International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.9, No.4, pp 254-260, 2016

Statistical optimization of the recombinant L-asparaginase from *Pseudomonas fluorescens* by Taguchi DOE

Rati Kumari Sinha, H. R. Singh*

Department of Bio-Engineering, BIT Mesra, Ranchi-835215, Jharkhand, India

Abstract: L-Asparaginase has been one of the naturally occurring enzymes which are known for its anti-tumor potential. It has proven to be an effective curative agent in the treatment of acute lymphocytic leukemia. In the present report, optimization of the concentration of media components of the recombinant L-asparaginase from *Pseudomonas fluorescens* was done by applying Taguchi Design of Experiment (M-9) method based on orthogonal array. The four factors at three concentration levels were considered for the optimization. The interaction pair of Lactose and $MgSO_4$ showed the most significant interaction effect. The ANOVA data revealed maximum F ratio of 274.108 in case NH_4Cl and hence proved to be the most influential factor with percentage contribution of 71.259%. The optimum condition for for the enhanced production of recombinant L-asparaginase was found to be lactose at 1% (w/v), Tryptone at 1 % (w/V), Ammonium chloride at 0.25%(w/v) and magnesium sulphate at 0.25%(w/v). The expected enzyme production of 271.663 U/ml was proposed by the model. After validation, an enhanced enzyme production of 270.122U/ml was obtained with 35% increase in the yield.
Key words : L-asparaginase, Pseucomonas fluorescens, Taguchi, orthogonal array.

Introduction:

L-asparaginase has achieved markedly increased attention for its chemotherapeutic potential and has been effectively used in the treatment of acute lymphocytic leukemia, a childhood cancer. It catalyzes the conversion of L-asparagine to L-aspartate and ammonia, and this catalytic reaction is essentially irreversible under physiological conditions[1] and also to a lesser extent, the hydrolysis of L-glutamine to L-glutamate[2]. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine[3-4]. Hence, they are not capable of producing L-asparagine, and mainly depend on the L-asparagine from the circulating plasma pools[3]. Supplementation of L-asparaginase results in continuous depletion of L-asparagine. Under such an environment, cancerous cells do not survive. This phenomenal behaviour of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase[5-6,3]. A potent source of L-asparaginase was found in *Escherichia coli*[7]. Since then its production using microbial systems has attracted considerable attention owing to their cost-effective and eco-friendly nature. However, a distinct toxicity profile of L-asparaginase, ranging from acute hypersensitivity (immunological sensitization) and hyperglycemia to hepatocellular dysfunction and pancreatitis (inhibition of protein synthesis)[8] suggest the need to explore new sources of L-asparaginase with high productivity and less antigenic effect in a cost effective manner. In order to achieve this recombinant technology can be employed to get new recombinant strains with enhanced productivity, reduced immunological effect and a cheaper treatment cost. At the same time, for effective utilization of any microbial system at bioprocess level, it is essential to screen and evaluate various nutritional and environmental requirements for the microbial growth and subsequent biocatalyst production[9-10], as culture conditions that promote optimum enzyme production differ significantly with the molecular nature of the

micro-organism[11]. Hence there is a need to optimize the medium components for enhanced L-asparginase production. For this either conventional method or statistical method can be employed. Optimization studies involving a conventional one-factor-at-a-time approach can prove to be tedious and time consuming. Moreover this approach tends to overlook the effects of interactions among factors, leading to misinterpretations of results. In contrast, statistically designed experiments minimize the error in determining the effect of parameters and their interactions, and the results could be achieved in an economical way.

Taguchi DOE is one of the statistical methods proposed by Dr. Genichi Taguchi of Japan based on orthogonal arrays. This method offers a main advantage of studying the combined effect of many factors simultaneously[12-13, 9]. In this method, investigation of the factors which directly affect the mean and variance of the process was the main emphasis.

