Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(12):839-843

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization of recombinant L-asparaginase purification by *Escherichia coli* K-12 using three phase partitioning system by taguchi design of experiment

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ABSTRACT

L-Asparaginase (E.C. 3.5.1.1) is the enzyme with anti-tumour activity and is well accepted as a chemotherapeutic agent against the acute lymphoblastic leukemia and lymphosarcoma. Present investigation revealed the application of three phase partitioning (TPP) along with Taguchi design of experiment to easier and scalable method for the enhanced purification of recombinant L-asparaginase. The recombinant L-asparaginase enzyme was produced by the over-expression of ansB gene of E. coli K-12 in E. coli BL21. The purification of enzyme from crude extract was carried out by TPP method. The purification level was further enhanced by using Taguchi design of experiment orthogonal array. The purification of recombinant enzyme under optimized condition has enhanced 67.26% yield with fold purification of 7.33. The maximum activity of 208.5 U and specific activity of 49.64 U/mg was identified in interfacial precipitate after the second round of partitioning. Three phase partitioning is an economical and easily scalable method for the purification of enzyme. The recombinant L-asparaginase was purified to higher homogeneity by this method. This method may be helpful in the overcoming the various problems associated with the cumbersome downstream processing.

Keywords: L-asparaginase**,** Three phase partitioning, Taguchi design of experiment, fold purification.

INTRODUCTION

L-Asparaginase (E.C. 3.5.1.1), also known as L-asparagine amidohydrolase is the enzyme with anti-tumour activity and is well accepted as a chemotherapeutic agent against the acute lymphoblastic leukemia and lymphosarcoma. L-Asparaginase is a polymeric protein that deaminates asparagine and glutamine to asparatic acid and glutamic acid respectively, with the liberation of ammonia [1]. L-Asparaginase has its application in the food industry also. It helps in reducing the content of acrylamide in baked food products by hydrolyzing the L-asparagine [2].

The bioseparation of enzymes from the multi-component system viz. microbial fermentation broth is an expensive and time consuming practice. Most of the methods developed for this purpose are costly, time-consuming and need pre-treatment of broth. Present study focus on the application of three phase partitioning system (TPP) for the purification of recombinant L-asparaginase. This method may be considered as one alternative to the problems associated with the other methods. The TPP technique is based on the principles of salting out, co-solvent precipitation, isoionic precipitation, and kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [3].

EXPERIMENTAL SECTION

Bacterial Strain and Expression Vector: *Escherichia coli* K-12 was used as the source of L- asparaginase gene (ansB). The *Escherichia coli* BL21[F^-ompThsdS_B (rB^-mB^-) gal dcm, DE3] and pET 29a(+) (5.4 Kb, Kan^r, T7

promoter, lacO.C-terminal 6x His-tag) were used as the expression host and plasmid system for the over-expression of ansB gene under the T7 inducible promoter. *Escherichia coli* DH5α and pUC19 [2.6 Kb, Amp^r, *lacZ* (M13mp18/19),Isolated from *E. coli* (dam^+, dcm^+)] were used for maintenance of recombinant plasmid.

Preparation of recombinant construct and over-expression of enzyme: The complete cds (accession# M34234) of L-asparaginase II (ansB) gene sequence of *Escherichia coli K-12*, was retrieved from the gene bank database of NCBI (http://www.ncbi.nlm.nih.gov/nuccore/M34234). The forward (5'-GCGGATCCAACAGCCATCGCAGCAAT-3') and backward primer (5'-GGCGAATTCTGGTCGTAAGACCGATATG-3') pairs were designed on the basis of primer designing guidelines of Dieffenbach et al. (1995) [4] for the PCR amplification of the gene. The restriction site for BamH1and EcoR1, i.e. present at the multiple cloning site of expression vector is introduced in the sets of primer for the directional cloning. The positive clones were grown in shake flask in fermentation medium having NaCl (10 g/L), tryptone (10 g/L) and yeast extract (5 g/L) with Kanamycin (50µg/ml). The initial pH and temperature was maintained at 7.2 and 37 ^{0}C respectively. The culture was incubated for overnight with constant agitation of 200 rpm. The culture at O.D. 0.7-0.8 at 600 nm was used as a seed culture to inoculate in 50 ml of medium and grown at 37 ^{0}C for 24 h in orbital shaker at 200 rpm. The final concentration of 0.1mM IPTG was used to induce the culture for over-expression of the recombinant L-asparaginase. The cells were harvested after 20 h of post induction by centrifugation at 10000 rpm for 10 min at 4^{0}C. The enzymatic activity was analysed in extracellular fractions.

