Statistical Screening of Supplementary Nitrogen Source for Enhanced Production of L-Asparaginase by *Aspergillus terreus* 1782

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In the present work, Latin Square Design (LSD) was applied to find the best supplementary nitrogen source for extracellular L-asparaginase production by *Aspergillus terreus* MTCC 1782 using corn flour as substrate in submerged fermentation. The independent effect of supplementary nitrogen source such as ammonium chloride, urea and sodium nitrate was studied on growth and production of L-asparaginase by *A. terreus*. Statistical significance of the nitrogen sources was studied by performing analysis of variance (ANOVA) and graphical ANOVA for LSD using Data plot software. It was found that there is no significant difference on growth due to the change in supplementary nitrogen source studied. Urea was identified as best supplementary nitrogen source with mean L-asparaginase production of 33.25 IU mL^{-1} and mean biomass production of 12.99 mg mL^{-1}.

Key words:
L-Asparaginase activity, submerged fermentation, latin square design, supplementary nitrogen source

Introduction

The production of 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) has been used as an anti-tumor agent for the effective treatment of acute *lymphoblastic leukemia* and *lymphosarcoma*. It destroys asparagine external to the cell by hydrolysis into aspartic acid and ammonia.[1] Hence, normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. It is also known as Elspar, Kidrolase, Leunase, Colaspase and Crasnitin.[2]

In 1922, Clementi reported the presence of L-asparaginase in guinea-pig serum.[3] 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.[4,5] The importance of microorganisms as L-asparaginase sources has been focused and commercial production of L-asparaginase appeared desirable only after the L-asparaginase from *E. coli* and its antineoplastic activity was demonstrated in guinea-pig serum.[6,7] The production of L-asparaginase has been studied in various bacterial sources using different carbon and nitrogen sources.[8] However, L-asparaginase from bacterial sources causes hypersensitivity in the long-term, leading to allergic reactions and anaphylaxis.[9] 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. Yeast sources such as *Rhodotorula sp.,*[10] *Rhodosporidium toruloides,*[11] actinomycetes such as *Nocardia* sp.,[12] *Streptomyces longsporusflavus,*[13] 1998) and Fungal sources such as *Aspergillus tamari* and *Aspergillus terreus,*[9] *Aspergillus nidulans*[14] using different synthetic substrates and *Aspergillus niger*[15] using agro-wastes from three leguminous crops have been found to produce L-asparaginase.

Novozymes A/S Denmark (submitted by Novozymes Australia Pty Ltd) is now seeking an approval for recombinant L-asparaginases of *A. oryzae* and *A. niger* as processing aid in the food industry to reduce the formation of acrylamide.[16,17] Acrylamide is formed as a reaction product between asparagine and reducing sugars contained in starchy foods when heated above 120 °C during baking or frying. Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended reducing the concentration of acrylamide for its carcinogenicity and neurotoxicity. L-asparaginase cleaves the substrate and ultimately reduces the levels of acrylamide concentration in foods.[18,19]

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The demand for L-asparaginase will increase several fold in the coming years due to its potential application in food processing in addition to its clinical applications. The optimization of nutritional requirements and operating conditions is an important step in any bioprocess development. Statistical experimental designs have been used in several steps of optimization strategy and it is better acknowledged than the traditional one variable at a time method.[20,21] Statistical experimental design such as LSD minimizes the error in determining the effect of parameters, which allows simultaneous, systematic, and efficient variation of all parameters than the classical method. It was first used in agricultural research to adjust fertility differences in two physical directions.[22] L-Asparaginase production is under nitrogen regulation, therefore it requires supplementary nitrogen source in addition to the substrate. In the present work, LSD was used to evaluate and compare the effect of different supplementary nitrogen source, and find the single most supplementary nitrogen sources for production of extracellular L-asparaginase by *A. terreus* MTCC 1782 using corn flour as substrate. Urea was identified as the best supplementary nitrogen source for maximum L-asparaginase production.

