Research Journal of Mathematics and Statistics 1(2): 55-58, 2009
ISSN: 2040-7505
© Maxwell Scientific Organization, 2009
Submitted Date: September 02, 2009 Accepted Date: September 14, 2009 Published Date: November 16, 2009

Evaluation and Screening of Nitrogen Source for L-asparaginase Production by *Aspergillus terreus* MTCC 1782 using Latin Square Design

[1]G. Baskar and [2]S. Renganathan
[1]Department of Biotechnology, St.Joseph's College of Engineering, Chennai - 600119. India
[2]Department of Chemical Engineering, Alagappa College of Technology, Anna University Chennai, Chennai - 600025. India

Abstract: The manufacture of enzymes, like L-asparaginase with less adverse effects in the treatment of cancer is an important facet of pharmaceutical industry. Yeast and Filamentous fungi are commonly reported in scientific literature to produce L-asparaginase with less adverse effects than prokaryotic microorganisms. In the present study Latin square design was used to examine the effect of L-proline, L-glutamic acid and Sodium nitrate as nitrogen sources on production of extracellular L-asparaginase by *Aspergillus terreus* MTCC 1782 in order to obtain the best nitrogen source in shake culture fermentation. The experimental values of L-asparaginase activity were subjected to statistical analysis and statistical significance of the nitrogen sources was studied for the Latin-Square Design using *Dataplot software*. It was found that L-proline as a best nitrogen source for the production of L-asparaginase by *Aspergillus terreus* MTCC 1782 for maximum L-asparaginase activity.

Key words: Bioprocess, latin square design, optimization and shake culture fermentation

INTRODUCTION

The manufacture of an enzyme for use as a drug is an important facet of today's pharmaceutical industry. L-asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) is an anti-neoplastic agent (Wriston and Yellin, 1973), used in the *lymphoblastic leukaemia* chemotherapy. Neoplastic cells cannot synthesize L-asparagine due to the absence of L-asparagine synthetase. For this reason the commonest therapeutic practice is to inject intravenously free enzyme in order to decrease the blood concentration of L-asparagine affecting selectively the neoplastic cells (Mitchell *et al*., 1994). L-asparaginase belongs to an amidase group that produces aspartic acid and ammonia by L-asparagine hydrolysis. Although Clementi in 1922 had reported its presence in guinea-pig serum, the anti-tumour properties of the enzyme were only recognized some time later (Wriston and Yellin, 1973). 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 (Pritsa *et al*., 2001). Commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston showed that L-asparaginase from *E. coli* inhibits tumors in mice.

Various bacteria, such as, *Erwinia carotovora, Thermus thermophilus* (Pritsa *et al*., 2001). *Thermus aquaticus* (Curran *et al*., 1985), *Vibrio* succinogenes (David *et al*., 1974), *Citrobacter freundi* (Davidson *et al*., 1977), *Streptomyces griseus* (Dejong, 1972), *Escherichia coli* (Howard and James, 1968), *Erwinia aroideae* (Peterson and Ciegler, 1969), *Proteus vulgeris* (Tosa *et al*., 1971), *Enterobacter aerogenes* (Mukherjee *et al*., 2002), *Zymomonas mobilis* (Pinheiro *et al*., 2001), *Bacillus licheniformis* (Golden *et al*., 1985), *Pseudomonas aeruginosa* (Abdel-Fattah *et al*., 2002) have been found to produce L-asparaginase. However, L-asparaginase from bacterial origin can cause hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis. The search for other L-asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms like yeast and filamentous fungi have a potential for L-asparaginase production (Sarquis *et al*., 2004).

Recombinant L-asparaginase of *Aspergillus niger* and *Aspergillus oryzae* used in processing of starchy food products. It converts the amino acid asparagine to aspartic acid then reduces acrylamide formation during processing of high starch food products (Pedreschi *et al*., 2008). The demand for this enzyme is expected to increase several fold in coming years due to its potential industrial application as food processing aid besides clinical applications. Our preliminary study reported that among the filamentous fungal strains studied, *Aspergillus terreus* was found to be a potential microbial source for L-asparaginase production.

