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RESEARCH ARTICLE

Optimization of Three-Phase Partitioning system for Enhanced Recovery of L-Asparaginase from *Escherichia Coli* K12 Using Design of Experiment (DOE)

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Abstract

L-Asparaginase has been shown to possess anti-leukemic characteristic, mainly against Acute Lymphoblastic Leukaemia (ALL). L-Asparaginase produced by *Escherichia coli* K12 was purified by Three Phase Partitioning method of Iso-Octane in presence of ammonium sulphate. The factors like ammonium sulphate concentration, ratio of Iso-Octane to fermentation broth, temperature and pH which significantly affects the yield of desired enzyme during Three Phase Partitioning were selected for optimization. The purification of L-Asparaginase was optimized by use of Taguchi Design of Experiment methodology. L-16 array was selected for the purpose of optimization of TPP based purification. The interactions of different parameters were studied and they were used to formulate the optimized condition for the purification of L-Asparaginase. After the validation of result by performing the experiment under optimized condition there was 36.21% increase in the activity of the L-asparaginase, with 11 fold purification was achieved.

INTRODUCTION

L-Asparaginase (E.C.3.5.1.1.) is a naturally occurring enzyme which catalyzes the hydrolysis of asparagine into aspartic acid and ammonia. L-Asparaginase has multiple uses in human life ranging from food industry, analytical bio-chemistry, field of medicine etc. (Jha et al 2012) Most important use of L-Asparaginase is in the treatment of Acute Lymphoblastic Leukaemia (ALL), 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 according to Wriston *et al.* 1973. Thus the administration of L-Asparaginase to ALL patients creates a resource scarcity for the leukemic cells.

Bracewell *et al.* 2009 illustrated that currently the downstream processing techniques followed in the industry, especially in the case of therapeutic enzymes, is a tedious and cumbersome process which requires a lot of time and resources. However Three Phase Partitioning (TPP) can provide an alternative solution to these problems. The factors like salting out, co-solvent precipitation, iso-ionic precipitation etc. are the principles on which TPP is based upon.

In present investigation TPP was used for enhanced recovery of L-Asparaginase from the fermentation broth of *Escherichia coli* K12. Purification of L-Asparaginase by TPP can be enhanced by the analysis of different factors via conventional or statistical method. Conventional method involves interpreting each and every factor individually which is cumbersome and time consuming while in statistical method combined effect of many factors can be studied simultaneously as demonstrated by Abdel-Fattah *et al.* 2005. Here the sets of experiments can be designed by Taguchi DOE method, proposed by Dr. Genichi Taguchi of Japan. It 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. As in the case of any multivariable 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 ammonium sulphate concentration, ratio of Iso-Octane to the fermentation broth, pH and temperature were selected for optimization.

MATERIALS AND METHOD

Microorganism and Culture conditions: Isolate of *Escherichia coli* K12 (MTCC 1302) was obtained from IMTech, Chandigarh, India. The isolate was sub-cultured on Luria-Bertani agar (HIMEDIA, Mumbai, India) slants and stored at 4^0C for future purposes. The batch production of L-Asparaginase was carried out in 250 ml Erlenmeyer flasks containing 50 ml of sterile Luria Broth supplemented with 0.1 % L-Asparagine. Broth was inoculated from freshly sub-cultured *Escherichia coli* K12 from LB agar slants (not more than 1 day old). The fermentation process was carried out at 37^0C and 180 rpm agitation for a fermentation period of 24 hours.

Estimation of enzymatic activity and protein concentration: L-Asparaginase activity in the fermentation broth and during TPP was carried out by the L-Asparaginase (E.C.3.5.1.1) assay method of Shirfrin *et al.* 1974 by nesslerization. Protein content in the fermentation broth was estimated by Lowry's method of protein estimation as described in Lowry *et al.* 1951 using BSA (Bovine Serum Albumin) as standard.

Three Phase Partitioning (TPP) of L-Asparaginase: Fermentation broth of *Escherichia coli* K12 was centrifuged at 10000 rpm at 4^0C. The pellets were discarded and the supernatant was kept as crude sample. For performing the TPP, 5ml of crude sample were taken and desired amount of ammonium sulphate was added according to Jha *et al.* 2013. It was followed by adjusting the pH to the required value. Then the required amount of Iso-octane was added in order to achieve desired ratio of Iso-octane to broth. The experimental setup was then incubated at 37^0 C for 1 hour and then centrifuged (2000 rpm for 10 minutes) at 4^0C in order to separate the phases. Activity measurement was done for both the interfacial (middle) and aqueous (bottom) phases. After that protein estimation was done in order to calculate the specific activity.

Optimization by Taguchi Design of Experiment (DOE): Optimization of TPP was done by using Taguchi DOE methodology using Qualitek-4 software. L-16 orthogonal array was selected for the optimization of ammonium sulphate concentration, Iso-octane to broth ratio, pH and Incubation temperature. As per Sharma *et al.* 2001, the 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 during three phase partitioning as shown in Table-1.

