



Statistical screening of process variables for the production of L-asparaginase from cornflour by *Aspergillus terreus* MTCC 1782 in submerged fermentation

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Abstract: L-asparaginase has been used as anti-tumor agent for the effective treatment of acute *lymphoblastic leukemia* and as food processing aid to reduce the formation of acrylamide during frying of starchy foods at high temperature. Objective of the present work was to study the effect of process variables such as cornflour (as natural substrate), urea (as supplementary nitrogen source), L-asparagine, glucose, di-potassium hydrogen phosphate, potassium chloride, inoculum size and pH on the production of extracellular L-asparaginase by *Aspergillus terreus* MTCC 1782 in shake culture fermentation. The effect and significance of these variables was studied using 2-level Plackett-Burman statistical design. The experimental L-asparaginase activity was subjected to statistical analysis using MINITAB 14 software. It was found that L-asparagine, cornflour and urea significantly influenced as most important factors on L-asparaginase production.

Keywords: Extracellular L-asparaginase, acute *lymphoblastic leukemia*, food processing aid, Plackett-Burman design, fermentation.

Introduction

L-asparaginase (L-asparagine amidohydrolase; EC.3.5.1.1) catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia. Colaspase, Crasnitin and Porton asparaginase are synonyms and Elspar, Oncaspar, Erwinase and Kidrolase are trade names of L-asparaginase. Although Clementi in 1922 had reported its presence in guinea-pig serum, the anti-tumour properties of the enzyme were only recognized sometime later (Bilimoria, 1969). Tsuji first reported Deamidation of L-asparagine by extracts of *E. coli* in 1957. Broome in 1961 discovered that the regression of *lymphosarcoma* transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L-asparagine (Wriston & Yellin, 1973; Pritsa *et al.*, 2001). Commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston in 1973 showed that L-asparaginase from *E. coli* inhibits tumors in mice.

L-asparaginase is used as a chemotherapeutic agent for acute *lymphocytic leukaemia* and less frequently for acute *myeloblastic leukaemia*, chronic *lymphocytic leukaemia*, Hodgkin's disease, *melanosarcoma* and non-Hodgkin's lymphoma. Various bacteria such as, *E. coli* (Howard & Schwartz, 1968), *Erwinia aroideae* (Peterson & Ciegler, 1969), *Proteus vulgaris* (Tosa *et al.*, 1971), *Streptomyces griseus* (Dejong, 1972), *Vibrio succinogenes* (Kafkewitz & Goodman, 1974), *Citrobacter freundii* (Davidson *et al.*, 1977), *Thermus*

aquaticus (Curran *et al.*, 1985), *Enterobacter aerogenes* (Mukherjee *et al.* 2000), *Thermus thermophilus* (Pritsa *et al.*, 2001), *Zymomonas mobilis* (Pinheiro *et al.*, 2001) and *Pseudomonas aeruginosa* (Abdel-Fatta Yasser *et al.*, 2002) found to produce L-asparaginase. L-asparaginase has also been found in variety of fungi, yeasts and algae. L-asparaginase produced by bacteria leads to adverse side effects in human trials. Therefore, there is a search for the other sources of L-asparaginase production. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly reported in scientific literature to produce asparaginase with less adverse effects (Sarquis *et al.*, 2004). Since the optimization of nutritional requirements and operating conditions is an important step for bioprocess development. In addition, traditional method of bioprocess development by studying the effect of one variable at a time is tedious, time consuming and expensive. The demand for L-asparaginase will increase several fold in coming years due to its potential application in food processing addition to its clinical applications (Krishnan *et al.*, 1998; Soliman *et al.*, 2005; Pedreschi *et al.*, 2008). Developing an economically viable bioprocess using with low cost substrates is an important step in any bioprocess development.

This research work was aimed to evaluate the effect of medium components and operating conditions on the production of extracellular L-asparaginase by *Aspergillus terreus* MTCC 1782 in shake culture fermentation using two levels Plackett-Burman (PB) statistical design using cornflour as substrate.

Materials and Methods

Microorganism

The filamentous fungus selected to use throughout this study is *Aspergillus terreus* MTCC 1782 and was obtained from Institute of Microbial Technology, Chandigarh, India.

