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Batch production and media optimization of anti-leukemic L-asparaginase from *Pseudomonas fluorescens* by Taguchi DOE methodology

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ABSTRACT

L-Asparaginase isolated from bacterial sources has been shown to possess antileukemic characteristic, mainly against the acute lymphoblastic leukemia. Large number of bacterial strains have been reported which can produce it. The production level of the enzyme by the bacterial sources is very low and hence the factors have to be distinguished which effect the growth of cells and production of enzyme directly or indirectly, in order to decrease the cost of treatment. The production of L-Asparaginase was optimized in classical fermentation process by the use of Taguchi DOE methodology. L-18 array was selected for the purpose of media optimization. The five factors at three levels were considered for the optimization. L-Asparaginase production was significantly ($p<0.05$) affected by the interaction of two factors present in the culture broth. The factors interaction viz. energy source-nitrogen source, energy source-phosphate source etc. having pronounced effect on the production while individually they have the minimum impact on L-Asparaginase production. So the interactions of different parameters were studied and they were used to formulate the optimized condition for the production of L-Asparaginase. After the validation of result by performing the experiment under optimized condition there was about 28.48% increase in the production of the enzyme.

INTRODUCTION

The enzyme L-Asparaginase has the chemotherapeutic property against the tumor cells.L-asparagine amidohydrolase, EC 3.5.1 .1 is another name for enzyme, isolated from *E.coli*, reported later. It is an effective curable agent against the treatment of acute lymphoblastic leukemia (ALL) and lymphosarcoma in children. The enzyme catalyzes the hydrolysis of L-Asparagine into L-Aspartic acid and ammonia. The principle behind the use of Asparaginase as an antitumor agent is that it takes advantage of the fact that ALL leukemic cells are unable to synthesize the non-essential amino acid asparagines their own, which is very essential for the growth of the tumor cells, whereas normal cells can synthesize their own asparagine with the action of asparagine synthetase, thus leukemic cells require high amount of asparagine (Verma *et al.*,2007).

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L-Asparaginase enzyme mainly isolated from the two bacterial sources: *Escherichia coli* and *Erwinia caratovora.* Bacterial source was helpful in producing high yield of the enzyme and a series of preclinical and clinical studies was able to be conducted. But today a number of sources for the isolation of enzyme is present. They also include fungal sources of isolation.

Analysis of the different factors responsible for affecting the production level of L-Asparaginase can be done by either conventional method or statistical method. Conventional method involves interpreting each and every factor individually. By this method one cannot interpret the effect of one factor over the other or the effect of two factors on the production level simultaneously. Moreover, conventional method is cumbersome and time consuming (Oskouie *et al.*, 2007).

In statistical method sets of experiments can be designed by Taguchi DOE method, proposed by Dr. Genichi Taguchi of Japan. Taguchi method of DOE 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 (Abdel-Fattah *et al.,* 2005; Rao *et al.,* 2004, Jha *et al.,* 2014). He laid emphasis to investigate the factors which directly affect the mean and variance of the process. In the design of an OA, each column consists of a number of conditions depending on the levels assigned to each factor (Prasad *et al.,* 2005).

In this study we have optimized the different concentrations of media components (i.e. carbon source, nitrogen source, L-Asparagine concentration) at pH 7 for L-Asparaginase production by *Pseudomonas fluorescens*.

MATERIALS AND METHODS

Microorganism

Pseudomonas fluorescens NCIM 2100 was used in this study. The bacterium was confirmed for L-Asparaginase activity on the media agar plate having phenol red as an indicator. Change in color due to increase in pH on the release of ammonia was observed (Gulati *et al.,* 1997). The microorganism was sub cultured every 30 days. All the chemicals used in the study were analytical grade.

Shake flask production of L-Asparaginase

A loop full of culture was transferred in proposed seed medium: Glucose-1 g/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. After 16-18 hours of incubation 5% (v/v) of culture was transferred to production medium having same composition as seed medium with different concentration levels as designed by Taguchi DOE.

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. 18 different trial conditions were designed by QUALITEK-4 software for the further optimization of the media for the better production.

Standardization of Enzyme Assay

L-Asparaginase assay has been started by the Worthington manual protocol but few modifications have been done to optimize it in the laboratory conditions. The rate of hydrolysis of asparagine is determined by measuring the released ammonia.

One unit of L-Asparaginase releases one micromole of ammonia per minute at 37 °C and pH 8.6 under the specified conditions. The standardization of the method has been done by the pure L-Asparaginase purchased from SIGMA-ALDRICH (Mashburn and Wriston 1963).

After the age of 16-18 hours the culture was centrifuged and enzymatic assay through Nesslerization was continued with very small amount of supernatant. O.D. was taken at 480 nm to define the activity of enzyme (Mashburn and Wriston 1963).

