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Research Article

Statistical optimization of L-asparaginase production by *E. coli* K-12 MTCC 1302

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Abstract

L-Asparaginase (L-asparagine amidohydrolase E.C.3.5.1.1) are the enzymes that catalyze the hydrolysis of L-asparagine to L-asparatic acid and ammonia and are clinically important. Objective of the present work was to optimize fermentation conditions for L-asparaginase production by *E. coli* K-12 (MTCC 1302). The Plackett–Burman experimental design was used to screen selected five independent variables (Lactose, yeast extract, L-asparaginase, $CaCl_2$,and NaCl). The most significant independent variables found to affect enzyme production, namely Lactose, yeast Extract and L-asparagine. These independent variables were further optimized by the central composite design. It was found that optimized L-asparaginase production by *E. coli* K-12 MTCC 1302 was 3.08 U/mL using Central Composite Design.

Keywords: L-Asparaginase; *Escherichia coli* K-12; Plackett-Burman design; Response surface methodology.

Introduction

L-Asparaginase catalyzes the hydrolysis of L-asparagine to L-asparatic acid and ammonia and plays imperative task due as an essential component of chemotherapy combination against acute lymphoblastic leukemia [1] and also used in food industry for the reduction of acrylamide percentage. The action of L-Asparaginase plays a most vital role in the cellular nitrogen metabolism of both, prokaryotes and eukaryotes [2].

Various microorganisms are able to produce commercially important L-asparaginase. But, *Escherichia coli* and *Erwinia chrysanthemi* derived L-asparaginases are presently used as effective drugs for various leukemia diseases due to less toxic side effects [3]. Cytoplasmic L-asparaginase I and periplasmic L-asparaginase II are the two types of enzyme produced by *E. coli.* L-Asparaginase II type has anti-cancer activity [4]. Many studies have been proposed to improve production of L-Asparaginase with increased characteristics [5]. Composition of the medium greatly influences the growth of the microorganism present in the fermentation which consequences in the yield of the product.

Optimization of fermentation conditions is a vital stage in bioprocess. Plackett–Burman design and response surface method are the valuable statistical methods for the optimization of production parameters. Recently, enhanced production of L- Asparaginase by *Streptomyces* species was reported by using combined method of Plackett-Burman design and central composite design. L- Asparaginase production was greatly influenced by fermentation substrate of combined soybean meal and wheat bran, L-Aspargine and K_2HPO_4 [6] and productivity was increased with 3.15 fold. The goal of the present study was to optimize fermentation conditionsfor the production of L-Asparaginase. Factors influencing the production were identified by Plackett– Burman design and optimized levels of significant variables were detected by using statistical approach.

Material and methods

Microorganism

The bacterial strain used throughout the study for the production of L-asparaginase was *E. coli* K-12 (MTCC 1302). It was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The obtained bacterial strain was maintained on Nutrient Agar

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medium slants and stored at 4°C and sub-cultured frequently.

Determination of L-Asparaginase activity agar plate method

E. coli K-12 (MTCC 1302) cultures were spot inoculated on L-asparagine containing minimal agar plates incorporated with phenol red pH indicator. The plates were incubated at 37°C for 48 h. Formation of pink zones around the colonies were tested to confirm the presence of L-asparaginase activity

Estimation of L-asparaginase activity

The presence of L-Asparaginase activity was determined according to method described by Imada et al [7] by using L-asparagine as a substrate and the product, ammonia, released during the catalysis was measured by using Nessler' reagent. The reaction mixture was prepared by adding 0.5 mL of 0.04 M L-Asparagine, 0.5 mL of 0.5 M Phosphate buffer, 0.5 mL of Enzyme solution and 0.5 mL of distilled water and incubated for 30 min at 30 °C. Then the reaction was added with 0.1 mL of 1.5 M tricholoroacetic acid. To 0.1 mL of the above mixtures, 3.7 mL of distilled water and 0.2 mL of Nessler's reagent were added. This mixture was incubated at 20°C for 20 min. The absorbance was recorded at 450 nm. Blank tube was prepared similarly without adding L-asparagine. One unit of enzyme activity was expressed as the amount of enzyme that liberates one micromole of ammonia per minute under optimum conditions. One mole of ammonium sulphate corresponds to two moles of ammonia.

