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Research Article

Enhanced Recovery of L-Glutaminase by the Optimization of A Three-Phase Partitioning System Using the Taguchi Doe Methodology

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ABSTRACT

L-Glutaminase is an anti-leukemic enzyme produced by number of microbes. The three-phase partitioning method of Iso-octane followed by the ammonium sulphate precipitation was used to purify the L-glutaminase produced by *Pseudomonas fluorescens*. The selected process parameters viz. concentration of ammonium sulphate, Iso-octane to broth ratio, temperature and pH were optimized by Taguchi orthogonal array design of experiment (DOE). Overall, 28.19%, enhanced recovery of L-glutaminase was achieved after the optimization of selected factors.

Keywords: L-Glutaminase, Taguchi DOE, Acute Lymphoblastic Leukemia (ALL), Three Phase Partitioning, *Pseudomonas fluorescens*.

INTRODUCTION

L-Glutaminase (E.C.3.5.1.2) is a naturally occurring enzyme with anti-leukemic characteristics, mainly against acute lymphoblastic leukemia (ALL). The enzyme catalyses the hydrolysis of L-glutamine into L-glutamic acid and ammonia. The principle behind the use of L-glutaminase as an anti-tumor agent is that it deprives the leukemic cells from a non-essential amino acid glutamine, which they cannot synthesize by their own, whereas normal cells can synthesize their own glutamines with the action of glutamine synthetase[1]. The downstream processing of the bioproducts from the fermentation broth is a challenging and costlier affair. The present study aims towards the economical downstream processing of the enzyme. The downstream processing of therapeutic proteins followed in the industry is a cumbersome and tedious process, which requires a lot of resources, time and pre-treatment of broth. However, as an alternative solution to these problems the Three Phase Partitioning (TPP) method can be adopted[2]. The TPP is based upon the principles like co-solvent precipitation, salting out, kosmotropic precipitation, iso-ionic precipitation, etc. The three phase partitioning can be performed directly with the crude sample and it is also an easily scalable method. The performance of the process can be further improved by the optimization of the process parameters. Conventional optimization, based on "single factor at a time" is a shotgun approach where each parameter is considered insensitive to the other process variables. It is always beneficial to determine the most important factors, interaction and influences of different factors for any multivariable process or system. The conventional methods are cumbersome, time-consuming, requiring larger data sets or experimental trial conditions and do not provide any information regarding the mutual interactions between the factors [3]. In the present investigation, the TPP was used to recover of L-glutaminase from the fermentation broth of *Pseudomonas fluorescens* (MTCC 103). The analysis of selected factors via conventional and statistical method, the purification of L-glutaminase by TPP can be enhanced. Abdel-Fattah *et al.*, (2005)[4] demonstrated that the combined effect of many factors could be studied simultaneously using statistical methods. Among the various available methods, Taguchi DOE is a method of choice. Taguchi DOE can analyze the effect of several influential factors on the overall productivity of the process. The optimum quantitative information can be extracted by performing minimum experimental trials by the Taguchi DOE. Hence, in the present investigation the four factors like concentration of ammonium sulphate, ratio of Iso-Octane to the fermentation broth, pH and temperature were selected for optimization by using Taguchi DOE.

MATERIALS AND METHODS

Microorganism and culture conditions

Cultures of *Pseudomonas fluorescens* (MTCC 103) were obtained from the departmental culture collection of Birla Institute of Technology, Mesra, Ranchi, Jharkhand (India). It was sub-cultured on the Luria-Bertani agar and stored at 4^0C for further use. The batch production of L-glutaminase was carried out in 500 mL Erlenmeyer flasks containing 100 mL of sterile medium constituting 0.7% glucose, 0.1% K_2HPO_4, 0.5% tryptone and 0.5% yeast extract supplemented with 0.1% L-glutamine. The pH of the resulting medium was adjusted to 8. The production medium was inoculated by transferring the loopful of cells from freshly sub-cultured *Pseudomonas fluorescens* (MTCC 103). The fermentation was carried out at 37^0C on a rotary shaker at 180 rpm for 24 hours.

