Optimization of Asparaginase Production by *Pseudomonas aeruginosa* Using Experimental Methods

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Abstract: Evaluation of fermentation process parameter interactions for the production of l-asparaginase by *Pseudomonas aeruginosa*. Box-Behnken design of experimentation was adopted to optimize nutritional sources, physiological (incubation period) and microbial (inoculum level).The experimental results and software predicted enzyme production values were comparable. Incubation period, inoculum level and nutritional source (soybean) were major influential parameters at their individual level. Interaction data of the selected fermentation parameters could be classified as least and most significant at individual and interactive levels. All selected factors showed impact on l-asparaginase enzyme production by this isolated microbial strain either at the individual or interactive level. Incubation temperature, inoculums concentration, and nutritional source (soybean) had impact at individual level. Significant improvement in enzyme production by this microbial isolate was noted under optimized environment. [Nature and Science. 2010;8(2):1-6]. (ISSN: 1545-0740).

Key words: box-Behnken; *pseudomonas aeruginosa*; L- asparaginase; response surface

1. Introduction

L-Asparaginase has received increased awareness in current years for its ant carcinogenic potential. Cancer cells distinguish themselves from normal cells in diminished expression of l-asparagine (Swain *et al.* 1993; Manna *et al.* 1995). Hence, they are not capable of producing l-asparagine, and mainly depend on the l-asparagine from the circulating plasma pools (Swain *et al.* 1993).l-Asparaginase (l-asparagine amidohydrolase EC 3.5.1.1) catalyses the conversion of l-asparagine to l-aspartate and ammonium, and this catalytic reaction is essentially permanent under physiological conditions. If l-asparaginase is given to cancer patients then there will be nonstop reduction of l-asparagine. This extradinary behavior of cancerous cells was broken by scientific community (Story *et al.* 1993; Swain *et al.* 1993). Asparaginase is used for treating acute lymphoblastic leukemia, lymphosarcoma. This therapy brought a major breakthrough in modern oncology. With the development of its new functions, a great demand for l-asparaginase is expected in the coming years. The biochemical and enzyme kinetic properties vary with the microbial source. However, *Erwinia* asparaginase had a shorter half life than E. coli (Asselin et al. 1993); suggesting the need to discover new l-asparaginases that are serologically different, but have similar beneficial effects. This requires selection of soil samples from various sources for isolation of possible microbes, which have the ability to produce the most wanted enzyme.

Experimental designs nowadays have been regarded as one of the most favorable techniques in covering a large area of practical statistics and obtain unambiguous results with the least expense. Response surface method (RSM) designs help to quantify the relationships between one or more measured responses and the vital input factors. The most popular response surface methodologies are Central Composite, Box-Behnken designs.

Box-Behnken design is an efficient and creative three-level composite design for fitting second-order response surfaces. It is an independent quadratic design. The methodology is based on the construction of balance designs which are rotatable and enable each factor level to be tested several times. Each factor or

independent variable can be placed at one of three equally spaced values (coded as –1, 0, and +1). In this design the treatment combinations are at the midpoints of edges of the cubical design region and at the center. Box-Behnken designs provide excellent predictability within the spherical design space and require fewer experiments compared to the full factorial designs or central composite designs. The number of required experiments for Box-Behnken design can be calculated according to $N = k^2 + k + c_p$, where k is the factor number and c_p is the replicate number of the central point.

In the present investigation**,** we study about optimization of asparaginase production by *Pseudomona aeruginosa using* design of experiments by Box-Benhken Design.

2. Materials and methods:

2.1 Maintenance and cultivation of Microorganism

The strain *Pseudomonas aeruginosa* was obtained from NCIM, Pune, India. The strain was subcultured in nutrient broth. The broth was incubated in the shaker with 175 rpm and at 37°C overnight. Sterile plates containing nutrient agar of specified composition were streak plated with the overnight cultures. In 100 ml nutrient broth, the cultures are grown overnight. The culture on the broth was used as the source for the entire experiment. Cultivation was achieved by solid-state fermentation (SSF) as previously reported by Ramesh and Lonsane (1987).2.24 g of soyabean is moistened with 5 ml of phosphate buffer containing culture. The plates are incubated for 48 hrs & are checked for enzyme activity.

2.2 Estimation of L-asparaginase activity

Reaction mixture consisting of 0.5 ml of 0.08 mol/l of 1 l-asparagine, 1ml of 0.05mol/l borate buffer (pH 7.5) and 0.5 ml of enzyme solution was incubated for 10 min at 37 ^{0}C. The reaction was stopped by the addition of 0.2 ml of 15% trichloroacetic acid solution. The liberated ammonia was coupled with 1 ml Nessler's reagent & OD is measured at 500 nm, and was quantitatively determined using standard curve.

