Production of L-asparaginase by *Serratia marcescens* SB08: Optimization by response surface methodology

Chidambaram Kulandaisamy Venil*, Kuppanan Nanthakumar, Kannan Karthikeyan, Perumalsamy Lakshmanaperumalsamy

Department of Environmental Sciences, Division of Environmental Microbiology, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India

Abstract
This paper describes optimization method that combines the Plackett-Burman design, a factorial design and the response surface method, which were used to optimize the medium for the production of L-asparaginase by *Serratia marcescens* SB08. Four medium factors, from out of 11 medium factors, were screened by Plackett-Burman design experiments and subsequent optimization process to find out the optimum values of the selected parameters using central composite design was performed. Sucrose, peptone, KH_2PO_4 and incubation time were found to be the best medium factors for the optimization of L-asparaginase production and central composite design experiments indicated the optimal concentrations of sucrose 12.50 g/l, peptone 4.5 g/l, KH_2PO_4 4.0 g/l and incubation time 51h. The combined optimization method described here is the effective for screening medium factors as well as determining their optimum levels for the production of L-asparaginase by *Serratia marcescens* SB08.

Keywords: *Serratia marcescens* SB08; L-asparaginase; PBD; CCD

INTRODUCTION

L-asparaginase is an important chemotherapeutic agent used for the treatment of a variety of lymphoproliferative disorders and lymphomas, acute lymphoblastic leukemia in particular. In recent years, the use of L-asparaginase in the treatment of leukemia and other lymphoproliferative disorders has expanded immensely. For these reasons L-asparaginase has established itself to be an indispensable component (Umesh *et al*., 2007). Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine (Manna *et al*., 1995; Swain *et al*., 1993). Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from circulating plasma pools (Swain *et al*., 1993). Clinical trials indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man (Oettgen *et al*., 1967). The principle source of L-asparaginase for clinical trials is the bacterium *Escherichia coli* (Adamson and Fabro, 1968). Although production and purification techniques have been developed, they generally provide a quantity of enzyme sufficient for only limited trials. To overcome this constraint, production of L-asparaginase by other bacterium can be explored. Also medium factors for enhanced production of L-asparaginase can be investigated by statistical modeling.

There is little information available regarding statistical optimization of the medium factors for L-asparaginase production. Screening and selection of the optimum concentration of medium components are very important to determine the overall economic feasibility of the production process. The reach of optimized fermentation conditions, particularly associated to physical and chemical parameters, is of primary and great importance for the development of any process, due to their impact upon its economics and practicability.

The diversity of combinatory interactions among medium components, metabolism of cells and the large number of chemical requirements for processing

*Correspondence to: ***Chidambaram Kulandaisamy Venil****, Ph.D.
Telefax: +91 422 2424729
E-mail: ckvenil@gmail.com

metabolic products, do not allow satisfactory detailed modeling. Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked (Abdel-Fattah *et al*., 2005). Statistical experimental 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 (Kim *et al*., 2005; Lee and Gilmore, 2005; Nawani and Kapadnis, 2005; Senthilkumar *et al*., 2005; Wang and Lu, 2005).

No defined medium has been established for the optimum production of L-asparaginase from different microbial sources. Each organism has its own special conditions for maximum enzyme production. A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing L-asparaginase production by *Serratia marcescens* SB08. The levels of the significant variables were further optimized using response surface methodology.

MATERIALS AND METHODS

Enumeration and maintenance of enteric gut associated bacteria: Potent strains of bacteria were isolated from the gut of sulphur butterfly (*Kricogonia lyside*). The sulphur butterfly was washed with 70% ethanol and several times with sterile distilled water to eliminate surface bacteria. All dissections were performed under sterile conditions. After disrupting the walls, the contents of the stomach were collected in sterile eppendorf tubes, containing phosphate-buffered saline which were serially diluted, spread into the surface of nutrient agar plates and incubated for 48 h at 30ºC in order to record total colony forming units (CFU/ml) (Azambuja *et al.,* 2004). The potent bacterial culture was grouped to the genera based on their morphological and biochemical characteristics employing standard protocols (Buchanan and Gibbons, 1974). The identified strain, *Serratia marcescens* SB08 was maintained at 4ºC on nutrient agar slants and subcultured every 2 to 4 weeks.