Optimization of Media

Optimization has been done for five factors *i.e.* five different components of media. The method followed for the optimization is based on the Taguchi orthogonal array (OA) experimental design (DOE) (Chauhan *et al.*,2004, Beg *et al.*,2003, Dasu *et al.*,2003 and Roy 1990). L-18 array was constructed. 18 different trial experiments were designed on this basis at 3 different levels: maximum, minimum and intermediate level with same composition. The result of different trial conditions have been used to estimate the optimum condition for the better production of L-Asparaginase. Further analysis has been done to find out all the possible interactions between the different factors participating in the L-Asparaginase production.

Table 1: The three different levels of medium component selected for optimization:

S.No.	Component	Level 1	Level 2	Level 3
1.	Glucose (g/l)	0.8	1.0	1.5
2.	K_2HPO_4 (g/l)	0.7	1.0	1.2
3.	Yeast extract (g/l)	5.0	6.0	7.0
4.	Tryptone (g/l)	5.0	6.0	7.0
5.	L-Asparagine (%)	0.5	1.0	1.5

RESULTS AND DISCUSSION

Maximum production of L-Asparaginase has been seen after 16-18 hours of inoculation of media. The yield of enzyme in all 18 trial conditions are mentioned in fig-1. One unit of ammonia released can be defined in terms of mmol of ammonia released per ml of enzyme.

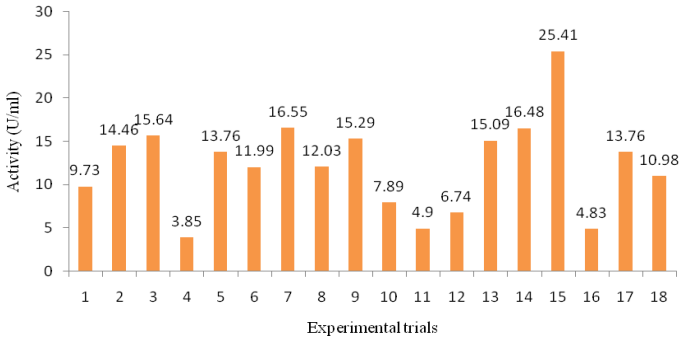

Fig. 1: Variability in enzymatic activity between designed trial conditions.

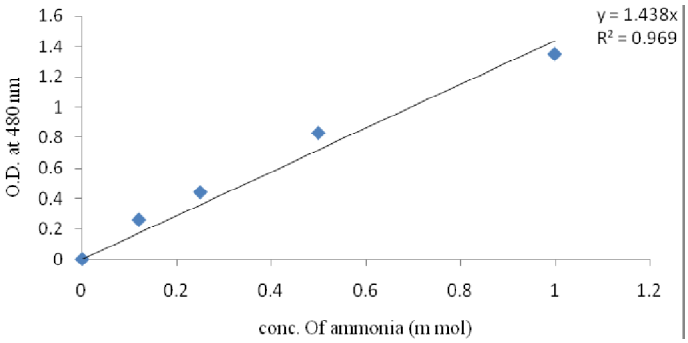

Fig. 2: Standard plot for ammonia released during enzymatic assay.

STANDARDIZATION OF L-ASPARAGINASE ASSAY

L-Asparaginase assay wasstandardized by Worthington manual protocol. Activity of the L-Asparaginase is measured in term of millimoles of ammonia released.

OPTIMIZATION OF MEDIA

Initially, for the optimization process five factors were under consideration at three different levels as shown in table 1. Eighteen different experiments were designed to optimize the media. Optimization was done on the results of these 18 experiments.

The interaction between two factors may be provide an understanding of overall process analysis. It has been observed that tryptone and K_2HPO_4 has maximum effect on enzyme production, rather yeast extract and tryptone has the minimum effect in case of *P. fluorescens.* Rest of the factors has intermediate effect on enzyme production. It can be easily shown through SI graph in fig 3 and table 2.

The maximum production of enzyme from *P. fluorescens* was obtained when the glucose level was 1.5 (g/l), K_2HPO_4 level was 1.2 (g/l), yeast extract level was 7 (g/l), tryptone level was 5 (g/l) and L-asparagine level was 1.0% (w/v) (table 3).

