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Value optimization of medium components for optimum L-asparaginase production by *Pseudomonas* sp. K2: An application of box-benken design

Kumar Gaurav, Rakesh Kumar*

Biotechnology Department, Dolphin P.G.College of Life Sciences, V.P.O., Chunni Kalan, Fatehgarh Sahib, Punjab, (INDIA)

E-mail : rakesh_panchal1@yahoo.co.in

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ABSTRACT

The present study aimed at understanding the effect of important process variables for L- asparaginase production from *Pseudomonas* sp. K2 using Box-Behnken design of response surface methodology. Maximum enzyme production (0.99 IU/ml) was obtained with medium constituents of temperature 37°C, pH 9.0 and asparagine concentration at 2%. Good fit of experimental data to the model was obtained with *p* value of 0.0152 and R^2, 88.16%. A low difference (11%) between experimental and observed values validates the reliability of model prediction. Five fold increase in enzyme production by this microbial isolate was noted under optimized environment.

KEYWORDS

L-Asparaginase;
Box-behnken design;
Pseudomonas sp. K2;
Optimization.

INTRODUCTION

L-asparaginase belongs to an amino acid group and catalyzes the irreversible conversion of L-asparagine to L-aspartate and ammonium ions[1]. It is an antineoplastic agent used in lymphoblastic leukemia chemotherapy and neoplastic cells cannot synthesize L-asparaginase due to lack of L-asparagine synthetase. For this reason, they are dependent upon the L-Asparaginase from circulating plasma pools[2,3]. Supplementation of L-asparaginase results in continuous depletion of L-asparagine. Under such conditions, cancerous cells do not survive. This phenomenon behavior of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase[4,5].

L-asparaginase produced by microorganisms has been widely used as effective therapeutic agent against acute lymphoblastic leukemia and lymphosarcoma. Ever since *Escherichia coli* L-asparaginase antitumor activity was first demonstrated by J.D.Broom[6] and L.T.Mashburn and J.C.Wriston[7], its production using microbial system has attracted considerable attention owing to their cost effective and eco-friendly nature. This requires screening of soil samples from various sources for isolation of potential microbes which have the ability to produce the desired enzyme. In this context, for effective utilization of any microbial system at bioprocess level, it is essential to screen and evaluate

various nutritional and environmental requirements for microbial growth and subsequent biocatalyst production[8,9], as culture conditions that promote optimum enzyme production differ significantly with the molecular nature of the microorganism.

Statistical experiment designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response. Recently the result analyzed by a statistical planned experiment is better acknowledged than those carried out by the traditional one-variable-at-a-time method. In the present study, the investigation was done in optimal nutritional, physiological and culture requirements for maximum production of L-asparaginase, using an isolated microbial source by Response Surface Methodology.

MATERIALS AND METHODS

Screening of asparaginase producing microorganism

The bacterial strain used in this study was isolated from sub surface soil of campus, Dolphin PG College of Life Sciences, Chunni-Kalan, District-Fatehgarh Sahib, Punjab, INDIA. One hundreds ml of Czepak-Dox (CD, g/l: Glucose, 2.0; L-Asparagine, 10; KH_2PO_4, 1.52; KCl, 0.52 and $MgSO_4$ trace) broth was inoculated by the inoculum of each individual isolate and after incubation at 37°C for 24 h, 1.5ml broth was removed and centrifuged at 5000 rpm for 10 min, the clear supernatant was used as crude enzyme extract.

Identification of most efficient microorganism

The isolate was identified by microscopic observation and measurement of various biochemical parameters in accordance with the Bergey's Manual of Determinative Bacteriology.

Asparaginase assay

For enzyme assay spectrophotometric method was used to estimate ammonia produced using Nesselerization reaction and was followed according to the method of Mashburn and Wriston, 1963[7]. The reaction mixture containing 0.2ml of 0.05M Triss HCl (pH 8.6), 1.7ml of 0.01M L-asparagine and 1ml of crude enzyme extract were incubated at 37 °C for exactly 10 min. The reaction was stopped by adding 1.5M Trichloro acetic acid. After centrifugation at 5000×g for 10 min 0.5ml supernatant was removed and to this 7ml of water was mixed. To this reaction mix 1ml of Nesseler's reagent was added and mixture was incubated for 10 min at room temperature. The absorbance was read at 480 nm. The activity was calculated as IU/ml and one enzyme unit is defined as the amount of enzyme librating 1 µmol of the ammonia/min under optimal assay conditions.

