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# Optimization of Fed Batch Production of *E.coli* K-12 L-Asparaginase by Taguchi Orthogonal Array

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**Abstract :** L-Asparaginase is a potent anti-leukemic enzyme, as well as having potential application in food industry for the prevention of acrylamide formation. L-Asparaginase from *E.coli* K-12 is approved globally to use as anti-leukemic drug. Present study aims towards the development of fed-batch strategy for the enhanced production of L-Asparaginase. Process was optimized by using Taguchi orthogonal array based design of experiment (DOE) methodology. After the optimization of six factors viz. Glucose, Tryptone, Yeast extract, $K_2HPO_4$, L-asparagine and Hexane at four different level, there was 1.8 fold increase in enzyme yield.
**Key words:** L-Asparaginase, Taguchi Design of Experiment, DOE, Acrylamide, *E.coli* K-12.

## Introduction

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1.) has been widely found in biological world. In recent years, the use of L-asparaginase in the treatment of leukemia and other lymph proliferative disorders has expanded immensely[1]. For these reasons L-asparaginase has established itself to be an indispensable component in medicine. It is also used in the food industry to reduce the acryl amide formation in food products. L-asparaginase production using microbial systems has attracted considerable attention, owing to the cost-effective and eco Friendly nature.

L-asparaginase is a naturally occurring enzyme which converts L-asparagine to L-aspartic acid and ammonia, by hydrolysis which, proceeds in two steps via a beta-acyl-enzyme intermediate. Where L-Asparaginase is used to hydrolyze the available asparagine molecules in blood stream so that leukemic cells cannot use the asparagine for the purpose of protein synthesis as cancer cells lose the ability to synthesize asparagine hence making asparagine an essential amino acid whereas the non-cancer cells do not have any adverse effect as they retain the ability to synthesize asparagine. L-asparaginase is used in the treatment of acute lymphoblastic leukemia and in many other clinical experiments relating to tumor therapy[2]. With the development of its new functions, a great demand for L-asparaginase is expected in coming years. The enzyme has been extensively studied in mammals and micro-organisms because of its potential antineoplastic activity[3,4]. Microbial enzymes are preferred over plant or animal enzymes due to their economic production, consistency, ease of process modification and optimization. They are relatively more stable than corresponding enzymes derived from plants or animals. Further, they provide a greater diversity of catalytic activities. Microorganisms have been proved to be very efficient and inexpensive sources of L-asparaginase[5,6].

Fed-batch fermentation is a production technique biotechnological process where proper feed rate of one or more nutrients with the right component constitution is required during the process. Fed-batch process is a very effective technique as the yield of the product is many times higher than others fermentation system.

Optimization studies can be performed by two major methods, viz., classical and statistical. Statistical designs for optimization studies are definitely advantageous than the classical single factor at a time. This is mainly because; such methods are economical, less time consuming and accurate. Moreover, the role and interactions of the parameters can be determined, which may be overlooked in classical approach. However, classical approach gives some basic information on the levels of individual parameters for testing by statistical approach. In statistical method sets of experiments can be designed by Taguchi DOE method, proposed by Dr. Genichi Taguchi of Japan. The methods involves establishment of large number of experimental situation described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. The main advantage of this method is that the combine effect of many factors can be studied simultaneously[7,8].

In this study we have optimized the production of L-Asparaginase by *E. coli* K-12. As in the case of any process or system it is always beneficial to determine the most important factor, interactions and influences of different factors. Hence for this study the factors like Carbon sources, nitrogen sources, salts and additives were selected for optimization.

## Materials and Methods

### Microorganism and culture conditions

The cultures of *E.coli* K-12 were obtained from the departmental culture collection of Birla Institute of Technology, Mesra, Ranchi, Jharkhand (India). The isolates were maintained on Nutrient Agar medium slants having the composition (g/L): Peptic digest of animal tissue 5.0, Sodium chloride 5.0, Beef extract1.50, Yeast extract1.50, Agar15.00 in 1000ml distilled water and used as growth medium. The microbial strain was grown at 30°Cfor 4 days after which, it was stored at 4°C until further use and sub-cultured after every two weeks. All the chemicals used in the study were analytical grade.