L-Asparaginase assay

Growth media: The L-asparagine broth medium containing (g/l): Peptone: 0.5 g; Beef extract: 0.5 g; Yeast extract: 0.5 g; L-asparagine: 0.1 g was used for the production of L-asparaginase. The pH of the medium was adjusted to 7.0 with 1N NaOH or 1 N HCl and was autoclaved at 121ºC for 15 minutes. The *Serratia marcescens* SB08 was cultivated in L-asparagine broth at 30ºC and used as inoculum for enzyme production.

Production of L-asparaginase: 100 ml of L-asparagine broth media was inoculated with 1 ml of inoculum and was incubated at 30ºC. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10,000 $\times g$ for 10 min. The cell free supernatant which contains the enzyme was taken for L-asparaginase assay.

Enzyme assay: L-asparaginase activity was determined by measuring the amount of ammonia formed by nesslerization (Wriston and Yellin, 1973). A 0.5 ml sample of crude enzyme, 1.0 ml of 0.1 M sodium borate buffer (pH 8.5) and 0.5 ml of 0.04 M L-asparagine solution were mixed and incubated for 10 min at 37ºC. The reaction was then stopped by the addition of 0.5 ml of 15% trichloroacetic acid. The precipitated protein was removed by centrifugation, and the liberated ammonia was determined by direct nesslerization. Suitable blanks of substrate and enzyme-containing samples were included in all assays. The yellow color was read in a spectrophotometer (Hitachi-3210 UV-Vis) at 500 nm. One unit (U) of L-asparaginase is that amount of enzyme which liberates 1 µmole of ammonia in 1 min at 37ºC.

Optimization of process parameters

Screening of important nutrient components using Plackett-Burman design: This study was done by Plackett - Burman design for screening medium components with respect to their main effects and not their interaction effects (Plackett and Burman, 1946) on L-asparaginase production by *Serratia marcescens* SB08. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-). According to the Plackett-Burman design, the number of positive signs (+) is equal to (N+1)/2 and the number of negative signs (-) is equal to (N-1)/2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1)/2 positive signs and (N-1)/2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all negative signs. The experimental design and levels of each variable is shown in Table 1. The medium was formulated as per the design and the flask culture experiments for

enzyme were assayed as described earlier. Response was calculated at the rate of enzyme production and expressed as U/ml. All experiments were performed in triplicates and the average of the rate of enzyme production was considered as the response.

The effect of each variable was calculated using the following equation:

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

Where E is the effect of tested variable, M_{+} and M_{-} are responses (enzyme activities) of trials at which the parameter was at its higher and lower levels respectively and N is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level (p-value) of each variables calculated by using Student's t-test.

$$t = Exi / SE$$

where E_{xi} is the effect of tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

Optimizaion of concentrations of the selected medium components using response surface methodology: The screened medium components affecting enzyme production were optimized using cenral composite design (CCD) (Box and Wilson, 1951; Box and Hunter, 1957). According to this design, the total number of treatment combinations is $2^k + 2k + n0$ where 'k' is the number of independent variables and $n0$ the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:
where x_i is dimensionless coded value of the variable

$$x_i = X_i - X_0 / \delta X$$

X_i, X_0 the value of the X_i at the center point, and δX is the step change. A 2^k-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j$$

where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

Validation of the experimental model: The statistical model was validated with respect to L-asparaginase production under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and L-asparaginase assay was determined as described above.

RESULTS

Plackett-Burman design: The influence of eleven medium factors namely pH, temperature, agitation, inoculum concentration, incubation time, sucrose, peptone, KH_2PO_4, yeast extract, NaCl and $CaCl_2$ in the production of L-asparaginase was investigated in 12 runs using Plackett-Burman design. Table 1 represents the Plackett-Burman design for 11 selected variables and the corresponding response for L-asparaginase production. Variations ranging from 54.08 to 253.04 U/ml in the production of L-asparaginase in the 12 trials were observed by Plackett-Burman design.

The Pareto chart illustrates the order of significance of the variables affecting L-asparaginase production (Fig. 1). Among the variables screened, the most effective factors with high significance level indicated by Pareto chart were in the order of sucrose, peptone, KH_2PO_4 and incubation time.