Stock and inoculum culture conditions

The spores and mycelial fragments of *Aspergillus terreus* MTCC 1782 strain was cultivated in Czapek agar slants at 37°C for 4 days. Czapek-Dox medium contains the following ingredients (g/l of distilled water). Solution A: L-asparagine, 10.0; sodium nitrate 40.0; potassium chloride 10.0; MgSO₄·7H₂O, 0.52; FeSO₄·7H₂O, 0.2; dissolved in one litre of distilled water and stored in refrigerator. Solution B: K₂HPO₄ 20.0; dissolved in 1 litre of distilled water and stored in refrigerator. Solution C: one gram of ZnSO₄·7 H₂O dissolved in 100 ml of distilled water. Solution D: 0.5 gram of CuSO₄·H₂O dissolved in

100 ml of distilled water. For one litre of Czapek-Dox medium; 50 ml of solution A, 50 ml of solution B, 1 ml of both solution C and solution D, 900 ml of distilled water, 30 gram of Glucose and 20 gram of agar were taken. *Aspergillus terreus* 1782 strain was cultivated in Czapek agar slants at 37°C for 4 days.

Plackett-Burman design

PB design is an efficient screening design when main effects are to be considered. It is used for experiments confronted with two-factor interaction. This is a very economical design with the run number a multiple of four and comprises of two level screening designs. This

Table 1. Actual values of the process variables

Process variables, % (w/v)	Corn Flour (X ₁)	Urea (X ₂)	L-asparagine (X ₃)	Glucose (X ₄)	K ₂ HPO ₄ (X ₅)	pH (X ₆)	Inoculum size (X ₇) µl	KCl (X ₈)
Low Level (-1)	1	1	0.5	0.1	0.1	4.5	1000	0.025
High Level (+1)	2	2	1	0.2	0.2	6.5	2000	0.050

Production and isolation of crude enzyme

Spore suspension was prepared from the inoculum culture, using 10 ml of sterile water. This spore suspension with spore count of 2×10^7 to 10^8 per ml was transferred to Erlenmeyer flasks with 100 ml of liquid Czapek-Dox medium prepared based on PB statistical design (Table 2) and kept in orbital shaker at 160 rpm for 4 days. Then culture was filtered on Whatman 2 filter paper and the cell-free filtrate was used as crude enzyme solution (Prista *et al.*, 2001).

Assay of L-asparaginase activity

Enzyme activity of the culture filtrates was determined at the end of cultivation time by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent. Nesslerization is the most commonly used method for estimation of L-asparaginase activity. The quantity of ammonia formed during the hydrolysis of 0.04 M L-asparagine was estimated using Nessler's Reagent in spectrophotometric analysis at 480 nm (Wriston & Yellin, 1973).

design is extremely useful in finding importance of the factors affecting the production of the enzyme. PB design offers a good and fast screening procedures and mathematically computes the significance of a large number of factors in one experiment, which is time saving and gives the effect of change in more than one factors in single experiment (Krishnan *et al.*, 1998; Abdel-Fatta Yasser *et al.*, 2002; Soliman *et al.*, 2005; Pedreschi *et al.*, 2008). To evaluate the effect of 8 factors of medium components and operating conditions on L-asparaginase activity PB factorial design in 12 experimental run was carried out.

Eight assigned variables and three unassigned variables (commonly referred as dummy variables) were screened in PB design of 12 experiments. Dummy variable are used to estimate experimental errors in data analysis (Abdel-Fatta Yasser *et al.*, 2002; Soliman *et al.*, 2005). Eight factors consisting of medium components and operating conditions prepared at two levels -1 for low level and +1 for high level. The factors (in gm/100ml) such as Cornflour (X₁), Urea (X₂), L-asparagine (X₃), Glucose (X₄), K₂HPO₄ (X₅), pH (X₆), Inoculum size in µl (X₇), and KCl (X₈) and 3 unassigned variables at same level were studied. The actual values of the variables at low level (-1) and high level (+1) is given in Table 1. Table 2 shows the factors considered for investigation and the PB design for 12 experimental run and the experimental L-asparaginase activity. PB experimental design is based on the first order model as given in equation 1.

$$Y = \beta_0 + \sum \beta_i x_i \quad \text{.....(1)}$$

Where, Y is the response (enzyme activity), β_0 is the model intercept, β_i is variable estimates and X_i are independent variables. This model describes

Table 2. Plackett-Burman design and L-asparaginase activity

Exp. No.	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	X ₁₁	Activity IU/ml
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	28.955
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	21.916
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	31.355
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	28.635
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	19.676
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	26.235
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	28.155
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	19.996
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	11.678
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	18.877
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	12.957
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	13.597

no interaction among the factors that influence asparaginase production and enzyme activity. All experiments were carried out in duplicate and the averages of L-asparaginase activity was taken as responses. The variables whose confidence levels were higher than 90 % were considered that significantly influences the L-asparaginase activity.