### Inoculum and Shake Flask Production of L-Asparaginase

A loop full of culture was transferred in proposed seed medium: Glucose-7g/l, $K_2HPO_4$-1 g/l, Yeast Extract-5g/l,Tryptone-5g/l, L-Asparagine-1% (w/v).Culture was incubated overnight in orbital shaker at 37°C, at 150 rpm for 16 to 18 hrs.

The production of L-asparaginase under Fed batch system was carried out by transferring 10 % (v/v) inoculum to the production media. Production medium having same composition as seed medium were fed by concentrated media of 10% and 20%. The fermentation process was carried out at 37°C and 150 rpm agitation for a suitable fermentation period of 24 hours. Production medium was prepared in 250 ml Erlenmeyer flask; conditions for fermentation were kept same. After 18 hours of incubation culture was centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected for assay to check the enzyme activity.

### Standardization of Enzyme Assay

L-Asparaginase assay was performed by the Nesslerization method[9]. The activity of L-asparaginase was determined by estimating the amount of ammonia liberated from L-asparagine. The activity was measured by withdrawing the sample after the regular interval of 2 hrs and centrifuged at 1000 rpm for10 min at 4°C. The supernatant was used as a crude extract for the enzyme assay by using 0.2 ml of 0.05M of Tris-HCl, 1.7 ml of 0.01M L-Asparagine,0.2 ml of enzyme, 1 ml of Nesseler's reagent and 7 ml of distilled water. The reaction mixture was incubated at 37°C for 10 min, after that the reaction was stopped by adding 0.1 ml of 1.5M TCA. The substrate blank was prepared by same method except the enzyme. The optical density was measured at 480nm. The ammonium sulphate was used as the standard to assay the amount of ammonia evolved during the process. The one unit of enzyme activity was expressed as the amount of enzyme required to evolve 1μmol of ammonia per minute per ml under the experimental conditions.[10]

### Design of Experiment (DOE) by Taguchi Method

Taguchi DOE methodology was used for the optimization of various physiochemical parameters were carried out to enhance the production of L-asparaginase. Taguchi orthogonal array based design of experiment (DOE) was used to optimized the six factors, viz. Carbon source, Nitrogen source, Salts, additives etc at four different levels were selected for optimization as they significantly affect the yield of desire enzyme. Table-1 represents the different levels of selected factors. L-32 orthogonal array designed total 32 trial conditions for the

optimization. The enzymatic activity obtained during these trial conditions were used to analyze the optimum level through the severity index and ANOVA analysis. The optimum level predicted by DOE was validated by performing the experiment under the proposed conditions. All the experiments were carried out in triplicates.

## Results and Discussion

The production of L-asparaginase by 10% feed has given 1.44 U/ml was observed in comparison to 1.35 U/ml by 20% feed at regular interval. The maximum production of L-asparaginase by *E.coli* K-12 in Fed batch process has been observed after 18 hours of inoculation of media. Initially for optimization six factors were selected at four different levels as shown in (Table 1).

**Table 1: The different factors and their levels selected for optimization of selected factors.**

| S.No | Factors | Level-1 | Level-3 | Level-3 | Level-4 |
|---|---|---|---|---|---|
| 1 | Glucose (g/l) | 5 | 6 | 7 | 8 |
| 2 | Tryptone(g/l) | 3 | 4 | 5 | 6 |
| 3 | Yeast ext g/l) | 3 | 4 | 5 | 6 |
| 4 | $K_2HPO_4$ (g/l) | 0.5 | 1 | 1.5 | 2 |
| 5 | L-asn (g/l) | 0.5 | 1 | 1.2 | 1.4 |
| 6 | Hexane (v/v) | 1 | 2 | 3 | 4 |

