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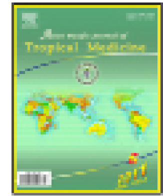

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L-asparaginase production by mangrove derived *Bacillus cereus* MAB5: optimization by response surface methodology

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ABSTRACT

Objective: To isolate marine bacteria, statistically optimize them for maximum asparaginase production. **Methods:** In the present study, statistically based experimental designs were applied to maximize the production of L-asparaginase from bacterial strain of *Bacillus cereus (B. cereus)* MAB5 (HQ675025) isolated and identified by 16S rDNA sequencing from mangroves rhizosphere sediment. **Results:** Plackett-Barman design was used to identify the interactive effect of the eight variables *viz.* yeast extract, soyabean meal, glucose, magnesium sulphate, KH_2PO_4, wood chips, aspargine and sodium chloride. All the variables are denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameters on L-asparaginase production was calculated. Soyabean meal, aspargine, wood chips and sodium chloride were found to be the significant among eight variables. The maximum amount of L-asparaginase produced (51.54 IU/mL) from the optimized medium containing soyabean meal (6.282 8 g/L), aspargine (5.5 g/L), wood chips (1.383 8 g/L) and NaCl (4.535 4 g/L). **Conclusions:** The study revealed that, it is useful to produce the maximum amount of L-asparaginase from *B. cereus* MAB5 for the treatment of various infections and diseases.

1. Introduction

Bacterial L-asparaginases, are enzymes of high therapeutic value due their use in leukemia treatment. *Escherichia coli (E. coli)* asparaginase, a high affinity periplasmic enzyme is particularly effective in certain kinds of cancer therapies. A number of bacteria possess L-asparaginase, although not all of these enzymes have anti-tumour properties. The variation in anti-tumour activity has been related to the affinity of the enzyme for its substrate and the clearance rate of the particular types of enzymes. Commercially used enzymes are obtained from *E. coli* and *Erwinia carotoiora*[1]. L-asparaginase was introduced in the therapeutics due to the fact that in a significant number of patients with acute leukemia, particularly lymphocytic, the malignant cells are dependent on an exogenous source of L-asparagine for survival. However normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion produced by treatment with the enzyme L-asparaginase. The general medical approach to leukemia therapy is therefore based on a metabolic defect in L-asparagine synthesis of some malignant cells[2,3]. The enzyme also inhibits protein synthesis by L-asparagine hydrolysis[1,3,4]. The most common therapeutic indications of L-asparaginase are: treatment of Hodgkin disease, treatment of acute lymphocytic leukemia mainly in children, acute myelocytic leukemia, acute myelomonocytic leukemia and chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma [3,5-7]. The amino acid sequence of several different asparaginases has been reported, including that of *E. coli* enzyme[8].

L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. As the several types of tumour cells require L-asparagine as an essential amino acid for protein synthesis; they are deprived of an essential growth factor in the presence of L-asparaginase. Effective depletion of L-asparagine results in cytotoxicity for leukemic cells[9].

Though several L-asparaginases of bacterial origin have been developed and their potential usages in clinical trials have been studied to prevent the progress of L-asparagine dependent tumours, mainly lymphsarcomas, the success hitherto has been rather limited, and most of the treatments must be interrupted due to severe side effects and immunological reactions in the patients. L-asparaginase or by exploring the exotic environment

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L-asparaginases with novel properties. The enzyme is produced throughout the world by both submerged and solid-state fermentations.

Optimization of medium by the classical method involves changing one independent variable, while unchanging all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables[10] and also may result in wrong conclusions[11]. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses[12]. Hence the present study details on the trials carried out to optimize the L-asparginase production by response surface methodology.