2.3 Optimization of the process parameters

Process optimization was carried out by conducting 17 experiments to identify the best combinations of the parameters which involved in the production biomass to obtain high yield of crude extract. The parameters, soybean (10, 12.5,15 gms), inoculums (300, 450, 600 µl) and incubation (48, 72, 60 h) were selected. 17 different cultures were obtained by varying the three parameters. The concentration of the enzyme was measured using standard plot. The data obtained from 17 experiments, were used to find out the optimum point of the process parameters by using Box-Behnken Design in Response surface methodology. All the data were treated with the aid of Design Expert from Stat-Ease.

3. Results and Discussion

3.1 Analysis of variance

Based on design of experiment, 17 combination were developed (Table 1) and processed to obtain asparaginase as mentioned in this paper. The data obtained from the experiments were used to the analysis of variance (Table 2 and 3). The Model F-value of 6.366E+007 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2, A^2B, A^2C, $AB^2$are significant model.

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Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Analysis of process variables by response surface plots

The optimum values of the selected variables were obtained by solving their regression equation and analyzing response surface contour plots. Response Surface plots as a function of two factor at a time maintaining all other factors at a fixed level (zero for instance) are more helpful in understanding both the main and interaction effects of the two factors. The plots can be easily obtained by calculating the data from the model. The values were taken by one factor, where the second varies with constant of a given Y -values. The yield values of the different concentrations of the variable can also be predicted from respective response surface plots. Figure 1 to 6 shows the relative effect of the two variables with protein concentration level. The coordinates of the central point within the highest

contour levels in each of these figures corresponded to the optimum concentrations of the respective components.

Figure 1 and 2 show their contour and response surface plot obtained as a function of incubation period vs. medium with asparaginase concentration, while all other variables are maintained at zero level (coded units). Figure 3 and 4 show their contour and response surface plot obtained as a function of volume of inoculum vs. medium with soybean concentration, while all other variables are maintained at zero level (coded units).Figure 5 and 6 show their contour and response surface plot obtained as a function of Incubation period vs. medium with asparaginase concentration, while all other variables are maintained at zero level (coded units).

Final equation in terms of terms of coded factors:

Asparaginase (mg/ml) = 5.56 + (0.4985 * A) - (0.544 * B) - (0.2155 * C) - (0.25825 * A * B) - (0.537 * A * C) - (0.092 * B * C) + (0.069375 * A^2) - (0.11963 * B^2) + (0.526125 * C^2) + (0.76725 * A^2* B) + (0.8625 * A^2* C) - (0.94925 * A * B^2)

Optimum values

The protein production was predominantly influenced by the amount of soybean, incubation period and inoculum. The contour plots show the region of the desirability for the production of protein content. The point prediction from the analysis of variable for response surface cubic model for the production of protein concentration (5.566 mg/ml) is 12.5 ml of medium, 450 μl of inoculum, and 60 h of incubation.

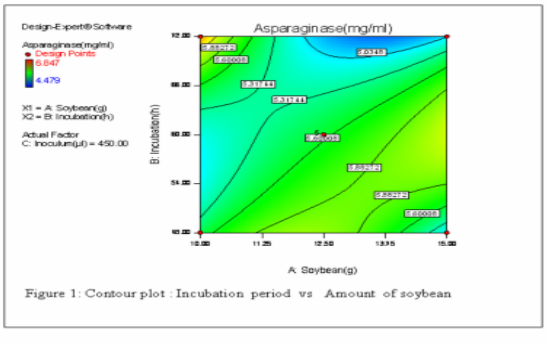

Figure 1: Contour plot : Incubation period vs Amount of soybean

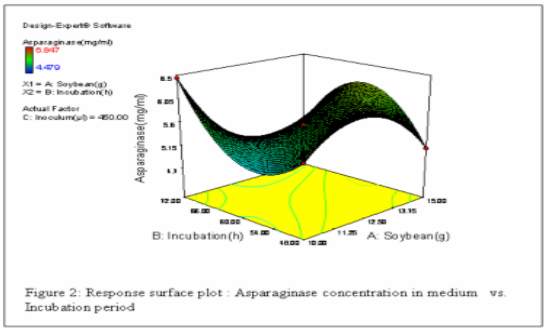

Figure 2: Response surface plot : Asparaginase concentration in medium vs. Incubation period

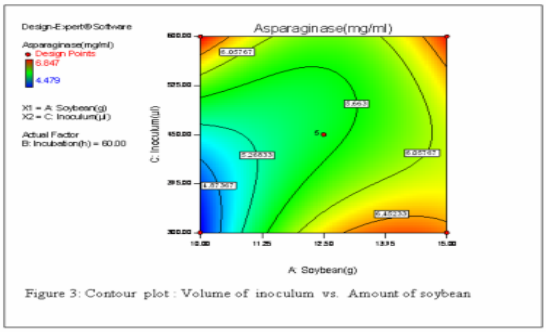

Figure 3: Contour plot : Volume of inoculum vs. Amount of soybean

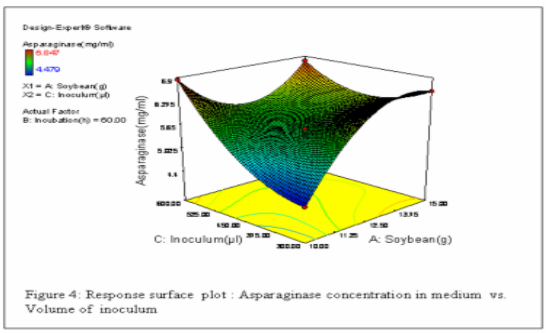

Figure 4: Response surface plot : Asparaginase concentration in medium vs. Volume of inoculum