In the present report, recombinant L-asparaginase production from *Pseudomonas fluorescens* was optimized by Taguchi DOE (M-9) orthogonal array method which is the first to be reported till now.

Materials and methods:

Chemicals:

Culture media and their constituents were purchased from Himedia. The cloning kit and IPTG were purchased from Fermentas Thermo Scientific. The Nessler's reagent was purchased from Sigma Aldrich, USA.

Strains and Plasmids:

Pseudomonas fluorescens NCIM 2100 strain were obtained from NCL, Pune, India. *Escherichia coli* DH5α (supE44 ΔlacU169 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1re/A1) as well as *Escherichia coli* BL21(DE3) (F^-ompThsdSB (rB^-mB^-) gal dcm(DE3) were obtained from Amersham and Novagen, USA respectively. The plasmid vectors pUC19 (2.6Kb, Ampr, *lacZ*(M13mp18/19), isolated from *E.coli* (dam^+, dcm^+)) and pET29a(+)(5.4 Kb, Kan^r, T7 promoter, lacO. C-terminal 6x His-tag) were obtained from BISR, Jaipur, India.

Cloning of *Pseudomonas fluorecens* ansB gene:

The extraction of DNA from *Pseudomonas fluorescens* was carried out by Fermentas DNA purification kit. The gene sequence of *Pseudomonas fluorescens* ansB was retrived from NCBI and based on the sequence the primers were designed by NCBI primer designing tool. The gene coding for the mature region of L-asparaginase II (ansB) was PCR amplified from the genomic DNA of strain *Pseudomonas fluorescens* (NCIM2100) using primers (forward) 5'- GGCGAATTCGTCGGTATCGAACAA-3'and (reverse) 5'-GCGGATCCTCAATACTCCCAG AAC-3'. EcoRI and BamHI restriction sites were incorporated in the primers to facilitate cloning of the structural asparaginase gene without its native signal sequence. The amplified product was cloned with the plasmid vector pUC19. The cloned vector was transformed to competent *E.coli* DH5α cells. The transformed *E.coli* cells were grown into LB agar plate containing 100µg/ml ampicillin, 0.1mM IPTG and 0.1mM X-gal. The recombinant clones were identified by blue/white selection and grown at 37ºC in 50ml LB medium containing 100µg/ml ampicillin. The plasmid was isolated from *E.coli* DH5α and was used to transform competent *E. coli* BL21 cells which were screened with blue/white selection.

Shake flask production of L-Asparaginase:

E.coli BL21(DE3) transformed with pET29a(+) recombinant construct was used for the shake flask studies. For the preparation of seed culture, the recombinant strain was grown in the specified Luria Bertani medium with kanamycin (50µg/ml) at pH 7 & temperature 37°C for overnight with constant agitation of 200 rpm. The OD was determined to be 0.7-0.8 at 600 nm. This culture was used as a seed culture to inoculate in 50 ml of production medium and grown at 37 °C for 24 h in orbital shaker at 200 rpm. The final concentration of 0.1 mM IPTG was used to induce the culture for the over expression of the recombinant L-asparaginase.

The composition of the proposed production medium consisted of Lactose (1%) as carbon source, Tryptone (1%) as organic nitrogen source, Ammonium chloride (NH_4Cl)(0.2%) as inorganic nitrogen source, Magnesium sulphate ($MgSO_4$) (0.2%) as mineral source, Glycerol (0.4%) as additive with pH 7.5 as screened by conventional one factor at a time approach.

Production medium was prepared in 250 ml Erlenmeyer flask; conditions for fermentation were kept same. After 24 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.