2. Materials and methods

2.1. Microorganism

A bacterial strain, MAB5 (HQ675025), was isolated from the mangroves Rizosphere soil, collected from Vellar estuary [11.29 lat (N) and 79.48 long (E)] Parangipettai. The soil sample were serial diluted up to 108, in that dilution factor 1ml of sample inoculated on zobell marine agar plates were incubated at 37 ℃ in an incubator. After purification, the culture was characterized using standard biochemical tests, and identified as *Bacillus cereus (B. cereus)*. The culture was maintained in the nutrient agar medium slants incorporated with antifungal agents for avoid the fungal contamination and stored at 4 ℃ after growth. The agar slants were sub cultured at fortnightly intervals.

2.2. Extraction of DNA and amplification of 16S ribosomal DNA gene fragments

The bacterial chromosomal DNA was extracted, using the method of Sambong and Rainey[13,14], and detected by 1% agarose electrophoresis. Primers were consensus primers[15]. The forward primer was 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′, and the reverse primer was 1495R: 5′-CTACGGCTACCTTGTTACGA-3′. The forward and reverse primers were respectively located at the 8-27 bp and 1495-1514 bp in the 16S rDNA. For the PCR reaction system, conditions were as follows: DNA (70 ng/μL) template 2 μL; dNTP mixture (2.5 mmol/L) 2.5 μL; 27 F (20 μmol/L) 1.5 μL; 1495 F (20 μmol/L) 1.5 μL; 10×Taq Buffer with ($MgCl_2$) 5 μL; Taq DNA polymerase 0.2 μL; bringing up ddH_2O to 50 μL. The PCR amplification conditions were as follows: force-degeneration at 94 ℃ for 3 min, degeneration at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min and at 72 ℃ for 3 min, 30 cycles, with another extension at 72 ℃ for 5 min. After purification, the PCR products were sent for sequencing.

2.3. Analysis of 16S rDNA sequences and drawing of phylogenetic tree

Resemblance analysis of the 16S rDNA sequence was done through the GenBank database using the BLAST method. Multiple alignments were carried out among the sequences with high resemblance in the Clustal X (1.8) program. Finally, a multiple alignment array was established, with gaps, and a phylogenetic tree was constructed using the Neighbor-Joining method[16].

2.4. Identifying the significant variables using Plackett-Burman design

The present study was aimed at screening the important medium components with respect to their main effects by Plackett-Burman design. The variables chosen for the present study were yeast extract, soyabean meal, glucose, magnesium sulphate, KH_2PO_4, wood chips, aspargine and sodium chloride. The experimental design for the screening of the variables is given in Table 1. All the variables are denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameters on asparginase production was calculated by the following equation:

$$E = (\Sigma M_{+} - \Sigma M_{-})/N \quad (1)$$

Where, E is the effect of parameter under study and M_+ and M_- are responses (alpha amylase activities) of trials, in which the parameter higher and lower levels respectively and N is the total number of trials.

2.5. Optimization by response surface methodology (RSM)

The next step in the formulation of the medium was to determine the optimum levels of significant variables for asparginase production. For this purpose, the RSM, using a central composite design (CCD), was adopted for the augmentation of total amylase production. The significant variables utilized were as follows: rice bran, wheat bran, Sodium chloride, Magnesium sulphate and incubation

Table 1

Plackett-Burman experimental design for screening of significant process variables affecting alpha amylase production.

Std	Yeast extract	Soya bean meal	Glucose	$MgSO_4$	KH_2PO_4	Wood chips	Aspargine	NaCl
1	1.0	1.0	5.0	0.5	0.5	1.0	5.0	5.0
2	1.0	5.0	1.0	1.0	0.5	1.0	1.0	5.0
3	0.5	5.0	5.0	0.5	1.0	1.0	1.0	1.0
4	1.0	1.0	5.0	1.0	0.5	1.5	1.0	1.0
5	1.0	5.0	1.0	1.0	1.0	1.0	5.0	1.0
6	1.0	5.0	5.0	0.5	1.0	1.5	1.0	5.0
7	0.5	5.0	5.0	1.0	0.5	1.5	5.0	1.0
8	0.5	1.0	5.0	1.0	1.0	1.0	5.0	5.0
9	0.5	1.0	1.0	1.0	1.0	1.5	1.0	5.0
10	1.0	1.0	1.0	0.5	1.0	1.5	5.0	1.0
11	0.5	5.0	1.0	0.5	0.5	1.5	5.0	5.0
12	0.5	1.0	1.0	0.5	0.5	1.0	1.0	1.0