Incubation period of 51h showed enzyme production ranging from 186.37 to 257.06 U/ml whereas when the incubation period is prolonged to 96h, the enzyme production was low ranging from 115.38 to 213.3 U/ml. Sucrose, peptone, KH_2PO_4 and incubation time were identified as most significant variables in L-asparaginase production and selected for further optimization while pH, temperature, agitation, inoculum concentration, yeast extract, NaCl and $CaCl_2$ which exhibited less significance level were omitted in further experiments.

Statistical analysis of the Plackett-Burman design demonstrates that the model F value of 10.64 is significant. The values of $p<0.05$ indicate model terms are significant (Table 2).

Regression analysis was performed on the results and first order polynomial equation was derived repre-

Table 1. Plackett-Burman experimental design for evaluating factors influencing L-Asparaginase by *Serratia marcescens* SB08.

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response
A: pH	B: Temp	C: Agi	D: noculum conc	E: Incubation time	F: Sucrose	G: Peptone	H: KH_2PO_4	J: Yeast extract	K: NaCl	L: $CaCl_2$	L-Asparagin ase
	ºC	rpm	%	h	g/l	g/l	g/l	g/l	g/l	g/l	U/ml
4	40	200	1	96	20	6	2	2	0.1	0.2	231.74
4	40	200	5	6	5	3	6	2	0.5	0.2	62.13
4	40	0	5	96	5	6	6	4	0.1	0.05	165.93
4	20	0	1	6	5	3	2	2	0.1	0.05	129.74
8	20	200	5	96	5	3	2	4	0.1	0.2	54.08
8	20	0	1	96	5	6	6	2	0.5	0.2	163.35
4	20	200	1	96	20	3	6	4	0.5	0.05	215.46
8	40	0	1	6	20	3	6	4	0.1	0.2	253.04
4	20	0	5	6	20	6	2	4	0.5	0.2	248.03
8	40	200	1	6	5	6	2	4	0.5	0.05	78.12
8	40	0	5	96	20	3	2	2	0.5	0.05	235.9
8	20	200	5	6	20	6	6	2	0.1	0.05	251.35

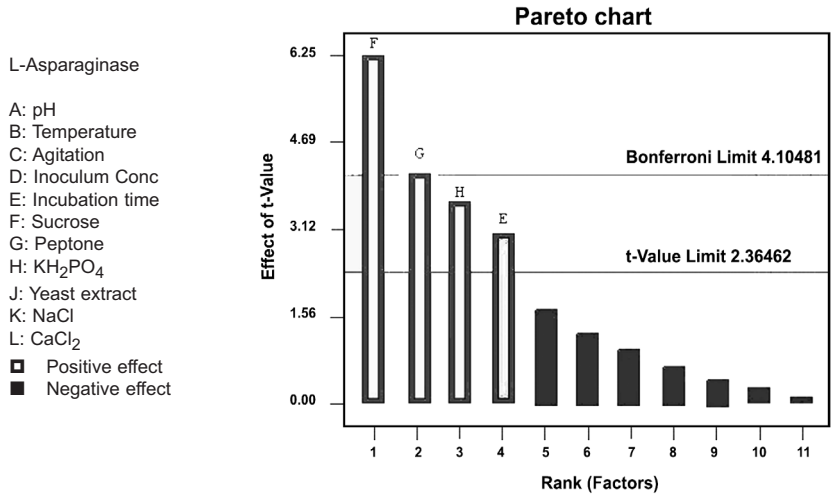

Figure 1. Pareto chart for Plackett Burman design for 11 medium factors on L-Asparaginase production by *Serratia marcescens* SB08.

senting L-asparaginase production as a function of the independent variables.

$$L-Asparaginase = 173.67 + 3.50\,E + 65.17\,F + 15.67\,G + 11.17\,H$$

The magnitude of the effects indicates the level of the significance of the variable on L-asparaginase production. Consequently, based on the results from this experiment, statistically significant variables i.e. incubation time, sucrose, peptone and KH_2PO_4 with positive effect were further investigated with central composite design to find the optimal range of these variables.

Central composite design: Based on Plackett-Burman design, incubation time, sucrose, peptone and KH_2PO_4 were selected for further optimization using response surface methodology. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of -2 to +2 in relation to production of L-asparaginase (Table 3).