Enzyme assay by Nesslerization method:

The enzyme activity was measured directly according to the method of Bergmeyer[14] by measuring the ammonia produced from the hydrolysis of L-asparagine with Nessler's reagent. The routine assay by ammonia nesslerization was performed in a total volume of 2.3 ml of buffer 0.05 M Tris- HCl, pH 8.6, containing 1.7 ml of 0.01M asparagine and 0.2 ml of enzyme preparation. The samples were incubated for 20 min. at 37°C. The reaction was stopped by the addition of 0.1 ml of 1.5 M trichloroacetic acid (TCA). The protein precipitate was removed by centrifugation. To 0.5 ml aliquots of the reaction mixture, 7.0 ml of distilled water was added, followed by 0.5 ml of Nessler's reagent, the mixture was left for 10 min. at room temperature. The absorbance was determined at 480 nm against the blank, using CE double beam digital U.V. Spectrophotometer.

Optimization of production medium components by Taguchi DOE method:

The optimization of the medium component was done selecting four factors i.e. Lactose, Tryptone, NH_4Cl and $MgSO_4$ by considering their concentrations at three different levels as designed by Taguchi DOE method[15-18]. L-9 array was constructed and 9 different trial experiments were designed on this basis at 3 different levels: maximum, minimum and intermediate level with the four selected factors. QUALITEK- 4 software was used for the designing of OAs and for the optimization based analysis of the media for the better production[19]. The result of different trial conditions was processed in the Qualitek-4 software with bigger is better quality characteristics for the determination of the optimum culture conditions for the fermentation, to identify individual factors influence on the L-asparaginase production, all the possible interactions between the different factors participating in the L-asparaginase production and to estimate the performance (fermentation) at the optimum conditions[20].

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

S.No.	Component	Level 1	Level 2	Level 3
1	Lactose (%)	0.5	1	1.5
2	Tryptone (%)	0.5	1	1.5
3	Ammonium chloride (%)	0.15	0.2	0.25
4	Magnesium sulphate (%)	0.15	0.2	0.25

Results and Discussion:

The L-asparaginase gene of *Pseudomonas fluorescens* strain was successfully cloned in the *E.coli* BL21 cells. The resulting recombinant strain was grown in the production media for about 24 hours at which maximum production of the recombinant L-asparaginase was obtained. The four medium components at four different levels were selected for the optimization using Taguchi DOE. The M-9 array was selected for the designing of experiment. On the basis of the M-9 array, total 9 trial conditions were designed. The yield of enzyme in all 9 trial conditions (Fig-1) were used for the analysis of optimum levels of the proposed factors. The enzymatic activity obtained during the different trial conditions are given in fig.1. The maximum activity was obtained in trial condition 5 (262.5 U/ml) and minimum at trial condition 1 and 6 (140 U/ml).

Table 2: Interaction Severity Index between the factors

S. No.	Interacting factor pairs (Order based on SI)	Columns	SI %	Optimum levels
1	Lactose x $MgSO_4$	1 x 4	73.46	[2,1]
2	Tryptone x $MgSO_4$	2 x 4	47.95	[2,1]
3	Tryptone x NH_4Cl	2 x 3	24.48	[2,3]
4	Lactose x NH_4Cl	1 x 3	11.22	[2,3]
5	Lactose x Tryptone	1 x 2	8.16	[2,2]
6	NH_4Cl x $MgSO_4$	3 x 4	0	[3,1]

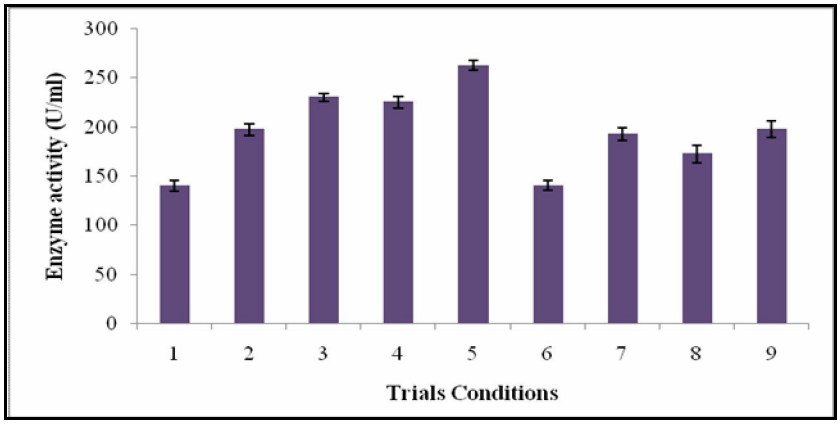

Fig. 1. Variation in the enzyme activity in different trial conditions designed by Taguchi DOE(M-9 array) method