Statistical optimization of culture conditions by box-behnken design

Optimization methodology adopted for this study was statistical technique using trial version, Design Expert 7.1.6 software. Response surface methodology by Box-Behnken design was used and the first screening step was to identify the variables which have significant effect on asparaginase production. Choice of these factors was based upon literature search. The variables to be evaluated include Asparagine, pH and Temperature. These factors were studied at two different levels High (+) and Low (-) of independent variables examined (TABLE 1).

EXPERIMENTAL

A design of experiments (matrix table) was generated and all the experiments were performed under shake flask conditions. For each set of experiments, 100ml of media was prepared having formulation as set by design. All flasks were incubated at incubation temperature as per the design. After incubation 1ml sample was removed and centrifuged at 5000×g for 10 min. Enzyme activity was calculated at every 24, 48 and 72 h. The mathematical relationship of the independent variables and the response was calculated by the quadratic polynomial equation:

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X^2_1+\beta_{22}X^2_2+\beta_{33}X^3_2$$

where Y is the predicted response, β_0 is the model constant, X_1, X_2, and X_3 are the independent variables, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the cross-product coefficients, and β_{11}, β_{22}, and

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β_{33} are the quadratic coefficients.

Verification of experimental data

The statistical model was validated with respect to L-asparaginase production under the conditions predicted by the model in shake flask. After inoculation, the flasks were incubated at 37°C for 48 h. Samples were withdrawn at specific interval and asparaginase activity was calculated as described earlier.

RESULTS

Selection of most efficient asparaginase producing microorganism

After primary screening of 86 bacterial strains, 16 isolates were found to have asparaginase producing ability and designated as K1 to K16. Maximum enzyme was produced by isolate K2 (0.475IU) with maximum growth (A_{600}, 2.919). Asparaginase production and the growth of 16 bacterial isolates selected during preliminary screening were represented in figure 1.

On further subculturing of 16 isolated bacterial strains, isolate K2 and K5 showed the maximum enzyme production. Repeated subculturing for enzyme production reveal the strain K2 as the most efficient asparaginase producer. The growth pattern and enzyme production was presented in figure 2. Further optimization study was conducted on strain K2 being the higher enzyme producer.

Identification of microorganism

The most efficient asparaginase producing K2 isolate was a Gram-negative, aerobic, rod shape with flagellum motility and non-spore former. Strain showed the positive results for catalase production, oxidase test and Citrate utilization while negative results were reported for Indole, methyle red and Voges-Proskauer test. Among colony characteristics, elevation was Convex, round in shape, creamish appearance and greenish on CD agar medium. On the basis of these results the strain was tentatively identified as *Pseudomonas* sp. strain K2.

Statistical optimization of asparaginase production

For optimization of medium components the response surface method with Box-Behnken Design was used. The model evaluates the effect of each independent variable to a response (enzyme production). The mathematical relationship of the independent variables and the response was calculated by the quadratic polynomial equation:

$$Y = 0.72 - 0.030A + 0.44B + 0.24C - 0.091A^2 - 0.028B^2 - 0.13C^2 + 0.03 \times AB + 0.020 \times AC + 0.034 \times BC$$

Where Y, A, B and C are predicted asparaginase production, temperature, pH and asparaginase, respectively. As evident from TABLE 2, the maximum enzyme activity (0.99 IU/ml) after 48 h was observed with 12th trial. The second close value of activity (0.81 IU/ml) was shown by 11th trial. The results showed that the production of asparaginase enzyme is significantly affected by high pH and high asparagine concentration while temperature had least effect on enzyme production.

The result of response surface model fitting in the form of analysis of variance (ANOVA) is given in TABLE 3. The significance of the model was tested at 95% confidence level (p-value > 0.05). The parameter estimation and corresponding *p*-value of linear, quadratic and mutual effect of temperature pH and asparagine had moderate effect on asparaginase production. These data analysis can be drawn from three dimensional contour plots (3D graphs) as shown in figure 3, 4 and 5.

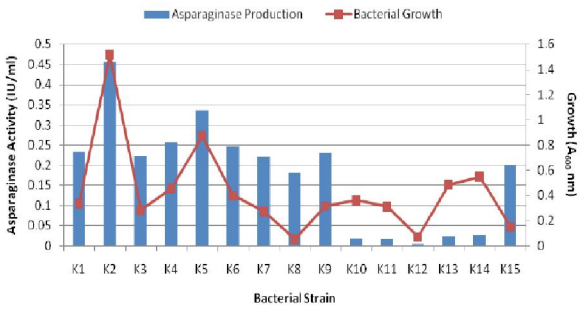

Figure 1 : Asparaginase producing ability of different soil isolates (K1 to K15) and their growth at 600nm