Screening of the best carbon and nitrogen sources and optimization of nutritional requirements and operating

Corresponding Author: S. Renganathan, Department of Chemical Engineering, Alagappa College of Technology, Anna University Chennai, Chennai - 600025. India

conditions is an important step in any bioprocess development. Statistical experimental design such as Latin Square Design (LSD) minimizes the error in determining the effect of parameters, which allows simultaneous, systematic, and efficient variation of all parameters than the classical method (Box *et al*., 1978; Torbjorn *et al*., 1988; Zheng *et al*., 2008). In addition, the statistical screening and optimization by experimental design enables finding out the best and optimal conditions. In the present work LSD was used to examine the effect of L-proline, L-glutamic acid and Sodium nitrate as nitrogen sources on production of extracellular L-asparaginase by *Aspergillus terreus* MTCC 1782 in shake culture fermentation.

MATERIALS AND METHODS

Fungal Strain and Stock Culture: The filamentous fungus *Aspergillus terreus* MTCC 1782 was obtained from Institute of Microbial Technology, Chandigarh, India. The spores and mycelial fragments of *Aspergillus terreus* MTCC 1782 strain was cultivated in Czapek agar slants at 37ºC for 4 days. Czapek agar slants were prepared using the Czapek-Dox medium contains the following ingredients in % (w/v). Solution A: L-asparagine, 1.0; Sodium Nitrate 4.0; Potassium Chloride 1.0; $MgSO_4.7H_2O$, 0.052; $FeSO_4.7H_2O$, 0.02; dissolved in distilled water and stored in refrigerator. Solution B: K_2HPO_4 2.0; dissolved in distilled water and stored in refrigerator. Solution C: 1 gm of $ZnSO_4$. 7 H_2O dissolved in distilled water. Solution D: 0.5 gm of $CuSO_4$. H_2O; dissolved in distilled water. For one litre of Czapek-Dox medium; contains 50 mL of solution A, 50 mL of solution B, 1 mL of solution C and solution D, 900 mL of distilled water, 30 gm of glucose and 20 gm of agar.

Production and Isolation of Crude Enzyme: The spore and mycelial fragments of strains was cultivated from the stock culture in modified Czapek-Dox medium used for stock culture without agar at 32ºC, 160 rpm and pH 6.2 for 7 days. 1 mL of spore suspension was transferred to Erlenmeyer flasks with 100 ml of liquid Czapek-Dox modified with different nitrogen sources (Sodium nitrate, L-proline and L-glutaminc acid) based on LSD experiment run order as given in Table 1 and submitted to an orbital shaker at 160 rpm, 30ºC and pH 6.2 for 48 h. Then culture suspension was filtered on Whattman 2 filter paper and cell-free filtrate was used as crude enzyme solution to estimate L-asparaginase activity (Wriston and Yellin, 1973).

Assay of L-asparaginase Activity: L-asparaginase activity was assayed by Nesslarization, a most common method for estimating L-asparaginase activity. L-asparaginase activity (production level) was estimated by quantifying ammonia formation by Nesslerisation using spectrophotometric analysis at 480 nm (Wriston and Yellin, 1973).

Comparison of Nitrogen Sources by Latin Square Design: Conventional methods of medium optimization are time consuming, expensive and inaccurate, when interactions between different variables are present. Therefore, statistical experimental designs were used to screen and optimize the carbon, nitrogen sources and other medium and operating conditions (Box *et al*., 1978; Torbjorn *et al*., 1988; Zheng *et al*., 2008). In particular the LSD was used to compare the effect of different carbon and nitrogen sources to find best carbon and nitrogen sources. Four level LSD for three factors at 4 levels (Table 1) were developed using *Dataplot software*. In the present research three nitrogen sources such as L-proline, Sodium nitrate and L-glutamic acid were explored each at four levels in both coded and actual units for comparing their effect on L-asparaginase production by *Aspergillus terreus* MTCC 1782 in shake culture fermentation. The block plot was developed to assess the statistical significance of the effect of factor of interest (L-proline) on L-asparaginase production for all combinations of other secondary (nuisance) factors.