This variability within the different trial conditions were further used to predict the severity index which may provide an understanding of overall process analysis. The interaction severity index between the factors was described in Table-2. Total 6 interaction pairs were identified. The maximum SI value of 73.46% was obtained in the case of Lactose and $MgSO_4$ and minimum SI value of 8.16% was observed in Lactose and Tryptone pair. All the other interaction pairs have an intermediate value of severity index.

Table 3: Analysis of variance of optimization by Taguchi DOE(M-9)

S.No.	Factors	Sum of squares (S)	Variance (V)	F-Ratio (F)	Pure sum (S')	Percent P(%)
1	Lactose	1744.468	872.234	24.922	1674.474	6.241
2	Tryptone	2211.098	1105.549	31.589	2141.103	7.981
3	NH_4Cl	19186.1	9593.05	274.108	19116.106	71.259
4	$MgSO_4$	3369.467	1684.733	48.139	3299.473	12.299
Other errors		2.22				
Total		26826.111				100.00%

The analysis of variance (Table-3) was used to analyze the significant levels of selected factors and their relative contribution of L-asparaginase production. The Analysis Of Variance (ANOVA) is a powerful and common statistical procedure to identify the effect of individual factors. The F-ratio was used to determine the degree of variation contributed by each factors[21]. Ammonium chloride has the maximum F-ratio of 274.108 and the minimum F-ratio of 24.922 was observed in case of Lactose. All factors and their respective interactions considered in the experimental design showed statistically significant effects at 95% confidence limit. The analysis of variance also revealed about the percentage contribution of selected parameters (Table-3) on enzyme production that varied from factor to factor. The percentage contribution of significant factors on performance was also graphically indicated in Fig.2 where Ammonium chloride (NH_4Cl) proved to be the most influential factor with percentage contribution of 71.259% , while Lactose showed minimum impact (6.241%) on the recombinant L-asparaginase production.

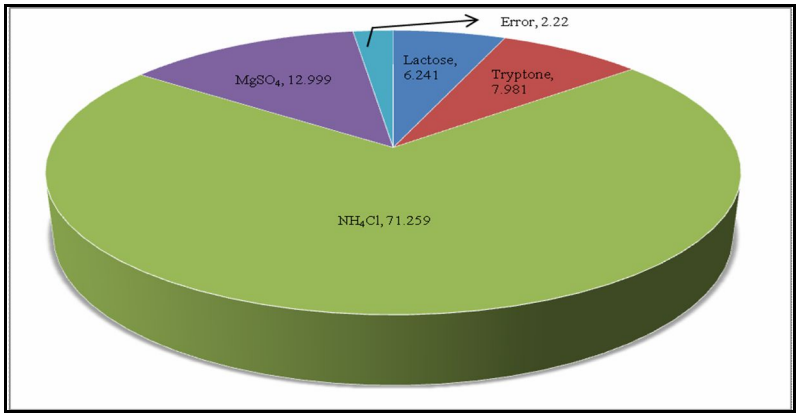

Fig. 2. Percentage contribution of significant factors on performance

The orthogonal array based analysis proposed the optimum conditions of various factors are given in table-4. As described in the Table-4, Lactose at 1%, Tryptone at 1%, Ammonium chloride at 0.25% and Magnesium sulphate at 0.25% gave the best result for the enhanced production of recombinant L-asparaginase. The expected enzyme production of 271.663 U/ml was proposed by the model. The proposed optimized conditions by factorial design were validated by performing the experiment according to the designed condition. After validation, an enhanced enzyme production of 270.122 U/ml was obtained with 35% increase in the yield (Fig.3).