Materials and methods

Fungal strain and inoculum culture

The filamentous fungus *A. terreus* MTCC 1782 was obtained from Institute of Microbial Technology, Chandigarh, India. The stock cultures were maintained in Czapek agar slants at 37 °C for 4 days, stored at 4 °C and monthly sub-cultured. Inoculum was grown in modified Czapek-Dox medium (standard Czapek-Dox medium with 1 % L-asparagine) containing the following ingredients in grams. Solution A: L-asparagine, 1.0; $NaNO_3$, 4.0; KCl, 1.0; $MgSO_4 \cdot 7\ H_2O$, 0.052; $FeSO_4 \cdot 7\ H_2O$, 0.02; were dissolved in 100 mL of distilled water and refrigerated. Solution B: K_2HPO_4 2.0; was dissolved in 100 mL of distilled water and refrigerated. Solution C: $ZnSO_4 \cdot 7\ H_2O$ 1.0; was dissolved in 100 mL of distilled water. Solution D: 0.5 g of $CuSO_4 \cdot H_2O$; was dissolved in 100 mL of distilled water. One litre of modified 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 g of glucose and 20 g of agar. The inoculum culture slants were incubated at 37 °C for 4 days.

Production and isolation of crude enzyme

Conidial suspension of the inoculum culture was prepared with 10^7 to 10^8 conidia per mL. 100 mL of liquid modified Czapek-Dox medium with w = 1 % L-asparagine, 1.5 % corn flour, 0.2 % glucose, 0.152 % K_2HPO_4, 0.052 % KCl, 0.052 % $MgSO_4 \cdot 7\ H_2O$, traces of $ZnSO_4 \cdot 7\ H_2O$ and $FeSO_4 \cdot 7\ H_2O$ and supplemented with different synthetic nitrogen sources (sodium nitrate, urea and ammonium chloride) based on LSD given in Table 1, initial pH was adjusted to 6.2 in 250 mL Erlenmeyer flask. The flasks were inoculated with 1 mL of conidial suspension and initial pH was adjusted to 6.2, incubated at 30 °C at 160 rpm for 72 h. Culture suspensions were filtered using Whatman-2 filter paper and cell-free filtrates were used as crude enzyme solution for estimation of L-asparaginase activity.

Table 1 – *Latin square experimental design with L-asparaginase and biomass production*

Std. run	Variables in actual unit, γ/mg mL^{-1}			L-Asparaginase activity/IU mL^{-1}	Biomass production, γ/mg mL^{-1}
	X_1	X_2	X_3		
1	0.0	0.0	0.0	7.83	11.00
2	0.3	0.0	0.4	12.26	10.29
3	0.6	0.0	0.8	21.72	10.00
4	0.9	0.0	1.2	16.69	12.01
5	1.2	0.0	1.6	11.54	19.41
6	0.6	0.4	0.0	9.59	16.17
7	0.9	0.4	0.4	14.02	18.06
8	1.2	0.4	0.8	13.27	18.27
9	0.0	0.4	1.2	15.03	16.04
10	0.3	0.4	1.6	25.16	14.57
11	1.2	0.8	0.0	13.49	6.24
12	0.0	0.8	0.4	40.15	12.40
13	0.3	0.8	0.8	37.48	16.45
14	0.6	0.8	1.2	27.62	17.89
15	0.9	0.8	1.6	20.90	10.21
16	0.3	1.2	0.0	8.79	9.71
17	0.6	1.2	0.4	8.26	15.94
18	0.9	1.2	0.8	18.55	7.86
19	1.2	1.2	1.2	16.37	9.54
20	0.0	1.2	1.6	24.20	16.37
21	0.9	1.6	0.0	33.43	17.42
22	1.2	1.6	0.4	30.12	24.56
23	0.0	1.6	0.8	21.27	4.75
24	0.3	1.6	1.2	55.88	6.06
25	0.6	1.6	1.6	25.54	8.68