period each of which was assessed at five coded levels (-2, -1, 0, +1 and +2) and a total of 32 experiments were conducted. All variables were taken at a central coded value, which was considered as zero. The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in actual and coded form is provided in Table 2. The response value (Y) in each trial was the average of the duplicates.

Table 2
Observed and predicted responses for the experiments performed using Plackett-Burman design.

Run no.	Observed (IU/mL)	Predicted (IU/mL)
1	23.235	23.251
2	27.324	27.307
3	27.111	27.294
4	16.218	16.401
5	29.198	29.214
6	29.739	29.555
7	24.245	24.061
8	18.326	18.309
9	22.322	22.338
10	29.205	29.188
11	34.758	34.941
12	25.204	25.024

2.6. Statistical analysis and modeling

The data obtained from RSM on asparginase production were subjected to analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta 0 + \Sigma i\ \beta iXi + \Sigma i\ \beta iiXi2 + \Sigma ij\ \beta ijXiXj \quad (2)$$

Where Yi is the predicted response, XiXj are independent variables, β0 is the offset term, βi is the ith linear coefficient, bii is the ith quadratic coefficient, and βij is the ijth interaction coefficient. However, in this study, the independent variables were coded as X1, X2, X3 and X4. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta 0 + \beta 1\ X1 + \beta 2\ X2 + \beta 3\ X3 + \beta 4\ X4 + \beta 11\ X12 + \beta 22\ X22 + \beta 33\ X32 + \beta 44\ X42 + \beta 12\ X1X2 + \beta 13X1X3 + \beta 14\ X1X4 + \beta 23\ X2X3 + \beta 24\ X2X4 + \beta 34\ X3X4 \quad (3)$$

The statistical software package, Minitab package version 15 was used for the regression analysis of the experimental data, and also to plot the response surface graphs. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

3. Results

3.1. Identification of the bacterial strain MAB5

The 16S rDNA sequenced from strain MAB5 was 1 461 bp long. Resemblance analysis was carried out between this sequence and other 16S rDNA sequences using the BLAST program. Strain MAB5 and the other nine strains together formed a cladogram according to resemblance with *Pseudomonas* as an out group. Based on the results shown in Figure 1, the MAB5 strain was identified as a member under *Bacillus*. Furthermore, the resemblance between the MAB5 strain and the strain *B. cereus* (EU675025) was 99.6% and was identified as *B. cereus*. This sequence has been received by GenBank (Accession number: HQ675025).

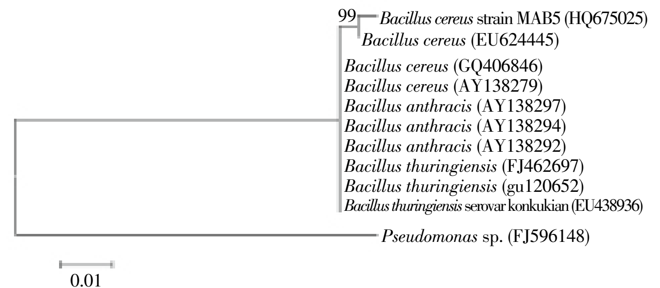

Figure 1. Phylogenetic tree of the 16S rDNA sequence of strain MAB5 and related strains.

3.2. Screening of parameters using Plackett-Burman design

The experiment was conducted in 12 runs to study the effect of the selected variables. Table 2 represents the results of the screening experiments using Plackett-Burman design. Statistical analysis of the responses were performed which is represented in Table 3. The model *F* value of 536.14 implies that the model is significant. The values of *Prob* < 0.05 indicate model terms are significant.