RESULTS AND DISCUSSION

L-asparaginase production level was estimated in L-asparaginase activity for all the 16 experiments developed by LSD. The experimental L-asparaginase activity was used to study the effect and screen and evaluate the nitrogen sources for the production of L-asparaginase by *Aspergillus terreus* MTCC 1782 using Data plot software. Table 2 gives for each level of each factor, the mean for the L-asparaginase activity, the effects of nitrogen sources, the standard deviation of the factor effect and the residual standard deviation. The smaller the residual standard deviation, the more we have accounted for the variance in the data. There was no effect on L-asparaginase production (effect = 0) due to changes in L-glutamic acid level. L-proline and Sodium nitrate gives lesser effect than mean at their lower levels (effect < 0) and increased effect at higher levels (effect > 0) with no standard deviation among the levels. Among the variables at their tested levels L-proline gives maximum L-asparaginase activity of 14.5 IU/ml at 2.25% (w/v).

The Dex Mean plot (Fig. 1) shows mean values for the four levels of each factor. A straight line connects the means for a single factor. In summary, factor X_2 (L-proline) was the clearly important, factors X_1 (L-glutamic acid) and X_3 (Sodium nitrate) are borderline factors whose inclusion in any subsequent models will be determined by further analyses. In block plots the 16 distinct positions along the horizontal axis correspond to all possible combinations of the nuisance factors. The block plot in Fig. 2 reveals that 9 of the 16 experiments give higher L-asparaginase production when L-proline was used as primary nitrogen source at its higher level than other nuisance factors at any level, therefore L-proline is highly significance than than L-glutamic acid and sodium nitrate. From a binomial point of view,L-proline was statistically significant as primary nitrogen source for the production of L-asparaginase by *Aspergillus terreus* MTCC 1782.

Table 1: Latin-Square Design for three variables at 4 levels and experimental L-asparaginase activity

Runorder	L-Glutamic acid (X_1)		L-Proline (X_2)		Sodium nitrate (X_3)		L-asparaginaseactivity (IU/ml)
	Level	Actual value % (w/v)	Level	Actual value % (w/v)	Level	Actual value % (w/v)	
1	1	0.00	1	0.00	1	0	17.653
2	2	0.10	1	0.00	2	1	13.493
3	4	0.30	1	0.00	3	2	11.893
4	3	0.20	1	0.00	4	3	10.453
5	4	0.30	2	0.75	1	0	15.413
6	3	0.20	2	0.75	2	1	24.586
7	1	0.00	2	0.75	3	2	27.413
8	2	0.10	2	0.75	4	3	11.413
9	2	0.10	3	1.75	1	0	1.493
10	4	0.30	3	1.75	2	1	31.413
11	3	0.20	3	1.75	3	2	18.453
12	1	0.00	3	1.75	4	3	42.133
13	3	0.20	4	2.25	1	0	42.240
14	1	0.00	4	2.25	2	1	36.926
15	2	0.10	4	2.25	3	2	65.506
16	4	0.30	4	2.25	4	3	39.626

Table 2: Effect of different nitrogen source on L-asparaginase activity

Factor	Level	Mean	Effect	Standard deviation
	1	8.5	0	0
L-glutamic	2	8.5	0	0
acid	3	8.5	0	0
	4	8.5	0	0
L-proline	1	2.5	-6	0
	2	6.5	-2	0
	3	10.5	2	0
	4	14.15	6	0
Sodium nitrate	1	7	-1.5	0
	2	8	-0.5	0
	3	9	0.5	0
	4	10	1.5	0

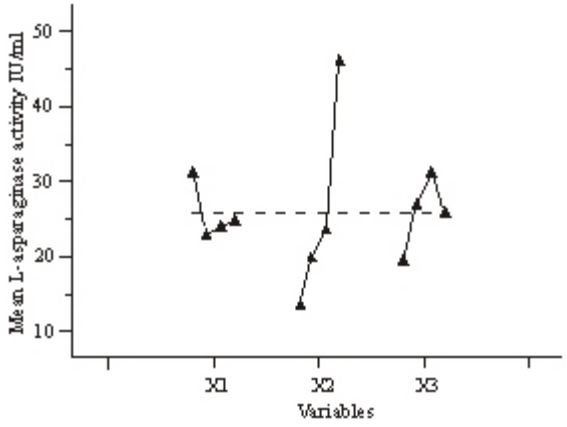

Fig.1: Effect of nitrogen sources on L-asparaginase production in Dex Mean Plot

Fig. 3 was plotted between L-asparaginase production level as L-asparaginase activity and factors (L-glutamic acid – X_1, L-proline – X_2 and Sodium nitrate – X_3) for the identification of the important factors. For each of the factors, as we go from the "-" setting to the "+" setting within the factor, the location shift in the body of the data determines the most important factor. In Fig. 3, there are three factors and each factor has four levels.For each factor, we define a distinct x coordinate for each level of the factor. The y coordinate is simply the