The results obtained from the central composite design were fitted to a second order polynomial equation to explain the dependence of L-asparaginase production on the medium components.

$$Y = 248.01 + 58.76\,A + 20.63\,B + 3.31\,C + 7.13D + 6.79AB + 0.66AC + 6.12\,AD + 2.73BC + 0.92BD - 2.38CD - 41.23A2 - 43.36\,B2 - 24.12C2 - 10.92\,D2$$

where Y is the predicted response of L-asparaginase production, A, B, C and D are the coded values of incubation time, sucrose, peptone and KH_2PO_4 respectively.

The analysis of variance of the quadratic regression model suggested that the model is very significant as was evident from the Fisher's F-test (Table 4). The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of R^2 (0.87) (multiple correlation coefficient) closer to 1 denotes better correlation between the observed and predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is

Table 2. Analysis of variance for L-Asparaginase production by *Serratia marcescens* SB08.

Source	Sum of square	Degree of freedom	Mean square	F-Value	*p*-Value
Model	55549	4	13887	10.643124	0.0042
E-Incubation time	147	1	147	0.1126601	0.0470
F-Sucrose	50960	1	50960	39.055764	0.0004
G-Peptone	2945.3	1	2945.3	2.2572899	0.0767
H-KH_2PO_4	1496.3	1	1496.3	1.146783	0.0198
Residual	9133.7	7	1304.8	-	-
Cor Total	64683	11	-	-	-

Table 3. Experimental plan for optimization of L-asparaginase production using central composite design.

Run	Incubation time h	Sucrose g/l	Peptone g/l	KH_2PO_4 g/l	L-Asparaginase (U/ml)	
					Experimental	Predicted
1	-1	-1	-1	-1	51.29	53.38833
2	1	-1	-1	-1	145.38	143.7742
3	-1	1	-1	-1	75.75	73.75083
4	1	1	-1	-1	194.66	191.3067
5	-1	-1	1	-1	55.91	57.98083
6	1	-1	1	-1	158.44	151.0017
7	-1	1	1	-1	63.35	89.28333
8	1	1	1	-1	205.68	209.4742
9	-1	-1	-1	1	41.83	58.34083
10	1	-1	-1	1	177.28	173.1867
11	-1	1	-1	1	73.1	82.37833
12	1	1	-1	1	216.16	224.3942
13	-1	-1	1	1	58.22	53.41333
14	1	-1	1	1	168.59	170.8942
15	-1	1	1	1	76.48	88.39083
16	1	1	1	1	213.3	233.0417
17	-2	0	0	0	0	0
18	2	0	0	0	208.33	200.6108
19	0	-2	0	0	34.74	33.2925
20	0	2	0	0	116.5	115.8025
21	0	0	-2	0	156.37	144.9075
22	0	0	2	0	168.83	158.1475
23	0	0	0	-2	203.75	190.0725
24	0	0	0	2	217.06	218.5925
25	0	0	0	0	239.11	248.0067
26	0	0	0	0	255.68	248.0067
27	0	0	0	0	249.75	248.0067
28	0	0	0	0	257.55	248.0067
29	0	0	0	0	237.46	248.0067
30	0	0	0	0	248.49	248.0067

usually indicated by high value of CV. In the present case a low CV (4.18) denotes that the experiments performed are highly reliable. The *p* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables.

The fitted response for the above regression model was plotted in Figure 2. 3D graphs were generated for the pair wise combination of four factors for L-asparaginase production. Graphs highlight the roles played by various factors affecting L-asparaginase production.

Validation of the model: The maximum experimental response for L-asparaginase production was 257.55 U/ml whereas the predicted value was 248.006 U/ml indicating a strong agreement between them. The optimum values of the tested variables are incubation time 51 h, sucrose 12.50 g/l, peptone 4.5 g/l and KH_2PO_4

Table 4. ANOVA for the experimental results of the central composite design (quadratic model).

