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Statistical assessment of media components by factorial design for L-aspraginase production by *Aspergillus niger* in surface fermentation

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ABSTRACT

L-asparaginase has been used as anti-tumour agent for the effective treatment of acute lymphoblastic leukemia. Objective of the present work was to study the effect of process variables such as cornflour, urea, L-asparagine, glucose, di-potassium hydrogen phosphate, potassium chloride, inoculum size and pH on production of extracellular L-asparaginase by Aspergillus niger in surface fermentation. The effect of significance of these variables was studied using 2-level Plackett-Burman statistical design because traditional method of bioprocess development by studying the effect of one variable at a time is tedious, time consuming and expensive. The experimental enzyme activity was subjected to statistical analysis using MINITAB 14 software. It was found that cornflour, urea and inoculum size significantly influenced as most important factors on L-asparaginase production.

Keywords: L-aspraginase, *Aspergillus niger* surface fermentation, Plackett-Burman statistical design, MINITAB 14 software

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INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase; EC.3.5.1.1) catalyses the deamidation of L-asparagine to L-aspartic acid and ammonia [1]. L-asparaginase produced by bacteria leads to adverse side effects in human trials [2]. Therefore, there is a need to explore for the other sources of L-asparaginase production Eukaryotic microorganisms like yeast and filamentous fungi are reported to produce L-asparaginases with less adverse effects [3].

The demand for L-asparaginase Is expected to increase several fold in coming years due to its potential application in clinical trials. Developing an economically viable bioprocess using with low cost substrates is an important step in any bioprocess development. Since the optimization of nutritional requirements and operating conditions is an important step for bioprocess development. [4,5,6]. This research work was aimed to evaluate the effect of medium components and operating conditions on the production of extracellular L-asparaginase by *Aspergillus niger* in surface culture fermentation.

MATERIALS AND METHODS

Microorganism

The filamentous fungus selected to use throughout this study is *Aspergillus niger*, isolated and identified in the laboratory, was cultivated in Czapek agar slants at 37^0C for 4 days.

Production of enzyme

Spore suspension was prepared from the inoculum culture, using 10 ml of sterile water. This spore suspension with spore count of 2 X 10^7 to 10^8 per ml was transferred to flasks with 100 ml of liquid Czapek-Dox broth prepared based on Plackett-Burman statistical design (Table 2) and incubated at 37^0C for 4 days. Then culture was filtered Using Whattman filter paper No.2 and the cell-free filtrate was used as crude enzyme solution [7].

Assay of L-asparaginase activity

Enzyme activity of the culture filtrates was determined at the end of reaction time by quantifying ammonia formation by spectrophotometric analysis using Nessler's Reagent at 500 nm [8].

Plackett-Burman design:

PB design is an efficient screening design when main effects are to be considered. This 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 of in more than one factors in one experiment.

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 (dummy variables) were screened in PB design of 12 experiments. Dummy variables are used to estimate experimental errors in data analysis. Eight factors consisting of medium components and operating conditions prepared at two levels -1 for low level and +1 for high level. The actual values of the variables at low and high level are given in table 1.Table 2 shows the factors considered for investigation and the PB design for 12 experimental L-asparaginase activity. PB experimental design is based on the first order model as given in following equation

$Y=\beta_0 + \beta_i X_i$

Where, Y is the response (enzyme activity), β_0 is the model intercept, X_i are independent variables. This model describes no interaction among the factors that influence asparaginase production and enzyme activity. The variables whose confidence levels were higher than 95% were considered that significantly influences the L-asparaginase activity.

RESULTS AND DISCUSSION

Evaluation of media components and operating conditions by Plackett-Burman design:

The data from Table 2 was subjected to multiple linear regression analysis using MINITAB 14 software to estimate t-value and p-value. The data on L-asparaginase activity using PB experiments showed a wide variation from 6.68 to 133.68 IU/ml of L-asparaginase activity.

As shown in Table 2, the mixture design no.3 that is high level concentration of Urea, L- Asparagine and K_2HPO_4 and low level concentration of corn flour, glucose, pH, inoculums size and KCl are found to give maximum production of L-asparaginase.

On analysis of regression coefficients and t-value of 8 factors cornflour (X1), Urea (X2) and L-asparagine (X3) showed a positive effect on L-asparaginase production, all other factors shown a negative effect on L-asparaginase production as shown in Table 3.

Table 3 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 follows equation

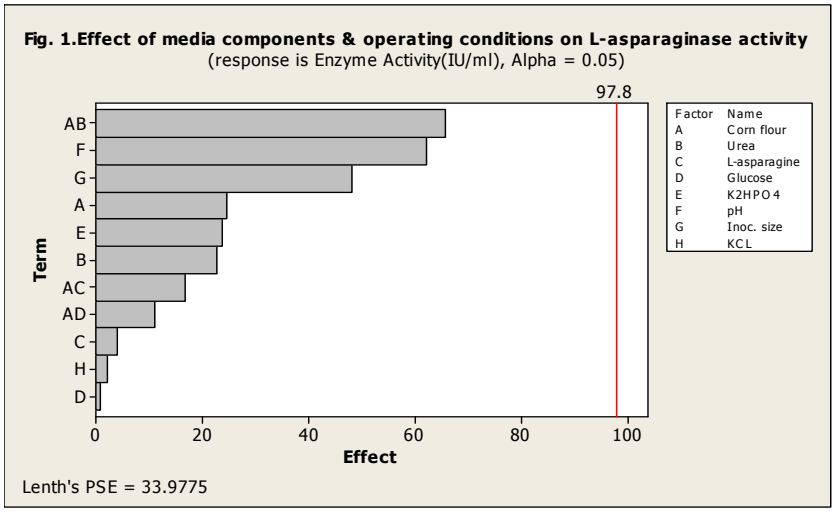
Y = 151 + 245 **X1** + 134 **X2** + 431 **X3** - 2674 **X4** - 0 **X5** - 15.6 **X6** - 0.282 **X7** - 5941 **X8.**