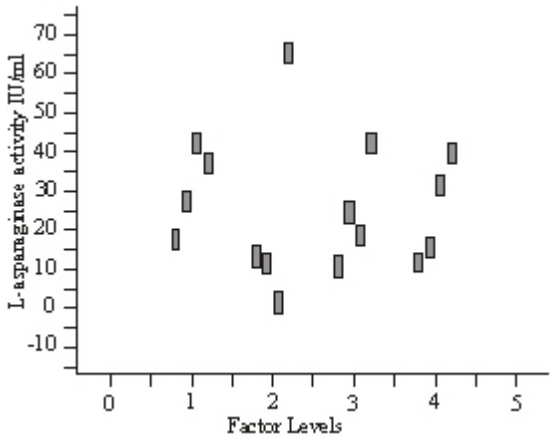

Fig.2: Effect of L-proline as primary nitrogen source on L-asparaginase production in Block Plot

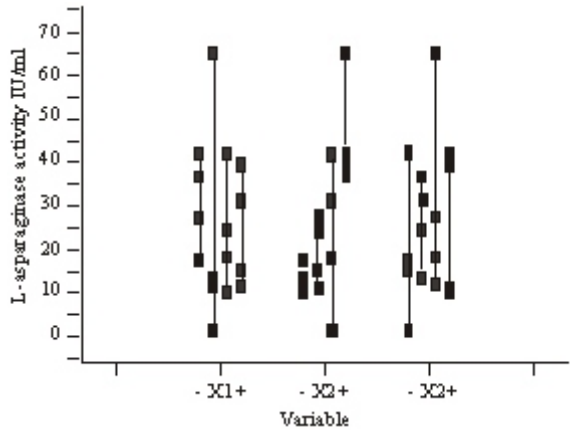

Fig.3: Main effects of nitrogen sources on L-asparaginase production asparaginase production in Scatter Plot

value of the response variable. The solid horizontal line is drawn at the overall mean of the response variable. Thus we can infer L-proline has the biggest location shift and hence the most important nitrogen sources while others

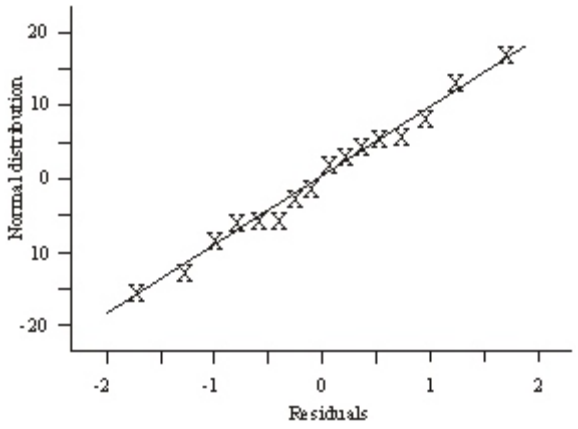

Fig 4: Normal Probability Plot on L-asparaginase production

are of lesser importance. The data was plotted against a theoretical normal distribution (Fig. 4) in such a way that the points should form an approximate straight line. Departures from this straight line indicate departures from normality. From the above Fig. 4, we are able to assess the assumption of a fixed distribution. As the residuals from the fitted model are normally distributed, none of the major assumptions of the model have been violated.

CONCLUSION

Latin Square Design a type of statistical design is very effective in screening of substrates and other media components. There was no interaction between the three different nitrogen sources namely L- glutamic acid, L-proline and Sodium nitrate based on statistical results and various data plots. L-proline was found as significant and best nitrogen source for the production of L-asparaginase by *Aspergillus terreus* MTCC 1782.