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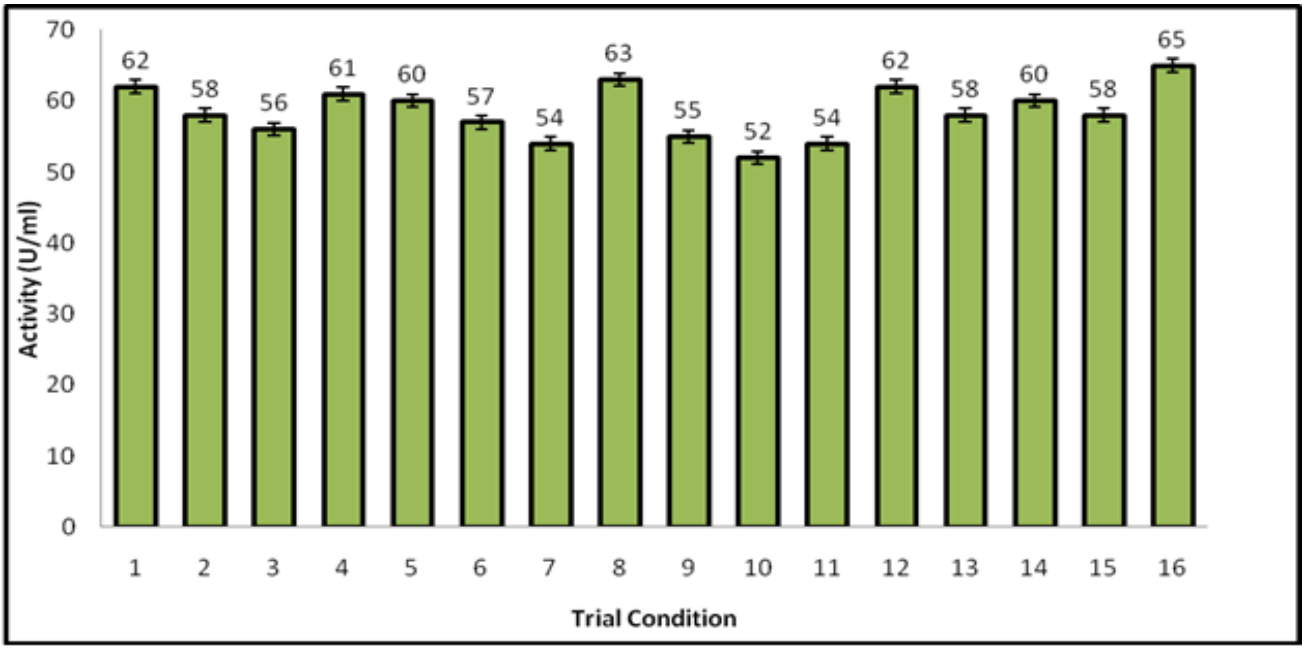

Fig. 1: Variability in enzyme activity between different trial conditions

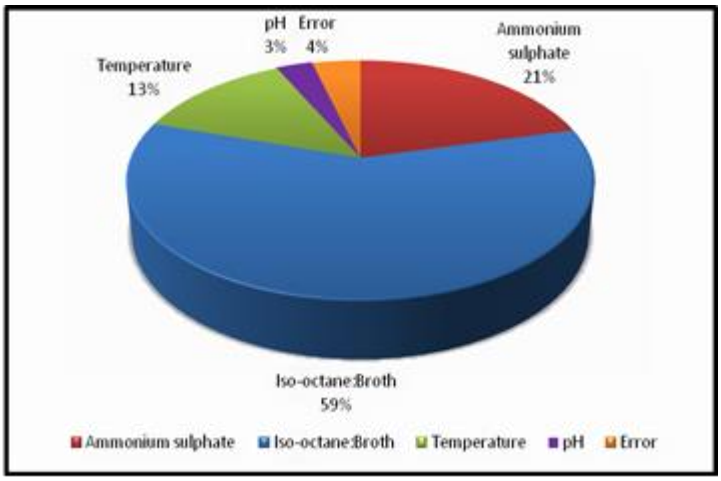

Fig. 2: The percentage influence of significant factor on process

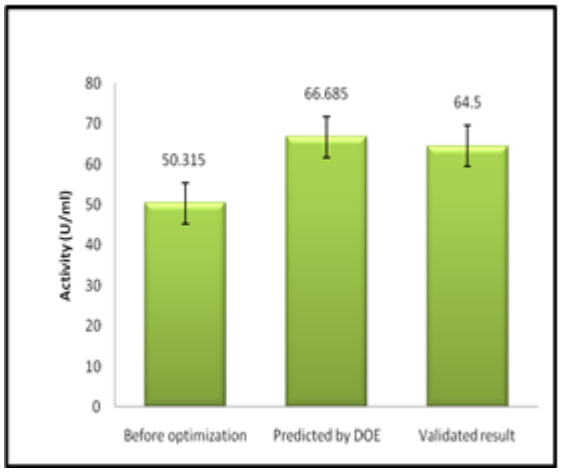

Fig. 3: Comparison of the enzyme activity before and after the optimization of TPP

Estimation of enzymatic activity

Activity of L-glutaminase in the fermentation broth and during TPP was estimated by Nesselerization assay method of Shirfrin *et al.,* (1974)[5]. The ammonium chloride solution was used as standard to estimate the amount of ammonia evolved during the L-glutaminase activity. Protein content in the fermentation broth was determined by dye binding method by using bovine serum albumin (BSA) as a standard[6].