Assay of L-asparaginase activity

Enzyme activity of the culture filtrates was determined at the end of cultivation time by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent. 0.1 mL of enzyme solution, 0.9 mL of 0.1 mol L^{-1} sodium borate buffer (pH 8.5), and 1 mL of 0.04 mol L^{-1} L-asparagine solution were taken and incubated for 10 min at 37 °C. The reaction was stopped by the addition of 0.5 mL of w = 15 % trichloroacetic acid. L-Asparaginase activity (production level) was estimated by quantifying the moles of ammonia formed using Nessler's Reagent and spectrophotometer analysis at 480 nm.[6]

Comparison of supplementary nitrogen sources by Latin Square Design

Design of Experiment (DOE) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. LSD defines the effect of the independent variables on one or more responses of a food, chemical or biological process. In addition, LSD generates a mathematical model that accurately describes the overall process. In particular, LSD is used to find the best source by evaluating and comparing the effect of different carbon or nitrogen source. The linear model shows the effect of independent variables on response which is given by eq. (1). The residual standard deviation reflects the effect of variables; the smaller the residual standard deviation of a variable, the greater the effect on response.

$$Y = \mu + RX_1 + CX_2 + TX_3 \qquad (1)$$

Where Y denotes any observation for which, X_1 and X_2 are blocking factors and X_3 is the primary factor. μ denotes the general location parameter, R denotes the residual standard deviation for X_1, C denotes the residual standard deviation for X_2 and T denotes the residual standard deviation for X_3.[21,22]

In the present investigation, three supplementary nitrogen sources such as urea, sodium nitrate and ammonium chloride were explored each at five levels to study their independent effect on L-asparaginase production by *A. terreus* MTCC 1782 in shake culture fermentation. The LSD for three factors at 5-level (Table 1) was developed using Data plot software and the experiments were conducted for L-asparaginase production as described in production and extraction step. The analysis of variance (ANOVA) was used to study the independent effect and significance of the factors by grand mean and factor effect.

Results and discussion

The experimental L-asparaginase activity and biomass production for all 25 experiments given in Table 1 was to study the effect of supplementary nitrogen source on production of L-asparaginase production by *A. terreus* MTCC 1782 using corn flour as substrate. The *Data plot* software was used for statistical analysis.

Effect of supplementary nitrogen sources on L-asparaginase and biomass production by *A. terreus* MTCC 1782 using corn flour as substrate

L-Asparaginase activity and biomass production given in Table 1 was subjected to ANOVA, a formal F-test to study the independent effect of urea, ammonium chloride and sodium nitrate on L-asparaginase production. ANOVA in Table 2 shows that urea has a higher effect (confidence level of 98.61 %) on L-asparaginase production than ammonium chloride (confidence level of 61.18 %) and sodium nitrate (confidence level of 60.69 %). ANOVA in Table 2 shows that urea (confidence level of 32.26 %) has higher effect than ammonium chloride (confidence level of 31.51 %) and sodium nitrate (confidence level of 20.13 %) on biomass production by *A. terreus* at their different concentration.

Tables 3 and 4 show the independent effect of supplementary nitrogen source at different concentration, mean and residual standard deviation for L-asparaginase and biomass production. Sodium nitrate and ammonium chloride decreases the

Table 2 – *ANOVA on LSD for L-asparaginase and biomass production*

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	Confidence level/%
L-asparaginase production					
X_1	4	353.856	88.214	1.118	60.69
X_2	4	1554.622	388.656	4.925	98.61
X_3	4	356.616	89.154	1.129	61.18
residual	12	946.956	87.913		
total	24	3211.049	133.794		
biomass production					
X_1	4	52.203	13.051	0.409	20.13
X_2	4	75.057	18.764	0.588	32.26
X_3	4	73.586	18.396	0.576	31.51
residual	12	382.667	31.888		
total	24	583.514	24.313		

Table 3 – *Effect of supplementary nitrogen sources on L-asparaginase production using corn flour by A. terreus*