Results and Discussion

Evaluation of medium components and operating conditions by Plackett-Burman design

The data on enzyme activity in Table 2

was subjected to multiple linear regression analysis using *MINITAB 14* software to estimate t-value, p-value and confidence level. The data on L-asparaginase activity using PB experiments showed a wide variation from 11.678 to 31.355 IU/ml of L-asparaginase activity.

On analysis of regression coefficients and t-value of 8 factors cornflour (X_1), urea (X_2) and L-asparagine (X_3) showed a positive effect on L-asparaginase production, all other factors shown a negative effect on L-asparaginase production. The t-test for any individual effect allows an evaluation of the probability of finding the observed effects purely by chance. In this work, variables with confidence levels greater than 90% were considered as significant. L-asparagine was found to be highly significant factor for L-asparaginase production (Table 3). Estimated t-value, P-value and confidence level for L-asparaginase activity was shown in Table 3. On the basis of the calculated t-values, p-values and confidence level given in table 3, L-asparagine (confidence level- 98.7%), cornflour (confidence level - 88.3%), urea (confidence level- 77.6%) and potassium chloride (confidence level- 77.6%) were found as most important factors influences the L-asparaginase production and the optimum level of these variables can be determined by response surface methodology in further study. Table 4 shows the Analysis of Variance (ANOVA) for linear model on effect of independent variables on L-asparaginase production.

The effect of significant and most important variables on L-asparaginase activity is given by the model in equation 2.

$$Y = 21.836 + 2.213X_1 + 1.546X_2 + 5.386X_3 - 1.547X_8 \quad \dots(2)$$

The effect of medium components and operating conditions on L-asparaginase activity was also studied using pareto chart (Fig. 1). L-asparagine (X_3) was found to be the significant factor and the cornflour (X_1), urea (X_2) and potassium chloride (X_8) were found to be other most important factors on L-asparaginase production.

Fig. 2, the normal probability plot of the residuals is

Table 3. Statistical analysis of Plackett-Burman design on L-asparaginase activity

Variable	Main effect	Coefficients	t-value	p-value	Confidence level, %
Intercept		21.836	21.56	0.000	
X_1	4.426	2.213	2.18	0.117	88.3
X_2	3.093	1.546	1.53	0.224	77.6
X_3	10.772	5.386	5.32	0.013	98.7
X_4	-0.320	-0.160	-0.16	0.885	11.5
X_5	-1.066	-0.533	-0.53	0.635	46.5
X_6	-1.493	-0.746	-0.74	0.515	48.5
X_7	-0.800	-0.400	-0.39	0.719	18.1
X_8	-3.093	-1.547	-1.53	0.224	77.6

Table 4. Analysis of variance (ANOVA) for linear model on effect of independent variables on L-asparaginase production

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	p-value
Main effects	8	476.57	59.57	4.84	0.111
Residual error	3	36.94	36.94		
Total sum of squares	11	513.51			

an important diagnostic tool to detect and explain the systematic departures from the assumptions. The residual was plotted against normal distribution of the model and it is approximate linear line for -asparaginase production. That the errors are normally distributed and are independent of each other and that the error variances are homogenous. An excellent normal distribution confirmed the normality assumption and the independence of the residuals. This indicates that the model was well fitted with the experimental results. As the residuals from the fitted model are normally distributed, all the major assumptions of the model have been validated. The residual plot in Fig. 3, shows equal scatter of the residual data above and below the x-axis indicates that the variance was independent of the L-asparaginase activity, thus supporting the adequacy of the model fit.