**Table 2- No of interaction between factors and Severity index (SI)**

| S.No | Interactions | Columns | SI(%) | Col | Opt |
|---|---|---|---|---|---|
| 1 | L-asp x Hexane | 6 x7 | 72.63 | 1 | [1,1] |
| 2 | Tryptone x yeast ext | 3 x 4 | 46.45 | 7 | [4,4] |
| 3 | Glucose x Yeast ext | 2 x 4 | 27.27 | 6 | [4,4] |
| 4 | Tryptone x Hexane | 3 x 7 | 22.92 | 4 | [3,1] |
| 5 | Glucose x Hexane | 2 x 7 | 21.57 | 5 | [4,1] |
| 6 | Glucose x Tryptone | 2 x 3 | 13.99 | 1 | [4,3] |
| 7 | Yeast ext x Hexane | 4 x 7 | 12.9 | 3 | [4,1] |
| 8 | Tryptone x $K_2HPO_4$ | 3 x 5 | 12.78 | 6 | [3,2] |
| 9 | Glucose x L- asp | 2 x6 | 12.69 | 4 | [4,1] |
| 10 | $K_2HPO_4$ x Hexane | 5 x7 | 8 | 2 | [2,1] |
| 11 | Glucose x $K_2HPO_4$ | 2 x 5 | 6.97 | 7 | [4,2] |
| 12 | Tryptone x L- asp | 3 x 6 | 6.27 | 5 | [3,1] |
| 13 | Yeast ext x L- asp | 4 x 6 | 3.6 | 2 | [4,1] |
| 14 | $K_2HPO_4$ x L- asp | 5 x 6 | 2.44 | 3 | [2,4] |
| 15 | Yeast ext x $K_2HPO_4$ | 4 x 5 | 2.22 | 1 | [4,2] |

**Table 3- Analysis of variance (ANOVA) for production of L-asparaginase**

| S.No | Factors | DOF(f) | Sum of Squares(s) | Variance(v) | F- Ratio (F) | Pure Sum(s') | Percent (%) |
|---|---|---|---|---|---|---|---|
| 1 | Glucose | 3 | 6.356 | 1.118 | 4.126 | 3.543 | 29.41 |
| 2 | Tryptone | 3 | 4.768 | 1.256 | 4.632 | 2.054 | 21.943 |
| 3 | yeast Ext | 3 | 2.415 | 0.138 | 0.51 | 1.780 | 15.23 |
| 4 | K2HPO4 | 3 | 2.042 | 1.347 | 4.969 | 1.229 | 11.948 |
| 5 | L-As | 3 | 1.112 | 0.37 | 1.367 | 0.299 | 1.106 |
| 6 | Hexane | 3 | 3.129 | 0.749 | 2.617 | 1.916 | 18.812 |
| 7 | Other/Error | 45 | 12.201 | 0.271 | | | 1.734 |
| 8 | Total | 63 | 27.027 | | | | 100.00% |

**Table 4: Favorable levels for maximum enzyme production**

| S.No | Factors | Level | Level | Contribution |
|---|---|---|---|---|
| 1 | Glucose | 6 | 2 | 0.414 |
| 2 | Tryptone | 5 | 3 | 0.234 |
| 3 | Yeast Extract | 6 | 4 | 0.102 |
| 4 | $K_2HPO_4$ | 1 | 2 | 0.211 |
| 5 | L-Asparaginase | 2 | 4 | 0.19 |
| 6 | Hexane | 1 | 1 | 0.289 |
| Total contribution from all factors | | | | 1.44 |
| Current grade average of performance | | | | 2.683 |
| Expected result at optimum condition | | | | 4.12 |