Three-Phase Partitioning of L-glutaminase

The fermentation broth was centrifuged at 10000 rpm at 4^0C. The supernatant of fermentation broth of *Pseudomonas fluorescens* was used as a crude sample and the pellets were discarded. Three phase partitioning was performed by adding the desired amount of ammonium sulphate to the crude sample and was mixed gently to dissolve the salt completely followed by adjusting the pH to the required value. To achieve the desired ratio of Iso-octane to broth required amount of Iso-octane was added to it and was incubated at room temperature for one hour. In order to separate the phases the mixture was centrifuged at 4^0C (2000 rpm) for 10 minutes. The mixture was incubated till the formation of three phases. The enzymatic activity in the interface precipitate (middle), organic (top) and aqueous (bottom) phase was calculated.

Optimization by Taguchi Design of Experiment (DOE)

Taguchi DOE methodology was used for the optimization of TPP. L-16 orthogonal array was employed for the optimization of selected factors. The four factors like ammonium sulphate concentration, ratio of Iso-octane to broth, pH and temperature were selected for optimization as they significantly affect the yield of desire enzyme[7]. Table-1 represents the different levels of selected factors. L-16 orthogonal array designed total 16 trial conditions for the optimization of TPP (Table-2). The severity index, analysis of variance (ANOVA) etc. was used to predict the optimized condition. The proposed optimized

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

Factors	Level-1	Level-3	Level-3	Level-4
Ammonium sulphate (% w/v saturation)	30	40	50	60
Iso-Octane:Broth ratio	1:0.85	1:1	1:1.15	1:1.25
pH	5	6	7	8
Temperature (^{0}C)	27	37	45	55

Table 2: L-16 orthogonal array for different trial conditions

Factors	Trial Conditions															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ammonium sulphate	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	4
Iso-Octane:Broth	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
pH	1	2	3	4	2	1	4	3	3	4	1	2	4	3	2	1
Temp (^{0}C)	1	2	3	4	3	4	1	2	4	3	2	1	2	1	4	3

Table 3: Main effect of the selected factors at various levels

S. No.	Factors	Level 1	Level 2	Level 3	Level 4
1	Ammonium sulphate	59.25	58.5	55.75	60.25
2	Iso-Octane: Broth	58.75	56.75	55.5	62.75
3	Temperature	59.5	59.5	58.5	56.25
4	pH	59.5	58.25	58.25	57.25

conditions were validated under the same experimental protocol. All experiments were carried out in triplicates.

RESULTS AND DISCUSSION

The enzymatic activity obtained during the trial conditions were used to predict the optimum level of selected factors. The variability obtained during all the 16 trial conditions is represented in figure-1. It was found that the enzyme recovery was very much dependent on the trial conditions. These variabilities were analyzed by orthogonal array method to estimate the main effect of all the factors at different level (Table-3). At different levels the difference between the average values of each factor indicates the relative influence of the effect on the recovery of L-Glutaminase by TPP. An understanding of overall process analysis may be provided by interaction between the two factors. Severity index (SI) represents the significant interaction between the selected factors. Table 4 shows the estimated interaction between the factors by their severity index (SI).The SI value of 100% indicates a 90^0 angle between the factors interaction lines while 0% SI for parallel lines[8,9,10]. The maximum severity index of 50% for ammonium sulphate and pH indicated that these factors interact with each other most significantly in comparison to others during the partitioning of L-Glutaminase by TPP. The minimum severity index of 3.84% was estimated between ammonium sulphate and Iso-octane: broth. The influential effect of ammonium sulphate may be due to its kosmotropic effect. In the salting out or kosmotropic precipitation of the desired protein, the ammonium sulphate plays important role. The concentration of ammonium sulphate was selected such that it should be less than the concentration causes the 'salting out' of any protein, so as to obtain maximum recovery of the enzyme as interfacial precipitate[11]. The contribution of all factors to the performance of the process may be represented by sum of square and percentage through analysis of variance (ANOVA). ISO-octane: broth ratio having a maximum sum of square of 120.687, while temperature having least value of 6.687. All other factors having an intermediate value of sum of square (Table-5). The influences were predicted by using the analysis of variance. 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. The F-ratio was used to determine the degree of variation contributed by each factors[12]. The contribution of Iso-octane: broth ratio, ammonium Sulphate, temperature and pH on the recovery of L-glutaminase were 59 %, 21%, 13% and 3% respectively (figure-2). Table 6 shows the optimized conditions for purifying L-Glutaminase by TPP. After the optimization 28.19% enhanced recovery of L-glutaminase was observed. 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 the expected activity of 66.685 U/ml for *Pseudomonas fluorescens* which was found to be 64.5 U/ml after the validation of results. The optimum conditions show that 1:1.25 ratio of Iso-octane to crude in combination with 60% (w/v) ammonium sulphate at pH 5 and temperature 27^0C gave the best result. The result obtained after performing TPP in optimized condition was 64.5 U/ml of enzymatic activity with enhanced recovery of 28.19%.