Source	Sum of square	Degree of freedom	Mean square	F-Value	*p*-Value
Model	187328.8	14	13380.6	7.2714219	0.0002
A-Incubation time	82863.352	1	82863.4	45.030351	< 0.0001
B-Sucrose	10211.85	1	10211.9	5.5494158	0.0325
C-Peptone	262.9464	1	262.946	0.1428927	0.7107
D-KH_2PO_4	1220.0856	1	1220.09	0.6630299	0.4282
AB	738.2089	1	738.209	0.4011641	0.5360
AC	6.943225	1	6.94322	0.0037731	0.9518
AD	598.2916	1	598.292	0.325129	0.5770
BC	119.6836	1	119.684	0.0650395	0.8022
BD	13.505625	1	13.5056	0.0073393	0.9329
CD	90.6304	1	90.6304	0.0492512	0.8274
A^2	46622.884	1	46622.9	25.336228	0.0001
B^2	51579.57	1	51579.6	28.029835	< 0.0001
C^2	15956.965	1	15957	8.671478	0.0100
D^2	3269.8849	1	3269.88	1.7769503	0.2024
Residual	27602.501	15	1840.17		
Lack of Fit	27258.89	10	2725.89	39.665341	0.0004

CV-4.18; R^2-0.87

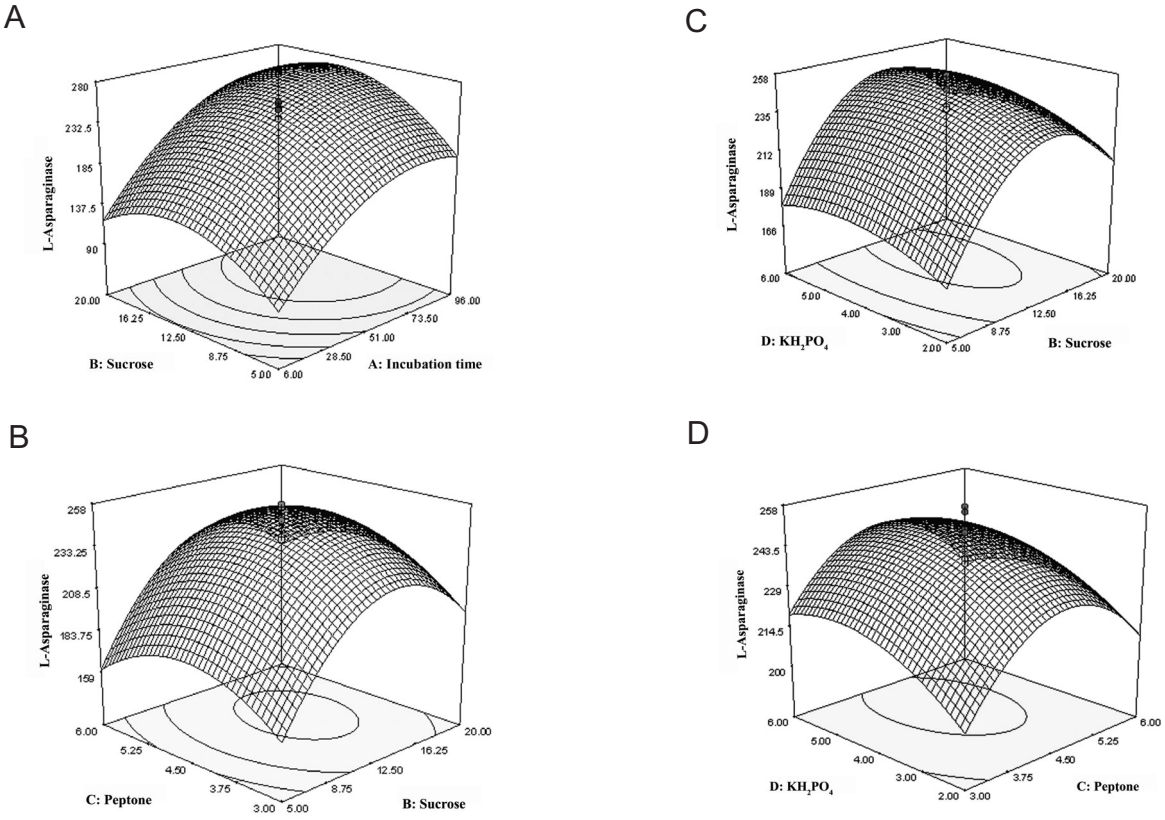

Figure 2. Three dimensional response surface plot for the effect of (A) incubation time, sucrose; (B) sucrose, peptone; (C) sucrose, KH_2PO_4; (D) peptone, KH_2PO_4 on L-asparaginase production by *Serratia marcescens* SB08.