Factor	Level	Mean	Effect	SD (effect)
	1	21.69	0.53	3.55
	2	27.91	6.74	3.55
sodium nitrate	3	10.55	–2.62	3.55
	4	20.74	–0.43	3.55
	5	16.96	–4.21	3.55
	1	14.03	–7.14	3.55
	2	15.41	–5.76	3.55
urea	3	27.93	6.76	3.55
	4	15.23	–5.94	3.55
	5	33.25	12.08	3.55
	1	14.63	–6.54	3.55
	2	20.96	–0.21	3.55
ammonium chloride	3	22.46	1.29	3.55
	4	26.04	5.17	3.55
	5	21.47	0.30	3.55

Table 4 – *Effect of supplementary nitrogen source on biomass production using corn flour by A. terreus*

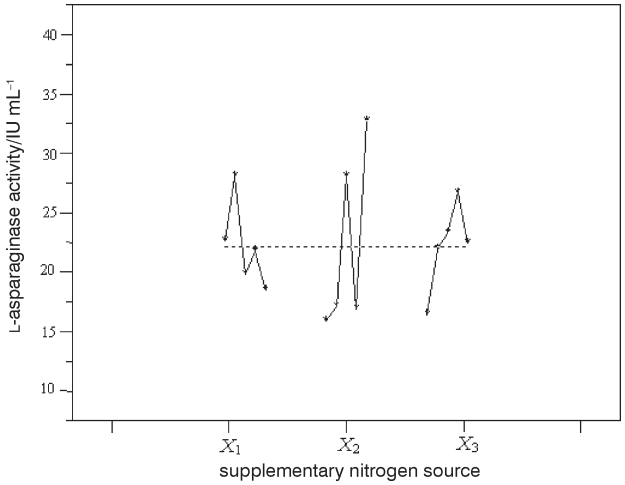
Factor	Level	Mean	Effect	SD (effect)
	1	12.11	–1.08	2.25
	2	11.41	–1.78	2.25
sodium nitrate	3	13.73	0.54	2.25
	4	13.11	–0.08	2.25
	5	15.60	2.41	2.25
	1	12.54	–0.65	2.25
	2	16.62	3.42	2.25
urea	3	12.63	–0.55	2.25
	4	11.88	–1.31	2.25
	5	12.29	–0.90	2.25
	1	12.10	–1.08	2.25
	2	16.25	3.05	2.25
ammonium chloride	3	11.46	–1.73	2.25
	4	12.30	–0.88	2.25
	5	13.84	0.652	2.25

L-asparaginase production at their low and high concentrations, and gives increased L-asparaginase activity (27.91 and 26.04 IU mL^{-1} respectively) at 0.3 % and 1.2 % respectively. Urea decreases the L-asparaginase production (22.01 IU mL^{-1}) at its low concentration; it was increased to 33.25 IU mL^{-1} at 1.6 %. The L-asparaginase activity of 27.91 and 26.04 IU mL^{-1} was obtained when modified Czapek-Dox medium was supplemented with sodium nitrate and ammonium chloride respectively, which was low when urea (33.25 IU mL^{-1}) was used as supplementary nitrogen source. Urea and ammonium chloride increased (16.62 and 16.25 mg mL^{-1}) the biomass production at their middle concentration (effect > 0) and decreased (11.88 and 11.46 mg mL^{-1} respectively) at their low and high concentration (effect < 0) with standard deviation between the supplementary nitrogen source and their levels. Sodium nitrate decreased the biomass production to its low concentration (16.62 mg mL^{-1}). Hence, among the supplementary nitrogen sources studied, the effect was very low on biomass production and there was no significant difference due to the type of nitrogen source used. Among the supplementary nitrogen source studied at different concentrations, modified Czapek-Dox medium with corn flour and supplemented with urea (0.4 %) gives maximum mean biomass production of 12.29 mg mL^{-1} with the maximum mean L-asparaginase production of 33.25 IU mL^{-1} (urea 1.2 %). The L-asparaginase production was found to be increased after applying LSD for screening of supplementary nitrogen source and comparatively higher than the reported activity of 16.2 IU mL^{-1} for *S. gulbargensis* using ground nut cake extract as the sole nitrogen source and 19.5 U mL^{-1} for isolated fungus using ammonium sulphate as an additional nitrogen source.[23,24] It is concluded that the increase in concentration of urea increases the L-asparaginase production.