Table 4: Optimum conditions and performance

S.No.	Factors	Level	Level description	Contribution
1	Lactose	2	1	13.888
2	Tryptone	2	1	15.555
3	NH_4Cl	3	0.25	33.055
4	$MgSO_4$	3	0.25	13.888
Total contribution from all factors		76.386		
Current Grand Average of performance		195.277		
Expected result at Optimum condition		271.663		

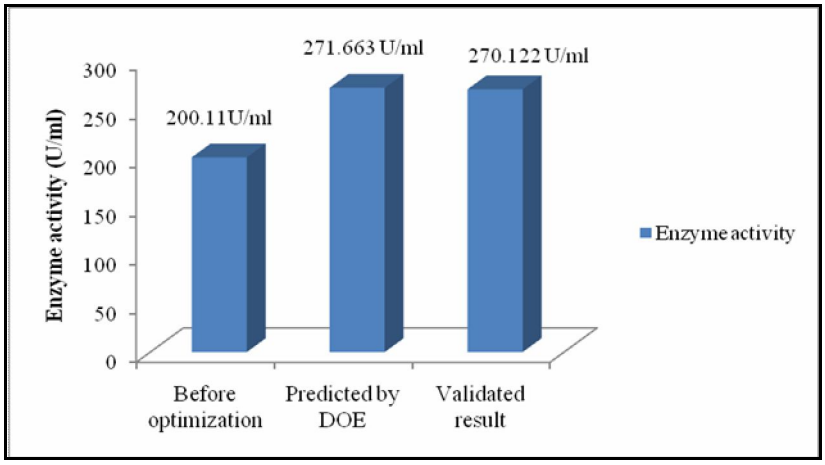

Fig. 3. Comparison of the recombinant enzyme yield before and after validation

This is the first report on media optimization of recombinant L-asparaginase production of *Pseudomonas fluorescens*. Till now only one report was available on the optimization of L-asparaginase production by *Pseudomonas fluorescens* utilizing this statistical method[13]. Prakasham et al.,[11] reported optimization of L-asparaginase production by *Staphylococcus sp*. using the Taguchi DOE method. However,

the application of Taguchi DOE to enhance the production of other enzyme has been reported in few literatures. Optimization of Laccase production[20] and Xylitol production[9] were some of the reports available on some of the other enzymes production.

Conclusion:

L-asparaginase has been known for its chemotherapeutic potential. Owing to its serious side effects and a quite expensive production, processes are needed to optimize the media components for the enhanced production of the recombinant version of this enzyme. In the present report the media components of the recombinant L-asparaginase from the bacteria *Pseudomonas fluorescens* were optimized by a statistical method of Taguchi DOE (M-9) array. In the Taguchi DOE method combined effect of many factors can be studied simultaneously. On the other hand optimization by conventional one factor at a time approach effect of only one factor can be studied at time which is quite a lengthy and time-consuming method. The analysis of the results of different trial condition designed by Taguchi DOE method gave a 35% enhanced production of the recombinant L-asparaginase production from *Pseudomonas fluorescens*.

Acknowledgement:

The authors are highly grateful to the Dr. Santosh Kumar Jha, Department of Bio-Engineering, BIT Mesra, Ranchi for providing the necessary support and to the Centre of Excellence (COE)-TEQIP-II for providing the financial assistance.