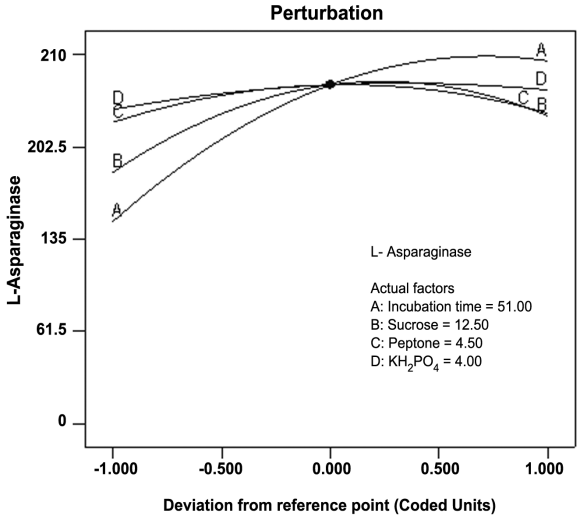

Figure 3. Perturbation graph showing the optimum values of the tested variables.

4.0 g/l as shown in perturbation graph (Fig. 3). The model was also validated by repeating the experiments under the optimized conditions, which resulted in the L-asparaginase production of 256.91 U/ml (Predicted response 248.006 U/ml), thus proving the validity of the model.

The scale-up study was carried out in a jar fermentor (5 lit.) by using medium under optimized conditions. The maximum production of 265.95 U/ml L-asparaginase was achieved in this scale-up study. The result of optimization study under flask conditions was 257.55 U/ml, whereas 265.91 U/ml was observed in the scale-up study with higher volume of fermentation.

DISCUSSIONS

Enzyme synthesis necessitates the presence of sucrose as enzyme substrate because of its inductive effect (Ray, 2001; Tani *et al*., 2000) and its remarkable efficiency in the production of enzyme being an inexhaustible source of carbon compared to other carbon sources (Le Mense *et al*., 1947; Mctigue *et al*., 1994) and also because of its role in stabilizing the enzyme (Aguilar *et al*., 2000; Santamaria *et al*., 1999). Nitrogen sources as a key factor support enzyme production (Mctigue *et al*., 1994). Peptone has been reported to be the best nitrogen source than yeast extract for enzyme production (Doull and Vining, 1989). This is in line with the role that peptone serves as better nitrogen source and it is responsible for terminal electron acceptor for this *Serratia marcescens* SB08.

Incubation time has a significant role in medium optimization for enzyme production. Incubation period of 51h showed enzyme production ranging from 186.37 to 257.06 U/ml. This is because *Serratia marcescens* SB08 achieves maximum growth at 51 h in stationary phase and its growth further declines in death phase, so the production was low in extended time period. Extended period of incubation might lead to the decomposition of enzyme due to interaction with other components in the media (Ramesh and Lonsane, 1987).

The results are encouraging for production of L-asparaginase from *Serratia marcescens* SB08. As this enzyme is already known for its medical applications, production of it from *Serratia marcescens* SB08 could be attempted in pilot scale for eventual utility in pharmaceutical industries.

CONCLUSIONS

In the optimization of bioprocess variables for enzyme production by *Serratia marcescens* SB08, the combination of Plackett-Burman design with central composite design is effective and reliable in selecting the statistically significant factors and finding the optimal concentration of those factors in fermentation medium. The work demonstrates the use of a central composite design by determining the conditions leading to the optimum yield of L-asparaginase production. This methodology could therefore be successfully employed to any process, where an analysis of the effects and interactions of many experimental factors are required. Thus, smaller and less time consuming experimental designs will generally suffice for the optimization of fermentation process.

Acknowledgements

The authors are thankful to Bharathiar University, Coimbatore, Tamil Nadu, India for providing the infrastructure facilities for this study.

References

Abdel-Fattah YR, Saeed HM, Gohar YM, El-Baz MA (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochem*. 40: 1707-1714.

Adamson RH, Fabro S (1968). Antitumor activity and other biologic properties of L-asparaginase. *Cancer Chemothe Rep*. 52: 617-626.