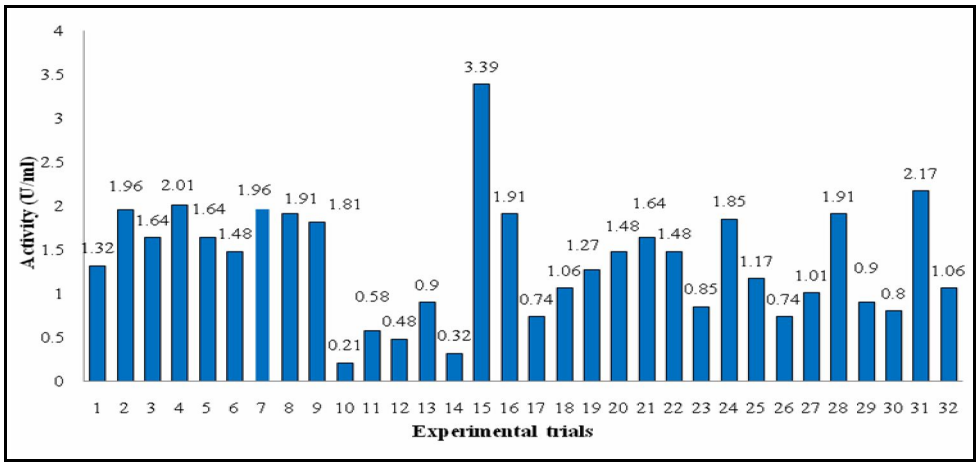


**Figure 1: Variability within and between trial results (Graphical Representation for *E.coli* K-12 )**

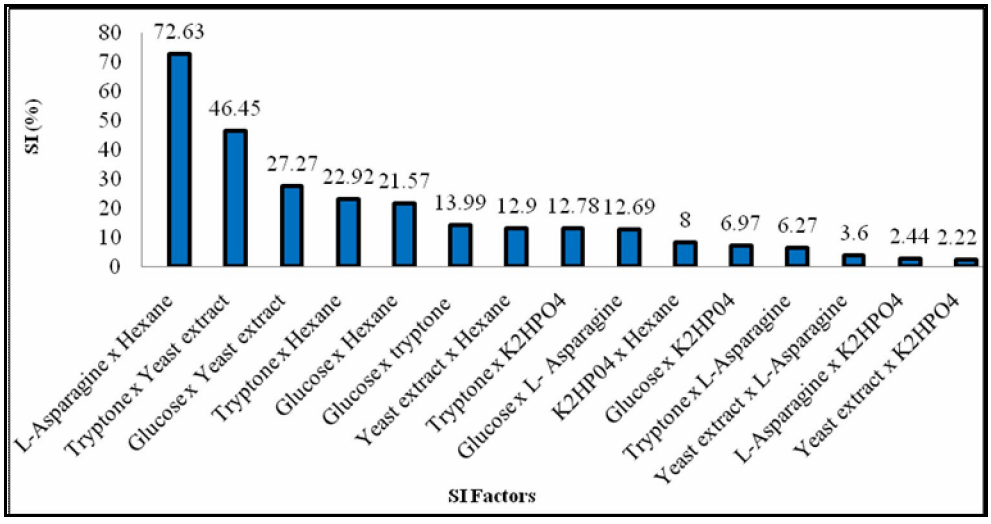


**Fig 2: Interaction between the factors and their severity index (SI)**

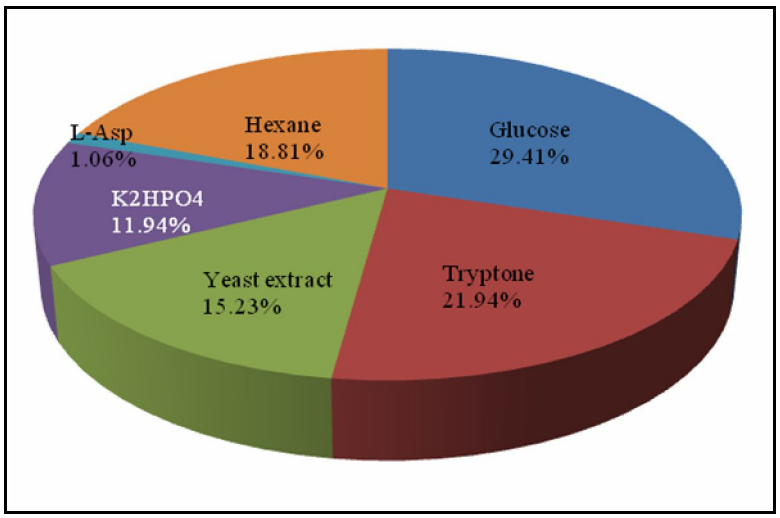


**Fig 3: Percentage contribution of various factors**

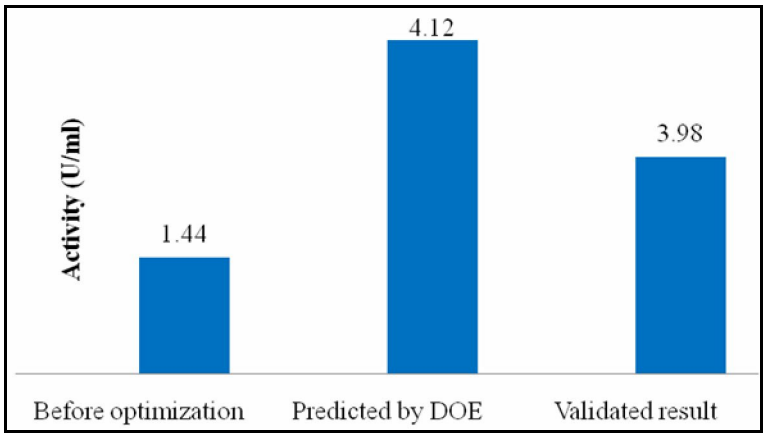


**Fig 4: Enzyme yield before and after the optimization**

Optimization of the process has been done by Taguchi method where L-32 array were designed for 32 different experimental conditions of L-asparaginase production in fed batch process. The enzymatic activity obtained during the trial conditions were used for predict the optimum levels of selected factors. The enzymatic activity obtained in all 32 trial conditions represented in figure 1.The maximum activity obtained at trial condition 15 was 3.36 U/mL while minimum activity of 0.21 U/mL was observed in trial condition of 10 ( Figure 1). All other trial conditions having intermediate value of L-asparaginase activity. This variability within the different trial conditions were further used to predict the severity index (Table-2).Severity index (SI) represents the significant interaction between the selected factors which gives an overall analysis of the process interaction between two factors. Total 15 interactions between factors were identified. The maximum SI value of 72.6% was obtained in the case of L-asparaginase and Hexane and minimum SI value of 2.22% was observed in Yeast extract and $K_2HPO_4$ (Figure 2). The analysis of variance is the most suitable method to analyze the more complex data set with the estimation of the effect of various factors and interaction between them. The statistically significant effect of each factor was estimated by mean square, F-ratio and percentage with three