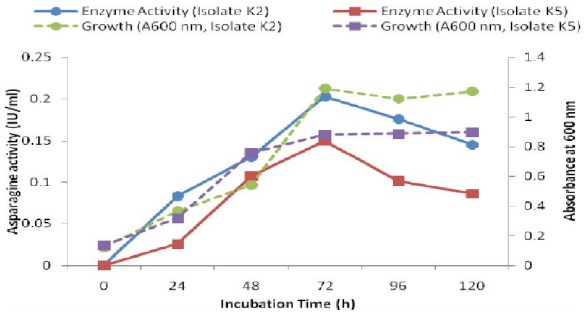

Figure 2 : Growth behavior and asparaginase production by Isolate K2 and K5

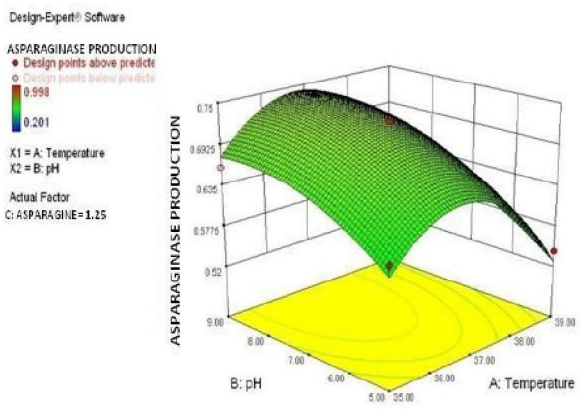

Figure 3 : Response surface and contour plot showing the effect of pH and temperature on enzyme activity. Maximum asparaginase production was achieved at medium temperature value (37°C) and at higher pH value (pH 9)

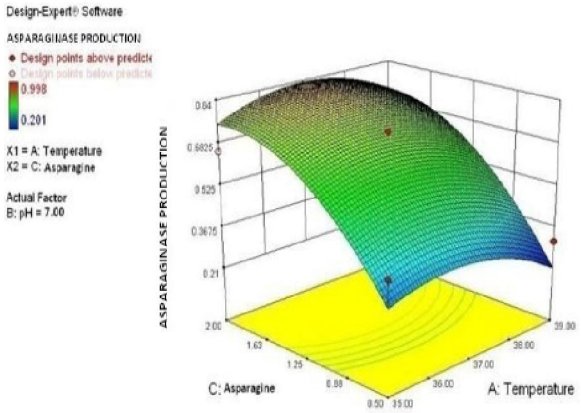

Figure 4 : Response surface and contour plot showing the effect of asparagine and temperature on enzyme activity. Moderate temperature (37°C) and maximum asparaginase concentration (2%) is the favorable combination which enhanced the asparaginase production

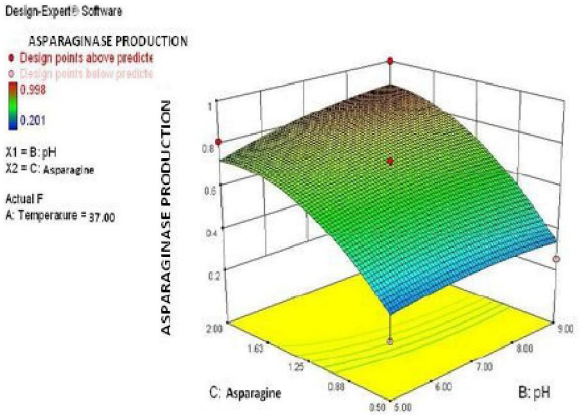

Figure 5 : Response surface and contour plot showing the effect of asparagine and pH on enzyme activity with other variable at constant. Higher asparagine concentration and moderate pH enhances the asparaginase production

The values of "Prob > F" less than 0.0500 indicates model terms are significant. In this study model *p* value, 0.0152 and F-value of 5.79 implies that the model is significant which implies that there is 1.52% chance that a "Model F-value" this large occur due to noise.

The value of R^2 indicates good correlation between experimental and predicted values and closer the value of R^2 to 1, better the correlation between experimental and predicted values. In our study the value of R^2 is 0.8816 which mean that 88.16 of variability can be explained and 11.84% of total variations are not explained by the model (TABLE 3). In addition, the value of adjusted R^2 (0.7293) is also high which advocate for a good significance of the model. The coefficient of variation (C.V.) Indicates the degree of precision with which the treatments are compared. Usually the higher the value of C.V., the lower is the reliability of the experiment. In this case the value of C.V. is 17.79 indicates good reliability of the experiment.