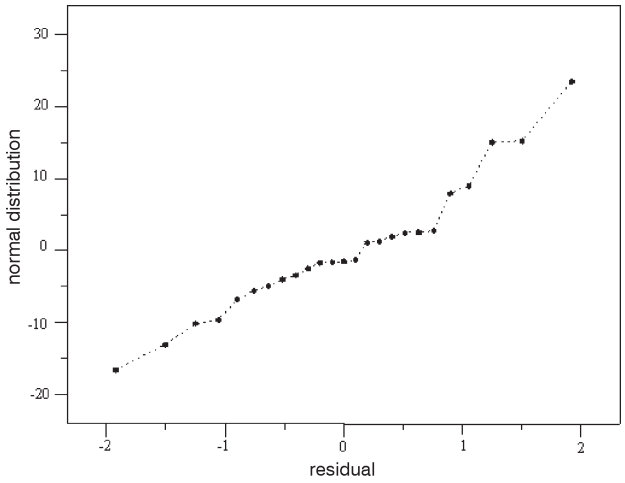
The residual standard deviation (RSD) on supplementary nitrogen sources as model parameters on L-asparaginase activity and biomass production is given in Table 5. Since urea was found to be the best supplementary nitrogen source, the linear model given in eq. (1) was reduced to eqs. (2) and

Table 5 – *RSD of model parameters nitrogen sources on L-asparaginase and biomass production*

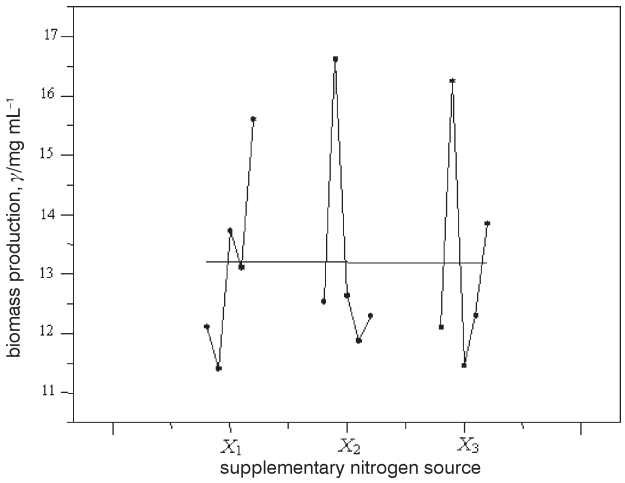
Model parameter	RSD	
	L-asparaginase activity/$Y_{activity}$	Biomass production/$Y_{biomass}$
constant/μ	11.57	4.93
sodium nitrate/X_1	11.95	5.15
urea/X_2	9.11	5.04
ammonium chloride/X_3	11.95	5.05
constant and all factors	8.88	5.64



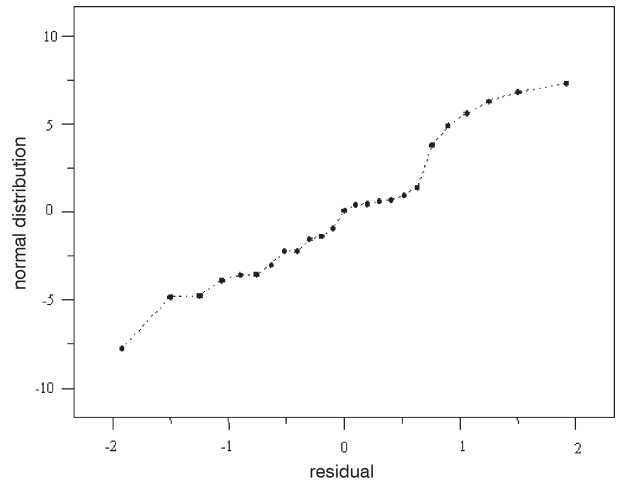
F i g . 1 – *Effect of supplementary nitrogen sources on* L*-asparaginase production using corn flour by A. terreus*



