisture content and inoculum concentration (Fig. 2c) where supplementation of selected higher level of inoculum was expected to yield the maximum enzyme if moisture content is maintained in the range of 80–90% during fermentation. However, the interaction between moisture content and asparagine concentration denoted that optimum L-asparaginase was expected with moisture content in the range of 85–110% if asparagine is supplemented in the range of 0.8–1.5 g (Fig. 2d). Similar trend was noticed with studied parameters. Such data further revealed that each parameter interact differently with others and regulate the L-asparaginase production by *B. circulans* (MTCC 8574).

The predicted maximum enzyme production was 2,356 U/gds when fermentation was performed at 36.3 °C with the supplementation of 99.5% of moisture content, 1.17 gm of glucose and 1.24 gm of asparagine for 5 gm of solid material using 2.8 ml of inoculum.

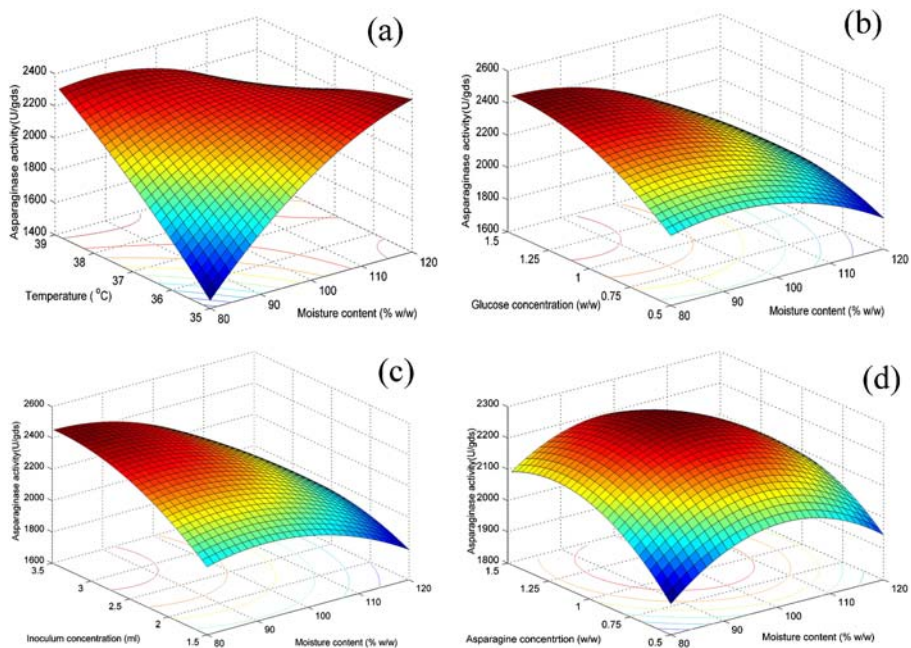


Fig. 2 Surface plots for interaction moisture content vs. temperature (a), moisture content vs. glucose concentration (b), moisture content vs. inoculum concentration (c), moisture content vs. asparagine concentration (d)

Experimental validation indicated 2,322 U/gds of L-asparaginase production revealing more than a 300% improvement. Such modelling defined improved enzyme productions were reported in literature for *B. circulans* (MTCC 6811) under solid-state fermentation [15] in *Staphylococcus* sp. [23] under submerged fermentation.

Overall, our results indicate that the isolated *B. circulans* (MTCC 8574) revealed higher enzyme production compared to those reported in the literature. Optimization studies showed more than a threefold improvement in L-asparaginase production using SSF. Each selected fermentation factor showed its influence at individual or interactive level either in linear or quadratic terms. The study emphasizes the selection of different process parameter levels that would influence economic production of L-asparaginase production.

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