TABLE 1 : Variables to be monitored in box-behenken design for asparaginase production

S.N.	Factors	Unit	Low actual	High actual	Mean	Std. dev.
1	Temperature	ºC	35	39	37	1.372
2	pH	-	5	9	7	1.372
3	Asparagine	%	0.50	2.0	1.25	0.514

Verification of experimental data

The values of optimized factors, temperature; 37°C, pH; 9 and asparagine concentration; 2% were chosen to verify the statistical data. The experimental value of asparaginase production was 0.99 IU/ml which was in close agreement with predicted value of 0.88 IU/ml.

DISCUSSION

L- asparaginase production and optimization of medium components have been reported by many researchers[10-12]. In this study statistical optimization of L-asparaginase production by isolated bacterial strain was investigated under shake flask conditions using Box-Behnken design of Response Surface Methodology, to understand the imperative roles of various factors. It was insufficient to analyse how the factors might influence the microbial enzyme production by considering only individual factor effect at different levels. However, any biological system is a highly complex process

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TABLE 2 : Design of experiment and response for asparaginase production by box-behnken design

Std.	Temperature (°C)	pH	Asparigine (%)	Enzyme activity (IU/ml)		
				Actual	Actual	
				24h	24h	24h
1	35	5.0	1.25	0.39	0.61	0.60
2	39	5.0	1.25	0.42	0.54	0.53
3	35	9.0	1.25	0.32	0.66	0.68
4	39	9.0	1.25	0.39	0.61	0.62
5	35	7.0	0.50	0.36	0.41	0.31
6	39	7.0	0.50	0.24	0.31	0.21
7	35	7.0	2.00	0.29	0.65	0.75
8	39	7.0	2.00	0.32	0.63	0.73
9	37	5.0	0.50	0.32	0.20	0.32
10	37	9.0	0.50	0.23	0.25	0.34
11	37	5.0	2.00	0.22	0.81	0.73
12	37	9.0	2.00	0.45	0.99	0.88
13	37	7.0	1.25	0.34	0.72	0.72
14	37	7.0	1.25	0.34	0.70	0.72
15	37	7.0	1.25	0.34	0.73	0.72
16	37	7.0	1.25	0.34	0.72	0.72
17	37	7.0	1.25	0.34	0.71	0.72

and the product formation depends on the interaction of several factors (pH of the medium and incubation temperature and Asparagine). Interaction among factors has enormous impact on cellular metabolism and subsequent product/enzyme production.

It is assumed that L-asparaginase expression in *Pseudomonas* sp. K2 is mainly regulated by asparagine, an amino acid that provide nitrogen source along with pH and temperature. The linear and quadratic effect of asparagine had significant effect ($p<0.05$) on asparaginase production. The results are in accordance with the previous finding[13,14] on *Enterobacter cloacae* and *Aeromonas* sp., respectively who had reported enhanced asparaginase production using 0.1% L-asparagine as the sole source of nitrogen. In contrast to this the linear, quadratic and mutual effect of other factors (pH and temperature) is insignificant that does not invalidate the predictive purposes. Optimum L-asparaginase production was obtained at pH 9.0 and temperature 37°C.S.Amena et al.[15] have reported the maximum asparaginase yield (9.8 IU) at pH 8.5. B. Borkotaky and R.L.Bezbaruah[16] have also reported the maximum asparaginase at 8.5 pH.

TABLE 3 : ANOVA for experimental data on L-asparaginase production by pseudomonas sp. using box-behnken design

Source	SS	Df.	F-value	p-value
Model	0.61	9	5.79	0.0152*
A- Temperature	0.0074	1	0.64	0.4505
B- pH	0.016	1	1.35	0.2831
C- Asparagine	0.46	1	39.34	0.0004*
AB	0.0049	1	0.0042	0.9501
AC	0.0016	1	0.14	0.7153
BC	0.0047	1	0.40	0.5459
A^2	0.035	1	2.97	0.1286
B^2	0.0033	1	0.28	0.6126
C^2	0.072	1	6.19	0.0417*

***p<0.05. Std. Dev., 0.11; R-Squared, 0.8816; Mean, 0.61; Adj R-Squared, 0.7293; C.V. (%), 17.79; Pred R-Squared, -0.8951; Press, 1.31; Adeq Precision, 8.106**

In this study the results are encouraging for L-asparaginase production from *Pseudomonas* sp. K2. As this enzyme is already known for its therapeutic applications, production of it from *Pseudomonas* sp. K2 could be attempted for eventual utility in pharmaceutical industries.

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