Nomenclature:

i = variable number
IU =International unit
LSD =Latin Square Design
MTCC = Microbial Type Culture Collection
SD = Standard deviation
Xi =independent variables
Y =Predicted response
X_1 = Glutamic acid, g/100mL
X_2= L-proline, g/100mL
X_3 =Sodium nitrate, g/100mL
$Y_{Activity}$ =L-asparaginase production level in IU/ml

REFERENCES

Abdel-Fattah, Y.R. and Z.A. Olama, 2002. L-asparaginase production by *Pseudomonas aeruginosa* in Solid-State culture: evaluation and optimization of culture conditions using factorial designs. Process Biochem., 38: 115-122.

Box, G.E.P., W.G. Hunder and S.J. Hunder, 1978. Statistics for Experiments. John Wiley and Sons Inc., New York.

Curran, M.P., R.M. Daniel, G.R. Guy and H.W. Morgan, 1985. A specific L-asparaginase from *Thermus aquaticus*. Arch. Biochem. Biophys., 241: 571-576.

David, K. and G. David, 1974. Asparaginase production by the rumen anaerobe *Vibrio succinogenes*. Appl. Microbiol., 27: 206-209.

Davidson, L., M. Burkom, S. Ahn, L.C. Chang and B. Kitto, 1977. L-Asparagainases from *Citrobacter freundii*. Biochim. Biophys. Acta, 480: 282-294.

Dejong, P.J., 1972. L-asparaginase Production by *Streptomyces griseus*. Appl. Microbiol., 23: 1163-1164.

Golden, K.J. and R.W. Bernlohr, 1985. Nitrogen catabolite repression of the L-asparaginase of *Bacillus licheniformis*. J. Bacteriol., 164: 938-940

Howard, C. and H.S. James, 1968. Production of L-Asparaginase II by *Escherichia coli*. J. Bacteriol., 96: 2043-2048.

Mitchell, I., H. Hoogendoorn and A.R. Giles, 1994. Increased endogenous thrombin generation in children with acute *Lymphoblastic leukemia*: risk of thrombotic complications in L-asparaginase induced antithrombin III deficiency. Blood, 83: 386-391.

Mukherjee, J., S. Majumdar and T. Scheper, 2002. Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. Appl. Microbiol. Biotechnol., 53: 180-184.

Pedreschi, F., K. Kaack and K. Granby, 2008. The effect of Asparaginase on acrylamide formation in French fries. Food Chem., 109: 386-392.

Peterson, R.E. and A. Ciegler, 1969. L-asparaginase production by *Erwinia aroideae*. Appl. Microbiol., 18: 64-67.

Pinheiro, I.O., J.M. Araujo, E.C.P.A. Ximenes and J.C.S. Pinto, 2001. Production of L-asparaginase by *Zymomonas mobilis* strain CP4. Biomater. Diagnostic., 6: 243-244.

Pritsa, A.A., K.T. Papazisis, A.H. Kortsaris, G.D. Geromichalos and D. Kyriakidis, 2001. Antitumor activity of L-asparaginase from *Thermus thermophilus*. Anticancer Drugs, 12: 137-142.

Sarquis, M.I.M., E.M.M. Oliviera, A.S. Santos and G.L. Da-Costa, 2004. Production of L-asparaginase by filamentous Fungi. Mem. Inst. Oswaldo. Cruz., 99(5): 489-492.

Torbjorn, L., S. Elisabeth, A. Lisbeth, T. Bernt, N. Asa, P. Jarle and B. Rolf, 1998. Experimental design and optimization. Chemometr. Intell., Lab. 42: 3-40.

Tosa, T., R. Sano, K. Yamamoto, M. Nakamura and I. Chibata, 1971. L-asparaginase from *Proteus vulgaris*. Appl. Microbiol., 22: 387-392.

Wriston, J.C. and T.O. Yellin, 1973. L-asparaginase. A review. Adv. Enz., 39: 185-248.

Zheng, Z.M., Q.L. Hu, J. Hao, F. Xu, N.N. Guo, Y. Sun and D.H. Liu, 2008. Statistical optimization of culture conditions for 1,3-propanediol by *Klebsiella pneumoniae* AC 15 via central composite design. Bioresource Technol., 99: 1052-1056.