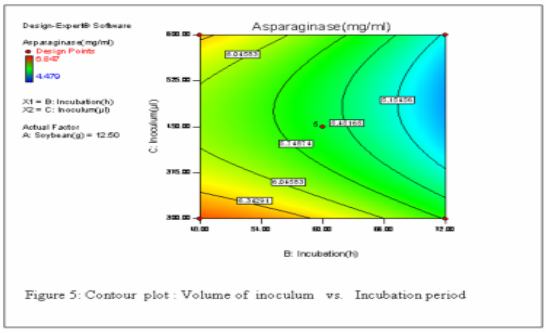

Figure 5: Contour plot : Volume of inoculum vs. Incubation period

Table 1. Combination of process variables

Run	A: Soybean (g)	B: Incubation (h)	C: Inoculum (µl)	Asparaginase (mg/ml)
1	10	72	450	6.448
2	10	48	450	5.485
3	15	60	600	6.770
4	10	60	600	6.847
5	12.5	60	450	5.566
6	12.5	60	450	5.566
7	12.5	60	450	5.566
8	12.5	48	600	6.393
9	15	72	450	5.030
10	15	60	300	6.550
11	12.5	72	300	5.736
12	12.5	48	300	6.640
13	10	60	300	4.479
14	12.5	60	450	5.566
15	12.5	60	450	5.566
16	12.5	72	600	5.121
17	15	48	450	5.100

Table 2. ANOVA for Response Surface Cubic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Model	7.741125	12	0.645094	6.366E+007	<0.0001	significant
A-Soybean(g)	0.994009	1	0.994009	6.366E+007	<0.0001	
B-Incubation(h)	1.183744	1	1.183744	6.366E+007	<0.0001	
C-Inoculum(µl)	0.185761	1	0.185761	6.366E+007	<0.0001	
AB	0.266772	1	0.266772	6.366E+007	<0.0001	
AC	1.153476	1	1.153476	6.366E+007	<0.0001	
BC	0.033856	1	0.033856	6.366E+007	<0.0001	
A^2	0.020265	1	0.020265	6.366E+007	<0.0001	
B^2	0.060253	1	0.060253	6.366E+007	<0.0001	
C^2	1.165505	1	1.165505	6.366E+007	<0.0001	
A^2B	1.177345	1	1.177345	6.366E+007	<0.0001	
A^2C	1.487813	1	1.487813	6.366E+007	<0.0001	
AB^2	1.802151	1	1.802151	6.366E+007	<0.0001	
AC^2	0	0				
B^2C	0	0				
BC^2	0	0				
A^3	0	0				
B^3	0	0				
C^3	0	0				
Pure Error	0	4	0			
Cor Total	7.741125	16				

Table 3. Regression Analysis

Std. Dev.	0	R-Squared	1
Mean	5.78	Adj R-Squared	1
C.V. %	0	Pred R-Squared	N/A
PRESS	N/A	Adeq Precision	0

Table 4. Coefficient value of the factor

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.566	1				
A-Soybean(g)	0.4985	1				2
B-Incubation(h)	-0.544	1				2
C- inoculum(µl)	-0.2155	1				2
AB	-0.25825	1				1
AC	-0.537	1				1
BC	-0.092	1				1
A^2	0.069375	1				1.005882
B^2	-0.11963	1				1.005882
C^2	0.526125	1				1.005882
ABC ALIASED Intercept						
A^2B	0.76725	1				2
A^2C	0.8625	1				2
AB^2	-0.94925	1				2

Table 5. Predicted value from Box - Behnken design

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding	
A	Soybean(g)	12.5	10	15	0	Actual	
B	Incubation(h)	60	48	72	0	Actual	
C	Inoculum (µl)	450	300	600	0	Actual	
Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Asparaginase (mg/ml)	5.566	0	5.566	5.566	0	5.566	5.566

PI - Prediction interval
CI - Confidence interval
SE Mean – Standard error of the mean.
SE Pred – Standard error of prediction

3. Conclusion

In this work the process parameters the amount of soybean, incubation time and inoculum were selected and optimized to produce asparaginase. Design Expert from Stat-Ease was used to develop design of experiment. Box Behnken design in Response surface method was used to optimize the process condition. Thus it has been concluded that the point prediction from the analysis of variable for response surface cubic model for the production of protein concentration (5.566 mg/ml) is 12.5 ml of medium, 450 µl of inoculum, and 60 h of incubation.

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