Total 16 trial conditions were designed for the optimization of TPP system which is illustrated in Table 2. The results from these trial conditions were then entered into the Qualitek-4 (Taguchi DOE) software in order to determine Analysis of Variance (ANNOVA), significant factor influences, Interaction severity indexes and optimized experimental conditions. The proposed optimized conditions were validated in same experimental protocol.

RESULTS AND DISCUSSION

The enzymatic activity in the interfacial phase obtained during the TPP in different designed trial conditions were indicated in Fig.1. The main effects of the different selected factors at various levels on recovery of enzyme were shown in Table 3. The difference between the average values of each factor at different levels indicates the relative influence of the effect on the recovery of L-Asparaginase by TPP.

The interaction between two factors may be provide an understanding of overall process analysis. Estimated interaction severity index (SI) of the factors under study were given in Table 4. The SI value of 100% indicates 90 degree angle between the lines while, 0% SI for parallel lines as mentioned by Koo *et al.* 2006; Dasu *et al.* 2003 and Han *et al.* 1998 . After the consideration on severity index (SI) it was found that ammonium sulphate and Iso-octane: Broth ratio both at level 4 showed highest interaction SI (31.03%). In the contrary ammonium sulphate and pH having least interaction SI (12.06%).

The ammonium sulphate concentration 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 (Dennison *et al.* 1997). The contribution of Ammonium Sulphate, Iso-octane: Broth ratio, pH and temperature on the recovery of L-Asparaginase were 38 %, 31%, 21% and 4% respectively in case of *Escherichia coli* K12. (Fig-2)

The results of the orthogonal array (OA) experiments were analyzed by the use of ANOVA (Table 5). The F-ratio was used to determine the degree of variation contributed by each factors as mentioned (Armstrong *et al.* 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.

The optimized conditions analyzed by the Qualitek-4 (Taguchi DOE) software for purifying L-Asparaginase by TPP (Table 6). The expected results after performing TPP in optimized conditions were 35.685 IU/ml for *Escherichia coli* K12. After performing TPP in optimized conditions the results obtained were 35.5 IU/ml (Fig. 3). Table 7 shows the overall summary of the purification of L-Asparaginase from *Escherichia coli* K12 in terms of specific activity and fold purification in the interfacial precipitate phase after optimization. The enhanced recovery of 36.21% with specific activity of 101.42 IU/mg and 11 fold purification was reported under the optimized conditions for Escherichia coli K12 fermentation system

CONCLUSION

The Three Phase Partitioning system is an economical and easily scalable method of purification of enzymes. The application of statistical principles like Taguchi DOE can further improved the purification process in terms of enhanced recovery. In present investigation suitability of Taguchi DOE was justified by the enhanced recovery of 36.21 % with 11 fold purification of the L-Asparaginase enzyme from *Escherichia coli* K12 fermentation broth. These facts indicate that TPP in combination with Taguchi DOE may be highly helpful to reduce the number of steps in downstream processing of therapeutic proteins by reducing the unwanted proteins in final interfacial precipitate of three phases.

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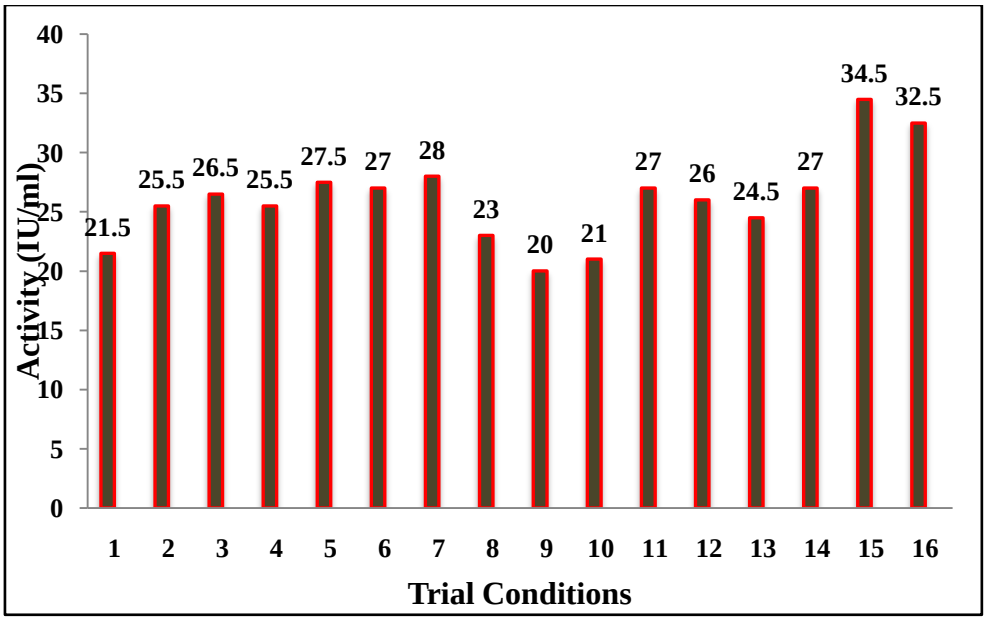