degree of freedom at 95% confidence limit. The results of the orthogonal array (OA) experiments were analyzed by the use of ANOVA. ANOVA was used to analyze the levels of selected factors and all relative contribution of L-asparaginase production (Table-3). The sum of square and F-ratio was used to analyze the percentage contribution of factors on the performance.(Armstrong and Hilton, 2004).Glucose having maximum 29.41% contribution on the sum of square with the F-ration of 4.126. L-asparagine having minimum contribution of 1.10% with the F-ratio of 1.367 and others having Tryptone 21.94% yeast Ext 15.23% $K_2HPO_4$ 11.94% Hexane 18.81 % (Figure 3). The comparison of predicted and validate result after performing the experiments under the designed trail condition is represented in figure-3. The Taguchi DOE has predicted optimum level of different factors was found to be as follow Glucose (6 gm/L), Tryptone (5 gm/L), Yeast

extract (6 gm/L), $K_2HPO_4$ (1 gm/L), L-asparagine (2 g/mL), Hexane (1g/L).(Table 4)The expected enzyme production was 4.12 U/mL proposed by Taguchi method. After the validation of result under the proposed optimized condition the enzyme yield of 3.98 U/ml was observed in fed batch system by *E.coli* K-12 (Figure 4). There was 1.8 fold increase in the production of L-aspraginase in fed batch fermentation by *E.coli* K-12.

## Conclusion

The fed batch strategy may be a better alternative than the batch production of L-Asparaginase by microbial source. Present study proved the potential of fed batch fermentation for the production of this anti-leukemic enzyme. In present study there was 176.3% increase in the production of L-Asparaginase by *E.coli* K-12. It may be a cost effective method for the production of enzyme and may cut down the higher cost of L-asparaginase therapy.

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