Enzyme assay: The recombinant L-asparaginase activity in fermentation broth and during the entire process of three phase partitioning was estimated by the nesslerization. The enzymatic activity was measured in term of micromoles of ammonia produced by the hydrolysis of L-asparagine by L-asparaginase [5]. Protein concentration was determined by the dye binding method of Bradford et al. (1976) [6]

Purification by TPP and Optimization by Taguchi DOE: The soluble fraction of fermentation broth was separated by the centrifugation at 10000 rpm at 4^{0}C. The pellets were discarded and supernatant was used as crude sample. The three phases partitioning of recombinant enzyme was initiated by adding desired amount of ammonium sulphate to 5 ml of crude extract with pH adjustment. The salt was dissolved completely by vortexing. The required amount of t-butanol was added to achieve the initial 1:1 ratio with fermentation broth (crude extract). The mixture was centrifuge at 2000 rpm for 10 minutes and incubated at 4^{0}C for one hour to develop the phases [7]. The enzymatic activity was assayed in interfacial precipitate and aqueous phase. Most of the enzymatic activity was recorded in aqueous phase, so to enhance the purification level the TPP was optimized by using Taguchi design of experiment (DOE) with repeated partitioning [8].

The optimization of L-asparaginase purification during the second round of TPP was optimized by using L-16 orthogonal array of DOE with fifteen degree of freedom. Four significant factors at four levels were selected for the optimization i.e. ammonium sulphate concentration, ratio of t-butanol-fermentation broth, pH and incubation temperature (Table-1). Total 16 trial conditions were designed for the optimization. The variability of enzyme activities within these designed conditions (Figure-1) were used to predict the optimum levels of selected factors. The analysis of variance (ANOVA), significant factors influence and interaction pattern were analysed. The proposed optimized experimental conditions were further validated to access the accuracy of the DOE.

RESULTS AND DISCUSSION

The first round of partitioning was carried out by using the 30% (w/v) ammonium sulphate saturation level in the fermentation broth with 1:1 ration of t-butanol:broth ratio. After the estimation of L-asparaginase activity, it was found that approximately 92% of activity was retained in aqueous phase. So the second round of partitioning was carried out under the designed trial conditions to precipitate most of the recombinant L-asparaginase at interphase.

A broad range of variability in enzymatic activity was observed in 16 trial conditions (Figure-1). The maximum enzymatic activity of 155 U/mL and minimum activity of 108 U/mL was observed in trial condition of 1 and 13 respectively. The variability in enzymatic activity within these trial condition indicates that the purification of recombinant enzyme govern by the number of interacting factors. The significant interaction between the selected factors may be represented in term of severity index (SI). Table-2 represents the estimated interaction between the factors by their severity index. The maximum severity index of 47.11% for ammonium sulphate and temperature indicated that these factors interact with each other most significantly in comparison to others during the partitioning of recombinant enzyme. The ammonium sulphate play important role in the salting out or kosmotropic precipitation of the desired protein. These phenomena are dependent on the temperature. The protein precipitation ability of ammonium sulphate is a complex phenomena associated with the interplay between solute-solvent interaction and

water perturbation effects [9]. The minimum severity index of 18.26% was estimated between ammonium sulphate and pH. All other interaction pairs having intermediate value of severity index.

The interaction between the pair of selected factors were further used to predict the influence of individual factor at the partitioning of recombinant L-asparaginase by three phase system. The influences were predicted by using the analysis of variance. The analysis of variance is the most suitable method to analyse the more complex data set with the estimation of effect of various factors and interaction between them [10]. The statically significant effect of each factors were estimated by, mean square, F-ratio and percentage with three degree of freedom (Table-3) at 95% confidence limit. pH having highest contribution of 40.836% in total sum of squares, whereas the ammonium sulphate and temperature having second and third highest contribution of 33.926% and 16.005% respectively. t-Butanol: Broth ratio having the lowest contribution (9.144%) in the total sum of squares. The highest percentage of pH reflects the most influential effect of it on the partitioning of recombinant L-asparaginase. While the t-Butanol: Broth ratio having the least ability to influence the process. Same conclusion may be withdraw from the F-ratio. The largest F-ratio (5.162) of pH again proved that it has largest effect on the process in comparison to the error mean square or variance. Although it has largest ability to influence the purification of enzyme, yet the other factors also play significant role it. The percentage contribution of all factors are represented as the pie chart in figure-2. The severity index and analysis of variance was further used to interpret the optimum level of all the selected factors. The 45% (w/v) ammonium sulphate (level-2), 1:1.2 ratio of t-butanol to broth (level-4) at pH 7 (level-3) and temperature 45 (level-3) were found optimum for the better partitioning of recombinant L-asparaginase. The proposed optimized condition was validated by performing the three phase partitioning of enzyme at the proposed level of selected factors. The orthogonal array based optimization proposed the 210.6 U of enzymatic activity after the second round of TPP. There was 60.76 % enhanced recovery was estimated. The validated result purify the enzyme till 208.5 U with 67.26% of enhancement of yield. After the complete optimization there was 9.33 fold increase in specific activity. The specific activity increases from 5.32 U/mg to 49.64 U/mg in the interfacial precipitate in comparison to crude extract (Table-5). Figure-3, gives the graphical comparison between the enzymatic activity in crude extract before and after the optimization with the result predicted by the DOE. The validated result (208.5 U) was found to be very close to the predicted result (210.6 U).