The effect of medium components and operating conditions on L-asparaginase activity was also studied using Pareto chart (Fig.1).Corn flour (X1) and urea (X2) was found to be the significant factors and pH (X6) and inoculums size (X7) were found to be other most important factors on L-asparaginase production.

Fig. 2, the normal probability plot of the residuals is 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 L-asparaginase production. 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 model fit.



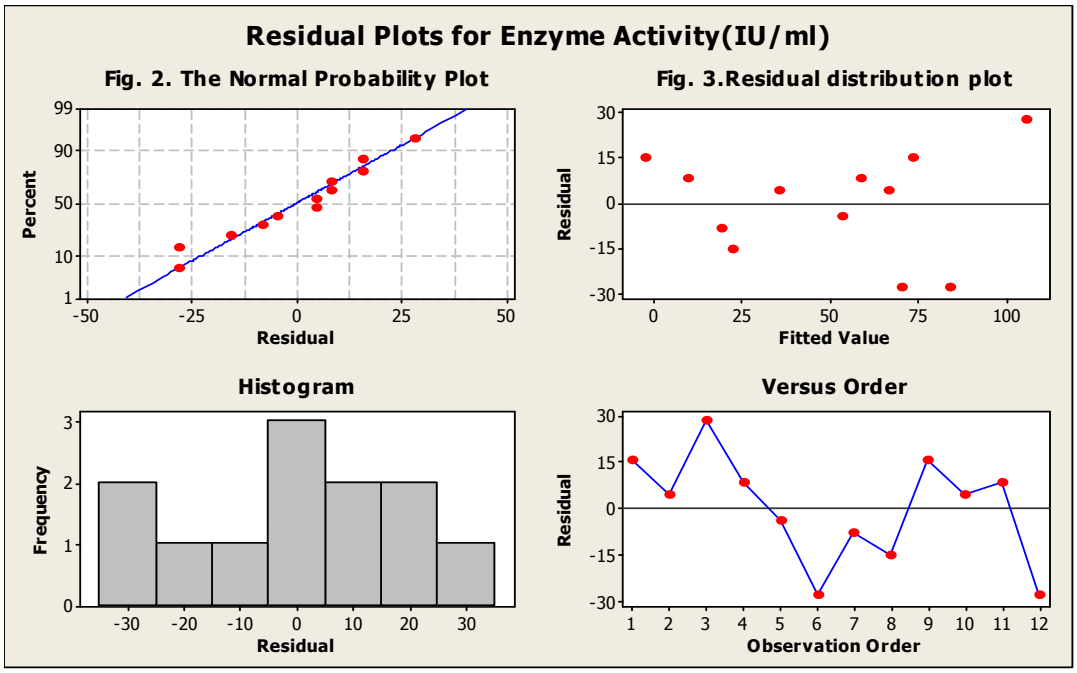


Table 1: Actual values of process variables

Process Variables % (w/v)	Corn Flour (X1)	Urea (X2)	L-Asparagine (X3)	Glucose (X4)	K_2HPO_4 (X5)	pH (X6)	Inoculum Size (X7) (µl)	KCL (X8)
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

Table 2: Plackett-Burman design and L-asparaginase Activity

Exp. No.	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Activity IU/ml
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	89.12
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	71.30
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	133.68
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	66.84
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	49.01
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	55.70
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	11.14
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	6.68
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	13.36
10	-1	-1	-1	-1	1	1	1	-1	1	1	-1	40.10
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	17.82
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	42.33

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

Variables	Coefficient's	SE Coefficient's	t-value	p-value
Intercept	151.13	94.05	1.61	0.206
X1	245.1	193.8	1.26	0.295
X2	133.7	193.8	0.69	0.54
X3	430.8	387.5	1.11	0.347
X4	-2674	1938	-1.38	0.261
X5	0	1983	0	1
X6	-15.597	9.688	-1.61	0.206
X7	-0.2822	0.1938	-1.46	0.241
X8	-5941	7750	-0.77	0.499

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	11846	1481	1.31	0.453
Residual error	3	3379	1126		
Total sum of squares	11	15224			

CONCLUSION

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 component and operating conditions on the production of L-asparaginase by *Aspergillus niger* were studied using PB design and it was found that cornflour, urea and inoculum size significantly influences and most important factors on the L-asparaginase production within their tested limits.

The decision making process for a hypothesis test can be based on the probability value (p value) for the given test. The p value is greater than the α value (0.05) and thus the null hypothesis is accepted.

However Y (Enzyme Activity) might depend on not all but some of the predictors i.e. media components. Because the value of r^2 is nearer to one (0.778) indicates a strong correlation between X (media components) and Y (Enzyme Activity). This is also evident from the regression equation and also as per the Pareto chart, the combination of cornflour (X1) and urea signicantly increase the L-asparaginase as compared to their application alone. The pH & inoculum size also significantly has impact at their tested levels.

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