CONCLUSION

The three phase based purification may be considered as a promising method for the enhanced recovery of protein and reducing the overall cost of the process. It was

Table 4: Estimated interaction between the factors (Severity Index)

S.No.	Interacting factor pairs (order based on SI)	Columns	SI (%)	Opt.
1	Ammonium sulphate x pH	1x4	50	[4,1]
2	Ammonum Sulphate x Temp.	1x3	26.92	[4,1]
3	Temp. x pH	3x4	15.38	[1,1]
4	Iso-Octane: Broth x Temp.	2x3	11.53	[4,1]
5	Iso-Octane: Broth x pH	2x4	7.69	[4,1]
6	Ammonium sulphate x Iso-Octane: Broth	1x2	3.84	[4,4]

Columns – represents column locations to which the interacting factors are assigned; SI – Interaction Severity Index; Opt. – Indicates factor levels desirable for the optimum condition

Table 5: Analysis of Variance (ANNOVA)

S.No.	Factors	DOF	Sum of Squares	Variance	F-ratio	Pure Sum	Percent
2	Iso-Octane:Broth	3	120.687	40.229	71.518	119	58.929
1	Ammonium Sulfate	3	44.684	14.895	26.481	43	21.293
3	pH	3	28.187	9.395	16.702	26.5	13.1722
4	Temperature	3	6.687	2.229	3.962	5	2.476
Other/Error		3	1.687	0.562			4.18
Total		15	201.937				100.00%

Table 6: Optimum condition for performance of TPP

Factors	Level Description	Optimum Level	Contribution
Ammonium sulphate	60	4	1.812
Iso-Octane: Broth ratio	1:1.25	4	4.312
Temperature (^{0}C)	27	1	1.062
pH	5	1	1.062
Total contribution from all factors			8.247
Current grand average of performance			58.437
Expected result at optimum condition			66.685
% increase in performance			28.19

estimated that 70-80% cost of the process is due to cumbersome and time consuming downstream processing. The present study is a justification of the TPP method by the 28.19% enhanced recovery of the L-glutamine. Along with a number of advantages, the process can be easily scalable.

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REFRENCES

1. Elshafei AM, Hassan MM, Ali NH, Abouzeid MA-E, Mahmoud AD, Elghonemy DH. Purification, Kinetic Properties and Antitumor Activity of L-Glutaminase from *Penicillum brevicompactum* NRC 829. British Microbiol Res J. 2014; 4(1):97-115.
2. Bracewell DG, Balasundaram B, Harrison S. Advances in product release strategies and impact on bioprocess design. Trends in Biotechnol. 2009; 27:477-485.
3. 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.
4. Abdel-Fattah YR, Saeed HM, Gohar YM, El-Baz MA. Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochem. 2005; 40:1707–1714.
5. Shirfrin S, Parrott CL, Luborsky SW. 4. Substrate binding and intersubunit interactions in L-asparaginase. J Biological Chem. 1974; 249:1335-1340.
6. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248–254.
7. Jha SK, Pasrija D, Singh HR, Nigam VK, Vidyarthi AS. Enhanced recovery of L-Asparaginase by the optimization of three phase partitioning system by Taguchi DOE methodology. Bio Processing Journal. 2013; 11:40-45.
8. Han JJ, Yang TH, Rhee JS. Optimization of reaction variables sucrose monoester production using lipase in a solvent free system by Taguchi's method. Biotechnol Tech. 1998; 12 (4):295-299.

9. Dasu VV, Panda T, Chidambaram M, (2003) Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's methods. Process Biochem38:877-880.
10. Koo TY, Lin IP, Liu HR, Chou CY. Determination of nattokinase production condition using Taguchi parameter design. Food Sci Technol Int. 2006; l12(3):215-22.
11. Dennison C, Lovrien R. Three phase partitioning: Concentration and purification of proteins. Protein Expr Purif. 1997; 11(2):149–161.
12. Armstrong RA, Hilton AC. The use of analysis of variance (ANOVA) in applied microbiology. Microbiologist. 2004; 5(4):18–21.