The magnitude of the effects indicates the level of the significance of the variable on asparginase production. Among the variables of screened soyabean meal, aspargine, wood chips and sodium chloride concentration were identified as most significant variables influencing asparginase production (Table 4).

3.3. Optimization of significant variables using response surface methodology

The experiments conducted in the present study were targeted toward the construction of a quadratic model consisting of thirty one trials. The design matrix and the corresponding results of RSM experiments to determine the effects of four independent variables (soyabean meal, aspargine, wood chips and sodium chloride) are shown in Table 5, along with the mean predicted values. The regression analysis of the optimization study indicated that the model terms, X1, X2, X3, X4, X12, X22, X32, X42, X2X3 and X2X4, were significant ($P < 0.05$). These results indicate that the concentration of the soyabean meal, aspargine, wood chips and sodium chloride bears a direct relationship to asparginase production. The interactions between soyabean meal and aspargine, soya bean meal and sodium chloride were significant, as was shown by the low *P*-value ($P<0.05$), ($P<0.01$) respectively. Analysis of variance (ANOVA) (Table 6) depicts the *P*-values for the model ($P<0.01$) and for lack

of fit (0.378) also suggested that the obtained experimental data was a good fit with the model.

The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation. The response, asparginase production (Y) by *Bacillus* sp. MAB5, can be expressed in terms of the following regression equation:

Y = −251.518 + 11.911 X1 + 26.556 X2 + 169.322 X3 + 31.771 X4 −0.765X12 −3.589X22 −70.935 X32 −4.591X42 + 0.093 X1X2 −1.509X1X3 −0.174 X1X4 + 4.502 X2X3 + 1.342 X2X4 + 2.653 X3X4

Whereas: Soya bean meal (X1), Aspargine (X2) Wood chips (X3), NaCl (X4), Soya bean*Soya bean (X1 X1), Aspargine*Aspargine (X2 X2), Wood chips*Wood chips (X3 X3) NaCl*NaCl (X4 X4), Soya bean meal*Aspargine (X1 X2), Soya bean meal*Wood chips (X1 X3), Soya bean meal*NaCl (X1 X4), Aspargine*Wood chips (X2 X3), Aspargine*NaCl (X2 X4), Wood chips*NaCl (X3 X4).

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.982 3 (a value >0.80 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 98.23% of the variation in response. The 'adjusted R^2' is 0.966 7 and the 'predicted R^2' is 0.922 1, which indicates that the model is good.

In order to determine the optimal levels of each variable for maximum asparginase production, three-dimensional response surface plots were constructed by plotting the response (asparginase production) on the Z-axis against any two independent variables, while maintaining other variables at their central levels (Figure 2). Maximum asparginase production was obtained at the middle level of each pair of factors at a constant middle level of the other factor. Further increase in these factors above the middle level showed a decrease in aspargine production. In order to determine the maximum aspargine production corresponding to the optimum levels of soyabean meal, aspargine, wood chips and NaCl, a second order polynomial model was used to calculate the values of these variables. Fitting of the experimental data to eqn. (2) allowed determination of the levels of soyabean meal (X1 = 6.282 8 g), aspargine (X2 = 5.5 g), wood chips (X3 = 1.383 8 g) and NaCl (X4 = 4.535 4 g), giving a maximum aspargine concentration of 48.289 4 IU/mL in shake flask culture.