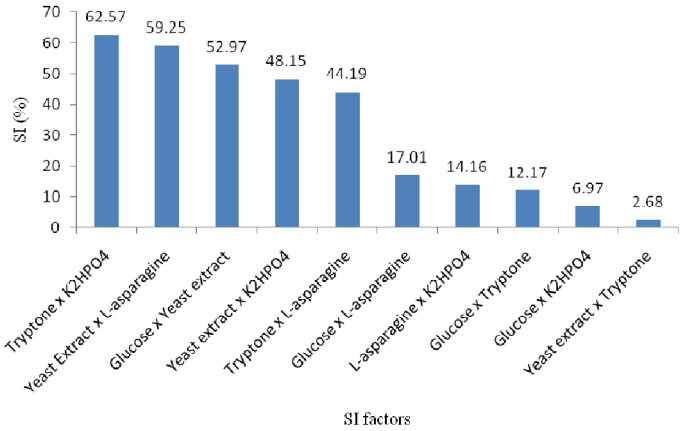

Fig. 3: Interaction between the factors and their severity index (SI) for *P. Fuorescens.*

Table 2: Estimated interaction between the factors (Severity Index) for *P. fluorescens.*

S. No.	Interacting Factor Pairs (order based on SI)	Columns	SI (%)	Optimum levels
1.	Tryptone x K_2HPO_4	4x6	62.57	1,1
2.	Yeast Extract x L-asparagine	3x5	59.25	1,1
3.	Glucose x Yeast extract	2x3	52.97	2,3
4.	Yeast extract x K_2HPO_4	3x6	48.15	3,3
5.	Tryptone x L-asparagine	4x5	44.19	1,2
6.	Glucose x L-asparagine	2x5	17.01	2,2
7.	L-asparagine x K_2HPO_4	5x6	14.16	1,1
8.	Glucose x Tryptone	2x4	12.17	2,1
9.	Glucose x K_2HPO_4	2x6	6.97	2,3
10.	Yeast extract x Tryptone	3x4	2.68	3,1

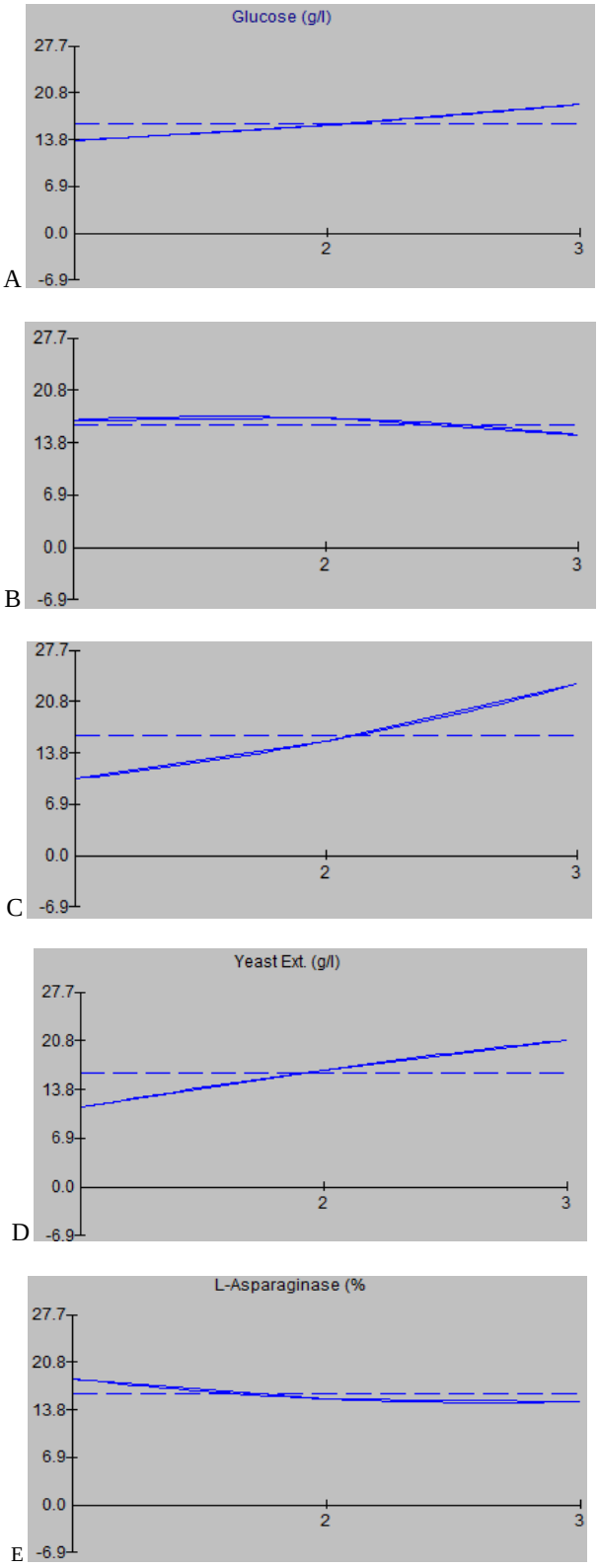

Fig. 4: Effect of Various Factors on enzyme production by *P. fluorescens* (A-glucose; B-K_2HPO_4; C- tryptone; D-yeast extract ; E-L-asparagine).