Optimization of L-asparaginase production

Plackett–Burman Design

The important step in the optimization stratagem was to categorize medium components that have a significant effect on L-asparaginase production. The significant variable affecting the production of L-asparaginase was identified by PB design [8]. For screening purpose, various components have been evaluated. A set of 12 experiments were employed using MINITAB 17 software for 5 components: Lactose, Yeast Extract, L-asparagine, $CaCl_2$ and NaCl. Each component was tested at two different concentration levels as represented by Table 1. The experiments were carried out in 250 mL conical flasks containing 100 mL media at 120 rpm and 37°C. The response was measured as L-asparaginase activity.

Table 1. Variable levels for PB Experiment

Medium Components	Symbol	Low level (-1)	High level (+1)
Lactose (g/L)	X_1	0.5	1.5
Yeast Extract (g/L)	X_2	0.5	1.5
L-Asparagine(g/L)	X_3	0.1	0.3
$CaCl_2$(g/L)	X_4	1.0	2.0
NaCl(g/L)	X_5	0.5	1.5

Central Composite Design

Response surface method using Central Composite design is an experimental design, useful for building a second order (quadratic) model for the response, for identifying feasible interactions, higher order effects and to determine the optimum operational conditions [9]. The three important parameters that have been proved to be significant from Plackett-Burman design were chosen as independent variable. Each parameter was studied at three different levels (-1, 0, +1). A matrix of 20 experiments [10] with three factors was generated using the software package MINITAB 17. The maximum L-asparaginase activity was taken as the dependent variable of response. These were the most significant parameters for L-asparaginase production as obtained by the Plankett-Burman method. Each parameter was studied at three different levels (-1, 0, +1). All the parameters were taken at central coded value considered as zero. The minimum and maximum ranges of parameters were investigated. For statistical analysis, a response surface central composite design have been employed. All statistic values and graphics were obtained with Minitab 17 software at a confidence level of 95% (α=0.05).

Table 2. Experimental range and variable levels for CCD Experiment

Medium Components	Symbol	Low level (-1)	Middle (0)	High level (+1)
Lactose (g/L)	X_1	0.5	1.0	1.5
Yeast Extract (g/L)	X_2	0.5	1.0	1.5
L-asparagine (g/L)	X_3	0.1	0.2	0.3

Results and discussion

Change of colour was observed on the plates containing minimal L-asparagine agar medium and thereby *E. coli* K-12 MTCC 1302

confirms the presence of L-Asparaginase activity.

Variables screened by PB Design

The experiment was performed in 13 runs to study the effect of selected variables. Statistical analysis of the responses were performed and represented in table 3. The data obtained from screening experiment by PB design showed a variation of L-asparaginase production. Estimated coefficient and the main effect of each variable were tabulated in table 4. Among the variables of screened, yeast extract was identified as the most significant medium component with the confidence of 98.1% followed by Lactose (97.3%) and L-asparagine (93.6%) on L asparaginase production. Hence it was identified that concentration yeast extract, lactose and L-asparagine as most significant variables for the production of L-asparaginase.

Table 3. Levels of variables, experimental design matrix for PB experiment with observed and predicted response

Exp. No.	X_1	X_2	X_3	X_4	X_5	L-Asparaginase (U/mL)	
						Observed	Predicted
1	0.5	0.5	0.1	1.0	0.5	0.67	0.86
2	1.5	1.5	0.1	2.0	1.5	0.91	0.94
3	1.5	1.5	0.3	1.0	1.5	0.82	0.90
4	1.0	1.0	0.2	1.5	1.0	0.3	0.42
5	0.5	1.5	0.3	1.0	1.5	0.92	0.92
6	0.5	0.5	0.3	2.0	1.5	1.08	0.90
7	1.5	1.5	0.1	2.0	0.5	1.1	0.78
8	1.5	0.5	0.3	2.0	0.5	1.07	1.21
9	1.5	0.5	0.1	1.0	1.5	0.55	0.54
10	0.5	1.5	0.3	2.0	0.5	1.23	0.81
11	0.5	0.5	0.1	2.0	1.5	1.38	1.21
12	1.5	0.5	0.3	2.0	0.5	0.68	1.01
13	0.5	1.5	0.1	1.0	0.5	0.53	0.71

Table 4. Estimated coefficients, calculated t value, p value and confidence level as per PB Experimental design for L-asparaginase production