Conclusion

Stepwise statistical optimization strategy such as Plackett-Burman offers a good and fast screening procedure and identifies the influence of more than one factor in single experiment. The effect of media components and operating conditions on the production of L-asparaginase by *Aspergillus terreus* MTCC 1782 were studied using PB design and it was found that L-asparagine, cornflour, urea and potassium chloride significantly influences and most important factors on the L-asparaginase production within their tested limits. The optimum level of these variables can be determined using response surface methodology in further research.

Nomenclature

ANOVA - Analysis of variance, F - Fishers's function, i - variable number, IU - International unit, p - corresponding level of significance, B - Plackett-Burman, t - student's

test, X_i - independent variables, Y - predicted response,
 β_0 - model intercept, β_i - variable estimate.

Fig. 1. Effect of medium components and operating conditions on L-asparaginase activity

(Response is L-asparaginase activity, IU/mL, Alpha = 0.10)

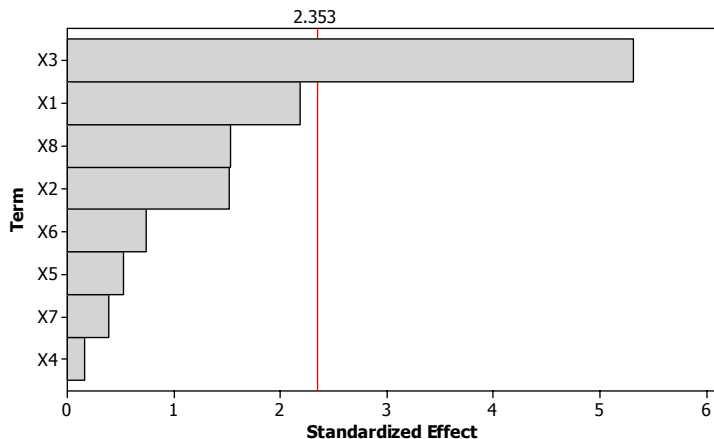


Fig. 2. The Normal probability plot for L-asparaginase activity

(Response is L-asparaginase activity, IU/mL)

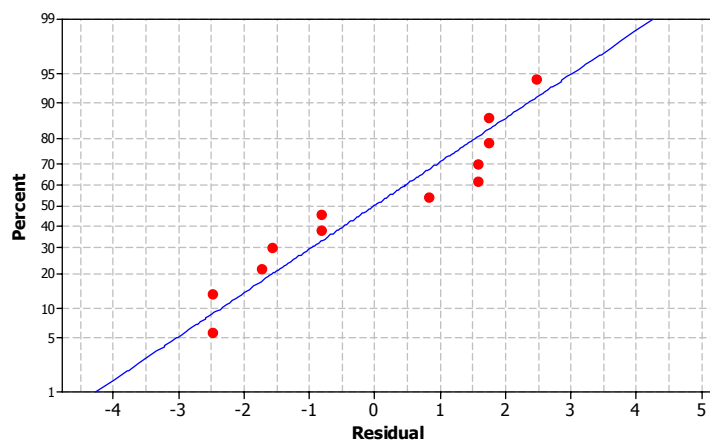
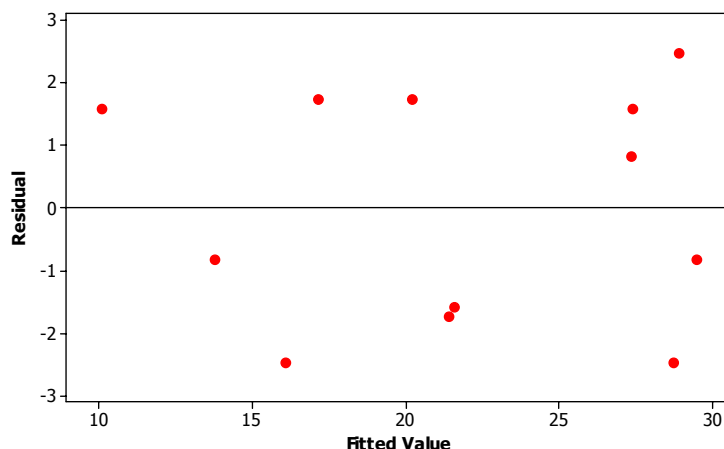


Fig. 3. Residual distribution plot for L-asparaginase activity

(Response is L-asparaginase activity, IU/mL)



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