Table 1: Factors and their level selected for the optimization

Factors	Level 1	Level 2	Level 3	Level 4
Ammonium Sulphate (% w/v saturation)	30	45	50	55
t-Butanol: Broth	1:0.9	1:1	1:1.1	1:1.2
pH	5	6	7	8
Temperature (^oC)	30	35	40	45

Table 2. Estimated interaction between the factors in term of Severity Index (SI)

Sl. No.	Interacting Factor Pairs (Order based on SI)	Columns	SI %	Opt.
1.	Ammonium Sulphate x Temperature	1 x 4	47.11	[2,3]
2.	t-butanol : Broth x pH	2 x 3	43.26	[4,3]
3.	pH x Temperature	3 x 4	33.65	[3,3]
4.	Ammonium Sulphate x t-butanol : Broth	1 x 2	24.03	[2,4]
5.	t-butanol : Broth x Temperature	2 x 4	21.15	[4,3]
6.	Ammonium Sulphate x pH	1 x 3	18.26	[2,3]

Table 3. Analysis of Variance (ANOVA)

Sl. No	Factors	Sum of Squares	Variance	F-Ratio	Pure Sum	Percent
1.	Amm. Sulphate	1389.5	463.166	4.288	1065.5	33.926
2.	t-Butanol: Broth	374.5	124.833	1.155	50.5	9.144
3.	pH	1672.5	557.5	5.162	1348.5	40.836
4.	Temperature	655.5	218.5	2.023	2.025	16.005
Other/Error		3.562	1.873	--	--	0.089
Total		4095.562				100.00%

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

Sl. No.	Factors	Level	Level Description	contribution
1.	Ammonium Sulphate (%w/v) saturation)	2	45	15.75
2.	t-Butanol: Broth	4	1:1.2	4.75
3.	pH	3	7.0	16.5
4.	Temperature (^{o}C)	3	45	7.102
	Total contribution from all factors			44.102
	Current Grand Average of Performance			131
	Expected Result at Optimum Condition			210.6
	Percentage Increase in Performance			60.76

Table 5. Summary of L-Asparaginase Purification by Three Phase Partitioning

Steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Fermentation Broth (crude)	310	58.2	5.32	100	1
Interfacial Precipitate-Round-01	24.4	6.7	16.87	7.9	-
Interfacial Precipitate-Round-02 (Under Optimized Conditions)	208.5	4.2	49.64	67.26	9.33
Aqueous Phase-Round-02	9.95	50.6	0.197	3.20	-

Note: The first round of precipitate was obtained by the 30% saturation and 1:1 ter-butanol and fermentation broth ratio. Second rounds were performed under the different trial conditioned. The reported result in the table represents the completely optimized process obtained by the validation of the result. Each experiments were carried out in triplicates.