F i g . 3 – *Normal probability plot for* L*-asparaginase production from corn flour by A. terreus*



F i g . 2 – *Effect of supplementary nitrogen sources on biomass formation using corn flour by A. terreus*



F i g . 4 – *Normal probability plot for biomass formation from corn flour by A. terreus*

(3), which represent the independent effect urea (X_2) on dependent variables L-asparaginase activity ($Y_{activity}$) and biomass ($Y_{biomass}$) respectively.

$$Y_{activity} = 11.57 + 9.11 X_2 \quad (2)$$

$$Y_{biomass} = 4.931 + 5.04 X_2 \quad (3)$$

The RSD for urea (X_2) was low on L-asparaginase activity (11.77 IU mL^{-1}) and biomass (5.04 mg mL^{-1}). The L-asparaginase activity of 26.15 IU mL^{-1} was predicted using eq. (2) for high level of urea (1.6 %) and it was closer to the mean L-asparaginase activity (33.25 IU mL^{-1}at high level of urea) reported in Table 3. The biomass production of 12.99 mg mL^{-1} was predicted using eq. (3) for low level of urea (1.6 %) and it deviated more from the mean biomass (16.62 IU mL^{-1} at high level of urea) reported in Table 3. Hence, these models validate that urea is the best supplementary nitrogen source for maximum L-asparaginase production. It was revealed from these results that the L-asparaginase production is independent of biomass production.

Dex-mean plot of graphical ANOVA was used to evaluate the effect of supplementary nitrogen sources on L-asparaginase and biomass production by *A. terreus* MTCC 1782. Figs. 1 and 2 show the effect of supplementary nitrogen sources (sodium nitrate – X_1, urea – X_2, ammonium chloride – X_3) as independent variables on L-asparaginase production and biomass production. The maximum L-asparaginase and biomass production is observed in Figs. 1 and 2 respectively when urea (X_2) is used as the supplementary nitrogen source.

It is confirmed with the results of ANOVA, that urea is the best supplementary nitrogen source for maximum L-asparaginase production by *A. terreus* MTCC 1782 using corn flour substrate. The normal probability plot was generated for the residuals from the fitted model for L-asparaginase activity

and biomass (Figs. 3 and 4). The residual was plotted against normal distribution and formed an approximate linear line for both L-asparaginase activity and biomass production, indicating that the model was well fitted to the experimental data. As the residuals from the fitted model were normally distributed, all the major assumptions of the model were validated.

Conclusion

It was found that the Latin Square Design is an effective statistical tool to identify the best carbon or nitrogen source for fermentative production of any product. In the present work, the effect of supplementary nitrogen sources such as sodium nitrate, urea and ammonium chloride was studied successfully on growth and production of L-asparaginase by *A. terreus* MTCC 1782 using LSD. There was no significant difference on biomass production among the supplementary nitrogen sources studied. Urea was found to be the best supplementary nitrogen source for maximum mean L-asparaginase production of 33.25 IU mL^{-1} by *A. terreus* MTCC 1782 using corn flour as substrate.

Nomenclature

ANOVA – Analysis of Variance
DF – degree of freedom
i – variable number
IU – international unit
JECFA – Joint FAO/WHO Expert Committee on Food Additives
LSD – Latin Square Design
MS – mean square
MTCC – microbial type culture collection
RSD – residual standard deviation
SD – standard deviation
SS – sum of square
X_i – independent variables
Y – predicted response

List of symbols

F – Fisher function
p – corresponding level of significance
R – residual standard deviation for X_1
C – residual standard deviation for X_2
T – residual standard deviation for X_3
X_1 – sodium nitrate, g/100 mL
X_2 – urea, g/100 mL
X_3 – ammonium chloride, g/100 mL
$Y_{activity}$ – L-asparaginase production level in IU mL^{-1}
$Y_{biomass}$ – biomass concentration in mg mL^{-1}
γ – biomass production, mg mL^{-1}
w – mass fraction, %
μ – constant

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