Components	Symbol	Main effect	Estimated co-efficient	Standard error	t-value	P-value	Confidence level
Lactose (g/L)	X_1	0.31	0.155	0.08763	1.77	0.27	97.3
Yeast extract (g/L)	X_2	0.12	0.06	0.8763	0.18	0.019	98.1
L-Aspargine (g/L)	X_3	0.08	0.04	0.08763	0.46	0.064	93.6
$CaCl_2$ (g/L)	X_4	0.0833	0.04167	0.08763	0.48	0.651	34.9
NaCl (g/L)	X_5	0.2733	0.13667	0.08763	1.56	0.170	83.0

CCD for optimization of L-asparaginase production

Table 5 summarizes central composite design of 20 runs with 3 axial points. Table 6 shows the estimated regression coefficients from the quadratic model. Factors considered were X_1: Lactose, X_2: yeast extract, X_3 : L-asparagine. The terms involving concentration of lactose and yeast extract were statistically significant ($p<0.05$), and it can be concluded that the identified respective concentration range were adequate to produce high response. But the term yeast extract (X_2) was not statistically significant; however it is not possible since there was no run without nitrogen source. Curvature in the response surface may result in very low p-values for the interaction terms. The regression coefficients were calculated and the data was fitted to a second order poly nominal equation. The response, L-asparaginase production (Y) can be expressed by the equation (1).

Table 5. Central composite design with observed and predicted L-asparaginase production

Run order	X_1	X_2	X_3	L-asparaginase activity (U/mL)	
				Response	Predicted
1	0.5	0.5	0.1	1.03	1.10
2	1.5	0.5	0.1	1.21	1.15
3	0.5	1.5	0.1	1.27	1.07
4	1.5	1.5	0.1	1.30	1.32
5	0.5	0.5	0.3	1.57	1.50
6	1.5	0.5	0.3	1.24	1.39
7	0.5	1.5	0.3	1.20	1.21
8	1.5	1.5	0.3	1.40	1.29
9	0.5	1.0	0.2	1.54	1.70
10	1.5	1.0	0.2	1.79	1.77
11	1.0	0.5	0.2	2.98	2.86
12	1.0	1.5	0.2	2.54	2.80
13	1.0	1.0	0.1	2.42	2.57
14	1.0	1.0	0.3	2.76	2.75
15	1.0	1.0	0.2	3.08	2.99
16	1.0	1.0	0.2	3.03	2.99
17	1.0	1.0	0.2	3.06	2.99
18	1.0	1.0	0.2	2.99	2.99
19	1.0	1.0	0.2	3.04	2.99
20	1.0	1.0	0.2	3.05	2.99

$$Y = -4.379 + 10.053X_1 + 1.263\ X_2 + 16.176\ X_3 - 5.003\ X_1^2 - 0.623\ X_2^2 - 32.591\ X_3^2 + 0.19\ X_1X_2 - 0.85\ X_1X_3 - 1.35\ X_2X_3 \quad \ldots..(1)$$

From ANOVA table 7, the P value for the model value indicates that the experimental data was a good fit with the model.

Table 6. Estimated regression coefficients for L-asparaginase production

Terms	Constant	Co-efficient	p-value	Significance
Constant	β_0	-4.379	0.000	Significance
X_1	β_1	10.053	0.000	Significance
X_2	β_2	1.2633	0.151	
X_3	β_3	16.176	0.003	Significance
X_1^2	β_{11}	-5.003	0.000	Significance
X_2^2	β_{22}	-0.623	0.125	
X_3^2	β_{33}	-32.590	0.006	Significance
$X_1 X_2$	β_{12}	0.190	0.405	
$X_1 X_3$	β_{13}	-0.850	0.454	
$X_2 X_3$	β_{23}	-1.350	0.245	

Tables 7. Analysis of variance (ANOVA) values for polynomial model for L-asparaginase production

Source	Degrees of freedom	Sum of squares	Mean square	F-value	p-value
Regression	9	12.576	1.3973	58.56	0.000
Linear	3	6.201	2.0670	86.62	0.232
Square	3	12.397	4.1325	173.18	0.000
Interaction	3	0.068	0.0229	0.96	0.704
Residual Error	10	0.238	0.0238		
Lack-of-Fit	5	0.233	0.0467	49.95	0.382
Pure Error	5	0.004	0.0009		
Total	19	12.814			

The response surface (3D) plots were constructed to identify the type of interaction between these three variables by plotting the response (L-asparaginase activity) on the Z axis against any two independent variables.