Aguilar G, Morlon-Guyot J, Trejo-Aguilar B, Guyot JP (2000). Purification and characterization of an extra cellular alpha amylase produced by *Lactobacillus manihotivorans* LMG 1801 (T), an amylolytique lactic acid bacterium. *Enzyme Microb Technol*. 27: 406-413.

Azambuja P, Feder D, Garcia ES (2004). Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. *Exp Parasitol*. 107: 89-96.

Box GEP, Hunter JS (1957). Multi-factor experimental designs for exploring response surfaces. *Ann Math Stat*. 28: 195- 241.

Box GEP, Wilson KB (1951). On the experimetal designs for exploring response surfaces. *Ann Math Stat*. 13: 1-45.

Buchanan RE, Gibbons NE (1974). Bergey's manual of determinative bacteriology. (Eighth edition), The Williams and Wilkins Co., Baltimore. PP. 747-842.

Doull JL, Vining LC (1989). Culture conditions promoting dispersed growth and biphasic production of actinorhodin in shaken cultures of *Streptomyces coelicolor* A3(2). *FEMS Microbiol Lett*. 65: 265-268.

Kim HO, Lim JM, Joo JH, Kim SW, Hwang HJ, Choi JW, Yun JW (2005). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresour Technol*. 96: 1175-1182.

Le Mense EH, Julian C, Van Lanen JM, Langlykke AF (1947). Production of mold amylases in submerged culture. *Biochemistry*. 54: 149-159.

Lee KM, Gilmore DF (2005). Formulation and process modeling of biopolymer (polyhydroxyalkanoates: PHAs) production from industrial wastes by novel crossed experimental design. *Process Biochem*. 40: 229-246.

Manna S, Sinha A, Sadhukhan R, Chakrabarty SL (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. *Curr Microbiol*. 30: 291-298.

Mctigue MA, Kelly CT, Fogarty WM, Doyle EM (1994). Production studies on the alkaline amylases of three alkalophilic *Bacillus* spp. *Biotechnology Lett*. 16: 569-574.

Nawani NN, Kapadnis BP (2005). Optimization of chitinase production using statistics based experimental designs. *Process Biochem*. 40: 651-660.

Oettgen HF, Old LJ, Boyae EA, Campbell HA, Philips FS, Clarkson, BD, Tallal L, Leper RD, Schwartz MK, Kim JH (1967). Inhibition of leukemias in man by L-asparaginase. *Cancer Res*. 27: 2619-2631.

Plackett RL, Burman JP (1946). The design of optimum multifactorial experiments. *Biometrika* 33: 305-325.

Ramesh MV, Lonsane BK (1987). A novel bacterial thermostable alpha amylase system produced under solid-state fermentation. *Biotechnol Lett*. 9: 501-504.

Ray RR (2001). Production of alpha-amylase xylanase by an alkalophilic strain of *Penicillium griseoroseum* RR-99. *Acta Microbiol Pol*. 50: 305-309.

Santamaria RI, Del Rio G, Saab G, Rodriguez ME, Soberon X, Lopez-Marguia A (1999). Alcoholysis reactions from starch with alpha-amylases. *FEBS Lett*. 452: 346-350.

Senthilkumar SR, Ashokkumar B, Raj K, Chandra, Gunasekaran P (2005). Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. *Bioresour Technol*. 96: 1380-1386.

Swain AI, Jaskolski M, Housset D, Mohana Rao JK, Wlodawer A (1993). Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. *Proc Natl Acad Sci USA*. 90: 1474-1478.

Tani S, Kawaguchi T, Kato M, Kobayashi T, Tsukagoshi N (2000). A novel nuclear factor, SREB, binds to a cis-acting element, SER, required for inducible expression of the *Aspergillus oryzae* taka-amylase A gene in *A. nidulans*. *Mol General Gene*. 263: 232-238.

Umesh K, Shamsher S, Wamik A (2007). Pharmacological and clinical evaluation of L-asparaginase in the treatment of Leukemia. *Crit rev oncol hematol*. 61: 208-221.

Wang YX, Lu ZX (2005). Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by *Boletus* spp. ACCC 50328. *Process Biochem*. 40: 1043-1051.

Wriston JC, Yellin TO (1973). L-asparaginase: a review. *Adv Enzymol*. 39: 185-248.