References:

1. Jha SK, Pasrija D, Sinha RK, Singh HR, Nigam VK, Vidyarthi AS. Microbial L-asparaginase: A review on current scenario and future prospects. Int J Pharma Sci Res., 2012, 3(9): 3076-3090.
2. Ebrahiminezhad, Rasoul-Amini S, Ghasemi Y. L-Asparaginase production by moderate halophilic bacteria isolated from maharloo salt lake. Ind J Microbiol., 2011, 51(3): 307–311.
3. Swain AI, Jaskolski M, Housset D, Mohana Rao JK, Wlodawer A. Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc Natl Acad Sci USA., 1993, 90: 1474–1478.
4. Manna S, Sinha A, Sadhukhan R, Chakrabarty SL. Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. Curr Microbiol., 1995, 30: 291–298.
5. Oettgen HF, Old LJ, Boyse EA, Campbell HA, Philips FS, Clarkson BD, Tallal L, Leeper RD, Schwartz MK, Kim JH. Inhibition of leukemias in man by L-asparaginase. Cancern Res., 1967, 27: 1619–1631.
6. Story MD, Voehringer DW, Stephens LC, Meyn RF. L-asparaginase kills lymphoma cells by apoptosis. Cancer Chemoter Pharmacol., 1993, 32: 129–133.
7. Mashburn LT, Wriston JC Jr. Tumor inhibitory effect of L-asparginase from *Escherichia coli*. Arch Biochem Biophys., 1964, 105: 451–452.
8. Oettgen HF, Stephenson PA, Schwartz MK, Leeper RD, Tallai L, Tan CC, Clarkson BD, Golbey RB, Krakoff IH, Karnofsky DA, Murphy ML, Burchenal JH. Toxicity of *E. coli* L-asparaginase in man, Cancer., 1970, 25: 253–278.
9. Sreenivas Rao R., Prakasham RS, Krishna Prasad K, Rajesham S, Sharma PN, Venkateswar Rao, L. Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. Process Biochem., 2004, 39: 951–956.
10. Prakasham RS, Subba Rao Ch, Sreenivas Rao R, Rajesham S, Sarma PN. Optimization of alkaline protease production by *Bacillus* sp. using Taguchi methodology. Appl Biochem Biotechnol., 2005b, 120: 133–144.
11. Prakasham RS, Subba Rao Ch, Sreenivas Rao R, Sarma PN. Enhancement of acid amylase production by an isolated *Aspergillus awamori*. J Appl Microbiol., 2007, 102(1): 204-211.
12. Abdel-Fattah YR, Olama ZA. L-asparginase production by *Pseudomonas aeruginosa* in solid state culture: Evaluation and optimization of culture conditions using factorial designs. Process Biochem., 2002, 38:115–122.

13. Jha SK, Pasrija D, Singh HR, Nigam VK, Vidyarthi AS. Batch production and media optimization of anti-leukemic L-asparaginase from *Pseudomonas fluorescens* by Taguchi DOE methodology. J Appl Pharma Sci., 2014, 4(12): 085-089.
14. Bergmeyer HU, Beutler HO. Ammonia, in: Bergmeyer HU, Bergmeyer J, Graßl M. (Eds.), Methods of Enzymatic Analysis, Vol.VIII, Academic Press, New York, 1985, pp. 454-461.
15. Chauhan B, Gupta R. Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Process Biochem., 2004, 39: 2115–2122.
16. Beg QK, Sahai V, Gupta R. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem., 2003, 39: 203–209.
17. Dasu VV, Panda T, Chidambaram M. Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's method. Process Biochem., 2003, 38: 877-880.
18. Roy RK. A primer on the Taguchi method. Society of Manufacturing Engineers, Michigan. 1990.
19. Karanam SK, Medicherla NR. Application Doehlert experimental design for the optimization of medium constituents for the production of L-asparaginase from Palm Kernal cake (*Elaeis guineensis)*. J Microbial Biochemical Technol., 2010, 2**:** 007-012.
20. Prasad K, Venkata MS, Sreenivas R, Pati BR, Sarma PN. Laccase production by *Leurotuso streatus*1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem Eng., 2005, 24: 17-26.
21. Armstrong RA, Hilton AC. The use of analysis of variance (ANOVA) in applied microbiology. Microbiologist., 2004, 18–21.

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