The results of the orthogonal array experiments were analyzed by the use of ANOVA (Table 3). The F-ratio was used to determine the degree of variation contributed by each factors (Armstrong and Hilton, 2004). The all factors and their respective

interactions considered in the experimental design were statistically significant effects at 95% confidence limit. By study of main effect of each factor the general trends of the influence of the factors towards the process can be characterized. Analysis of variance has given the percentage contribution of all factors on the performance of the process. In case of *P. fluorescens* glucose has contributed 46 %, K_2HPO_4 1 %, yeast extract 25 %, tryptone 21 %, L-asparagine 6 % can be seen in fig 5.

Table 3: Analysis of Variance (ANOVA) .

S. No.	Factors	Sum of Squares	variance	F-Ratio	Pure sum	Percent
1.	Glucose	494.024	247.012	18.923	467.918	46.412
2.	Yeast Extract	278.756	139.378	10.677	252.651	25.06
3.	Tryptone	91.369	13.052	9.567	211.454	21.215
4.	L-Asparaginase	85.64	42.82	3.28	59.535	5.905
5.	K_2HPO_4	40.303	20.151	1.543	14.198	1.408
	Other/Error	18.072	9.036			0.155
	Total	1008.164				100.00%

The proposed optimized conditions by factorial design were validated by estimating L-Asparaginase activity according to designed conditions. After the study of complete interactions among all factors, the optimized condition should give the 28.711U/ml of enzyme yield with 32.49% enhanced recovery (table 4). In case of *P. fluorescens* the 1.5 g/l glucose, 1.0 g/l K_2HPO_4, 7.0 g/l yeast extract, 7.0g/l tryptone, 0.5% (w/v) L-asparagine gave the best result (table 4). The L-Asparaginase activity estimated statistically showed 27.843 U/ml of enzymatic activity with enhanced recovery of 28.47 % (fig 6).

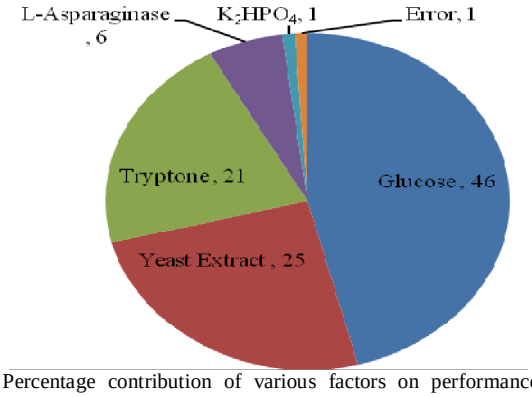

Fig. 5: Percentage contribution of various factors on performance for *P. Fluorescens.*

Table 4: Optimum condition and Performance for the production of L-asparaginase.

S.No	Factors	Level	Level Description	contribution
1.	Glucose	3	1.5	2.802
2.	Yeast Extract	3	7.0	4.609
3.	Tryptone	3	7.0	6.807
4.	L-Asparaginase	1	0.5	2.102
5.	K_2HPO_4	2	1.0	0.822
	Total contribution from all factors			17.141
	Current Grand Average of Performance			21.67
	Expected Result at Optimum Condition			28.711
	Percentage Increase in Performance			32.49

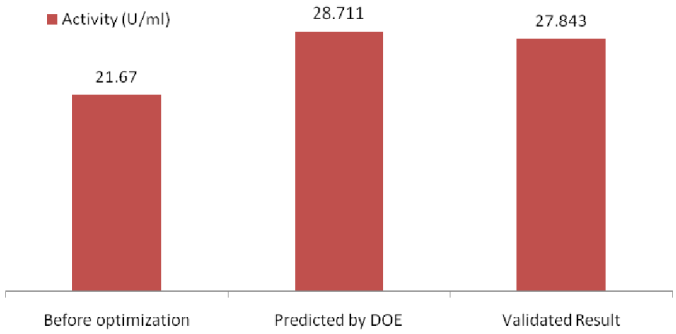

Fig. 6: Comparison of the enzyme yield before and after the optimization of media components by *P. Fluorescens.*

CONCLUSION

L-Asparaginase is a well-established chemotherapeutic agent against acute lymphoblastic leukemia. New sources for the isolation of enzyme have to be discovered to meet the demand of medicine industry. Optimization of the production medium is one of the key factors to maximize the yield of a product under study. Traditional methods of optimization involved changing one independent variable while fixing the others at a certain level. This single-dimensional search is laborious, time consuming, and incapable of reaching a true optimum due to interactions among variables. The Taguchi approach of OA DOE constitutes a simple methodology that selects the best conditions producing consistent performance. It has been observed that yeast extract and tryptone has the minimum effect in case of *P. fluorescens.*The L-Asparaginase activity estimated statistically showed the 27.843 U/ml of enzymatic activity with enhanced recovery of 28.47 % from *P. fluorescens.*

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