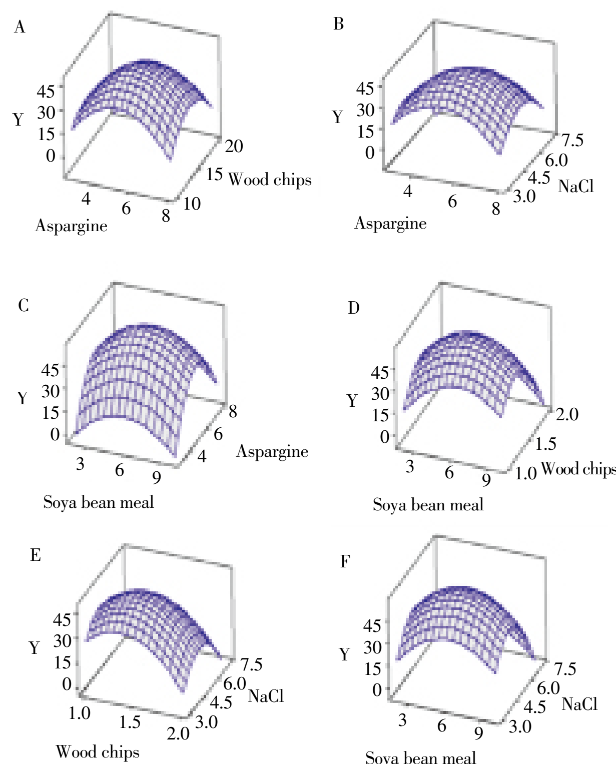

Figure 2. Statistical optimization of asparginase production using RSM. A: Aspargine and wood chips; B: Aspargine and NaCl; C: Soyabean meal and aspargine; D: Soyabean meal and wood chips; E: Wood chips and NaCl; F: Soyabean meal and NaCl.

Fermentation was performed using one liter synthetic medium containing the optimized level of soyabean meal (6.282 8 g), aspargine (5.5 g), wood chips (1.383 8 g) and NaCl (4.535 4 g). Maximum asparginase production (51.54 IU/mL) was obtained, which was slightly higher than the value given by the model.

Table 3
Statistical analysis of the model.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Main Effects	8	291.253	291.253	36.406 7	536.14	0
Residual Error	3	0.204	0.204	0.067 9	–	–
Total	11	291.457	–	–	–	–

Table 4
Statistical parameters for selected the linear polynomial model using Plackett-Burman design.

Term	Effect	Coef	SE Coef	*T*	*P*
Constant	–	25.574	0.075 23	339.96	0.000
Yeast extract	0.492	0.246	0.075 23	3.27	0.047
Soya bean meal	6.311	3.155	0.075 23	41.95	0.000
Glucose	−4.856	−2.428	0.075 23	−32.28	0.000
$MgSO_4$	−5.270	−2.635	0.075 23	−35.03	0.000
KH_2PO_4	0.820	0.410	0.075 23	5.45	0.012
Wood chips	1.015	0.507	0.075 23	6.75	0.007
Aspargine	1.842	0.921	0.075 23	12.24	0.001
NaCl	0.754	0.377	0.075 23	5.01	0.015

Table 5

Central composite factor experimental design along with experimental and predicted values.

Experimental number	Soya bean meal	Aspargine	Wood chips	NaCl	L-asparaginase activity (IU/mL)	
					Experimental	Predicted
1	4	4.25	1.25	4	36.56	37.624 2
2	8	4.25	1.25	4	38.53	39.822 1
3	4	6.75	1.25	4	34.49	33.732 1
4	8	6.75	1.25	4	36.15	36.862 5
5	4	4.25	1.75	4	29.25	27.737 1
6	8	4.25	1.75	4	26.15	26.917 5
7	4	6.75	1.75	4	28.64	29.472 5
8	8	6.75	1.75	4	30.75	29.585 4
9	4	4.25	1.25	6	25.65	25.997 1
10	8	4.25	1.25	6	25.63	26.802 5
11	4	6.75	1.25	6	27.58	28.817 5
12	8	6.75	1.25	6	29.86	30.555 4
13	4	4.25	1.75	6	17.47	18.762 5
14	8	4.25	1.75	6	16.61	16.550 4
15	4	6.75	1.75	6	29.32	27.210 4
16	8	6.75	1.75	6	24.99	25.930 8
17	2	5.50	1.50	5	33.50	33.897 1
18	10	5.50	1.50	5	36.40	34.815 4
19	6	3.00	1.50	5	23.00	21.412 1
20	6	8.00	1.50	5	26.50	26.900 4
21	6	5.50	1.00	5	38.40	36.112 1
22	6	5.50	2.00	5	20.50	21.600 4
23	6	5.50	1.50	3	35.89	35.867 1
24	6	5.50	1.50	7	21.75	20.585 4
25	6	5.50	1.50	5	45.49	46.590 0
26	6	5.50	1.50	5	46.26	46.590 0
27	6	5.50	1.50	5	48.26	46.590 0
28	6	5.50	1.50	5	49.26	46.590 0
29	6	5.50	1.50	5	45.35	46.590 0
30	6	5.50	1.50	5	46.25	46.590 0
31	6	5.50	1.50	5	45.26	46.590 0