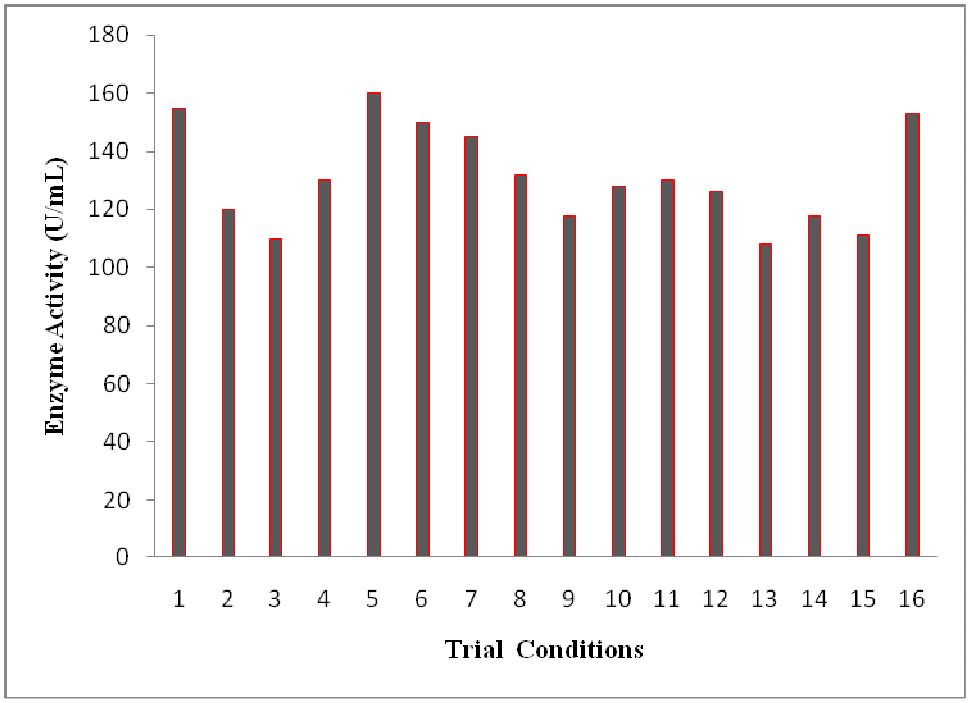

Figure 1- Variability in enzyme activity within designed trial conditions

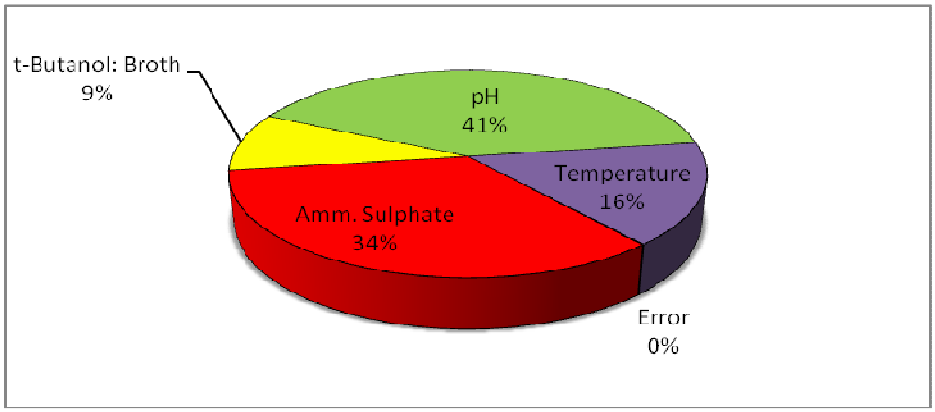

Figure 2- Percentage influence of selected factors on production of recombinant L-asparaginase

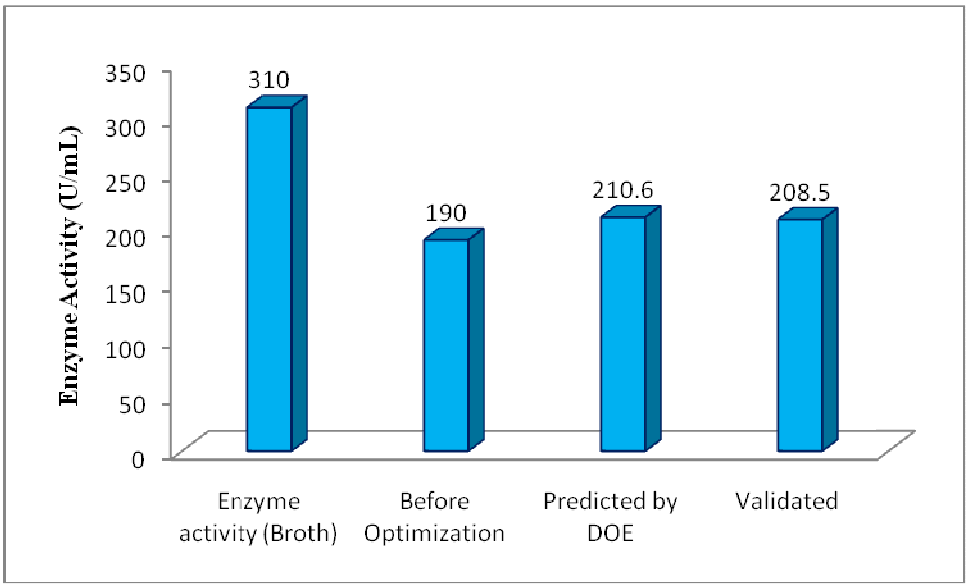

Figure 3- Comparison of recombinant L-asparaginase activity before and after the optimization

CONCLUSION

It was estimated that the 70-80% of the process cost is associated with the downstream processing of the product. The three phase partitioning based technique may be a better alternative for the economical and easily scalable method for the purification of recombinant enzyme to higher level of purity. The process can be further improve by the application of statistical methods viz. Taguchi design of experiment. Present investigation justify the applicability of the method by the 67.26% enhanced yield of enzyme with fold purification of 7.33. The higher yield and specific activity obtained during the process confirm that TTP in combination with Taguchi design of experiment may be a method of choice for the higher level of purification of recombinant enzyme. This method having an additional advantages of reducing the number of steps during the bioseparation of enzyme by reducing the unwanted proteins in broth.

Acknowledgement
The authors also gratefully acknowledge the Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi for providing infrastructure, chemicals and instrumentation facilities.

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