Effect of yeast extract and lactose of L-asparaginase activity

The response surface plot (Fig. 1) represents the maximum point of the response (L-asparaginase activity) with respect to concentration of yeast extract and Lactose by keeping the third variable as a constant at middle level. The predicted enzyme activity was greatly influenced by the selected factors namely yeast extract and Lactose. It was observed that the enzyme activity was high at middle Lactose concentration and reaches maximum value at middle level concentration of yeast extract. Therefore, the activity was high at middle Lactose concentration and middle yeast extract concentration.

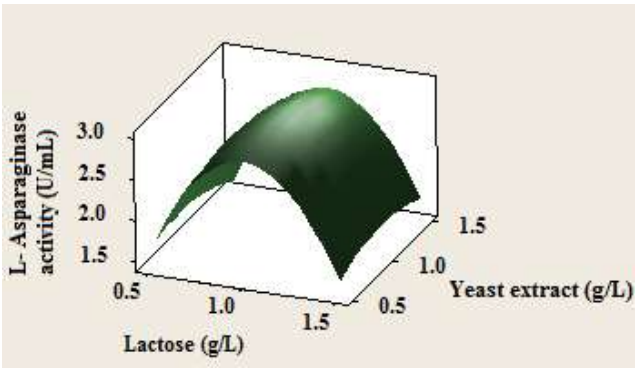

Fig. 1. Surface plot of yeast extract and lactose on L-asparaginase activity

Effect of L-asparagine and lactose on L-Asparaginase activity

The surface plot (Fig. 2) denotes the effect of L-asparagine and lactose on L-asparaginase activity. The third variable was kept constant at their middle level. It was observed that the enzyme activity was high at middle L-asparagine concentration and reaches maximum value at middle level concentration of Lactose. Therefore, the enzyme activity was high at middle L-asparagine concentration and middle lactose concentration. The results concluded that L-asparaginase activity was significantly influenced by L-asparagine and lactose.

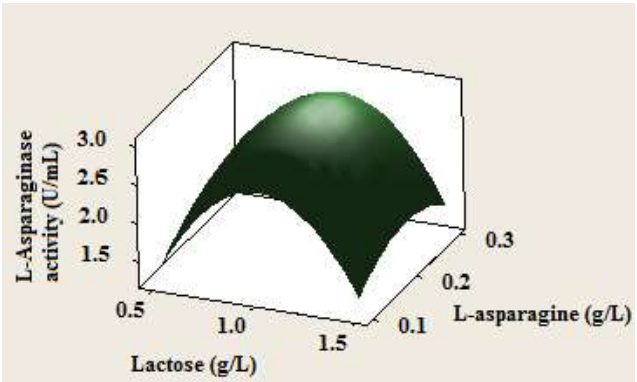

Fig. 2. Surface plot of L-asparagine and yeast extract on L-asparaginase activity

Effect of L-asparagine and yeast extract on L-Asparaginase activity

The response plot (Fig. 3) shows the variation in the enzyme activity, as a function of L-asparagine and yeast extract which demonstrated improved L-asparaginase activity. It was observed that the enzyme activity was high at middle L-asparagine concentration and

reaches maximum value at low concentration of Lactose. Therefore, the activity was high at middle L-asparagine concentration and low lactose concentration.

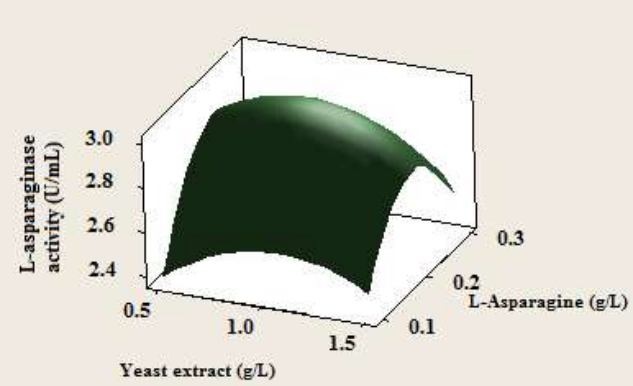

Fig. 3. Surface plot of L-asparagine and yeast extraction on L-asparaginase activity

Conclusions

The most significant independent variables influencing L-Asparaginase production by *E. coli* K12 (MTCC 1302) were found to be Lactose, L-asparagine and yeast extract using Plackett-Burman experimental design and these variables were optimized by response surface method. Production of L-asparaginase by *E. coli* K12 MTCC 1302 was 1.38 and 3.08 U/mL after statistical optimization by Plackett–Burman design and after CCD, respectively.

Conflicts of Interest

Authors declare no conflict of interest.

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