Table 6

Analysis for variance of asparginase production.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	14	2 609.91	2 609.91	186.422	63.27	0.000
Linear	4	712.62	358.82	89.705	30.45	0.000
Square	4	1 801.61	1 801.61	450.403	152.86	0.000
Interaction	6	95.68	95.68	15.946	5.41	0.003
Residual Error	16	47.14	47.14	2.946	-	-
Lack-of-Fit	10	32.48	32.48	3.248	1.33	0.378
Pure Error	6	14.66	14.66	2.443	-	-
Total	30	2 657.05	-	-	-	-

4. Discussion

The antilymphomic, anti-leukaemic and antineoplastic activities of L-asparaginase have been well documented[17,18]. L-asparaginase have been produced by *E. coli, Serratia marcescens, Erwinia carotovora, Pseudomonas acidivoras* and *P. geniculata* under aerobic conditions. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma[19,20]. Hence in the present study the bacteria isolated from the mangrove rizosphere sediment of Vellar estuary having the l-asperginase activity was identified and optimized for maximum enzyme production.

In recent years, the application of the industrial residue has provided an alternative way to replace the pure and costly raw materials. The use of such materials would help to solve many environmental hazards[21]. Several processes have been developed that utilize these as raw materials, including CFR for the production of value added products such as ethanol, enzymes, organic acids, *etc*[22].

The substrate employed in the present study has been reported as a potent substrate for the production for asparginase under SSF[23]. It is well documented that wood chips and soyabean meal is a rich source of carbon and nitrogen thus supplementation of other nitrogen sources in the medium does not show significant increase in enzyme

yield. The supplementation of sodium chloride has been reported to provide good growth and also influence higher enzyme production[24]. The tested ranges of other factors did not result in any significant variation in asparginase production. Thus the four variables soyabean meal, aspargine, wood chips and sodium chloride were selected and their optimal levels were identified using response surface methodology.

The RSM used in this investigation suggested the importance of various fermentation parameters at different levels. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of the RSM to optimize the process for enzyme production.

The present study involved the use of statistical experimental designs to optimize medium constituents of the fermentation medium for the production of L-asparaginase from *B. cereus* MAB5. Four variables: soyabean meal, aspargine, wood chips and NaCl were identified as significant by Plackett-Burman design for L-asparaginase production. These variables were further optimized using central composite design involving RSM. Among the four variables tested for the correlation between their concentrations and the production of L-asparaginase, all the four variables showed significant influence on the enzyme production. The significant interactions between the four variables were also observed from the surface plots. The maximum amount (51.54 IU/mL) of L-asparaginase was produced, when the fermentation medium constituents were set as follows: soyabean meal (6.282 8 g), aspargine (5.5 g), wood chips (1.383 8 g) and NaCl (4.535 4 g). Applying statistical experimental designs to optimize the selected factors for maximal production is an efficient method that tests the effect of factors interaction with minimum number of experiments. The methodology as a whole proved to be adequate for the design and optimization of the bioprocess for obtaining a therapeutically valuable product like L-asparaginase from an abundantly available, low grade industrial waste like wood chips. It is useful to produce the maximum amount of L-asparaginase from *B. cereus* MAB5 for the treatment of various infections and diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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