Figure 1: Variability within and between trial results

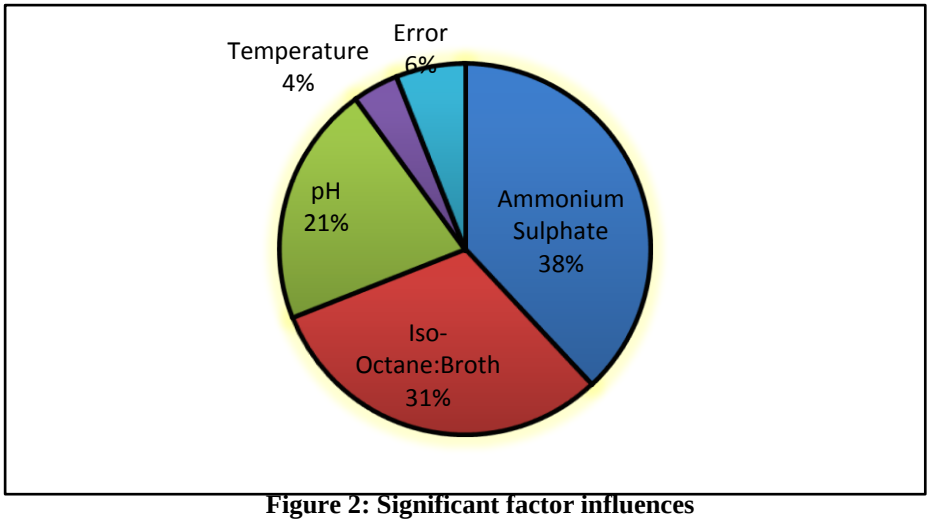

Figure 2: Significant factor influences

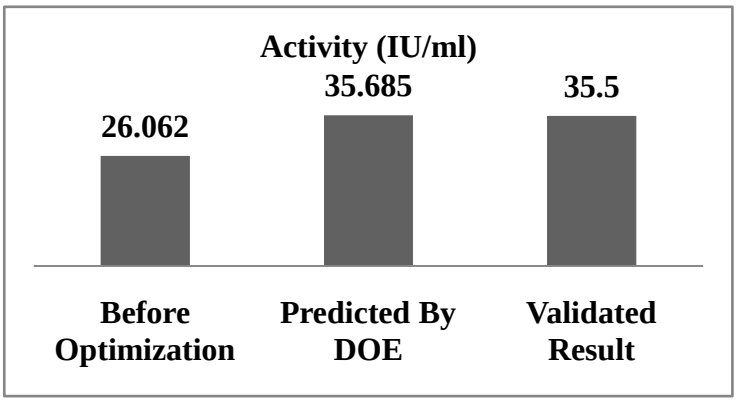

Figure 3: Comparison of the enzyme activity before and after the optimization of TPP

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

S.No	Factors	Level 1	Level 2	Level 3	Level 4
1.	Ammonium Sulphate (% w/v saturation)	20	30	40	50
2.	Iso-Octane: Broth Ratio	0.8:1	0.9:1	1:1	1.1:1
3.	pH	5	6	7	8
4.	Temperature (^{o}C)	28	30	37	45

Table 2: L-16 orthogonal array for different trial conditions according to Taguchi DOE

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 Ratio	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
Temperature (^{o}C)	1	2	3	4	3	4	1	2	4	3	2	1	2	1	4	3

Note: 1, 2, 3 and 4 correspond to the different levels of individual factors.

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	24.75	26.375	23.5	29.625
2.	Iso-octane : Broth	23.375	25.125	29	26.75
3.	pH	27	28.375	24.125	24.75
4.	Temperature	25.625	25	26.875	26.75

Table 4: Estimated interaction between the factors i.e. Severity Index

S. No.	Interacting Factor Pairs (Order based on SI)	Columns	SI %	Opt.
1.	Ammonium Sulphate x Temperature	1 x 4	31.03	[4,4]
2.	Iso-octane : Broth x pH	2 x 3	25.86	[3,2]
3.	pH x Temperature	3 x 4	20.68	[2,4]
4.	Ammonium Sulphate x Iso-octane : Broth	1 x 2	15.51	[4,3]
5.	Iso-octane : Broth x Temperature	2 x 4	15.51	[3,4]
6.	Ammonium Sulphate x pH	1 x 3	12.06	[4,2]

Note: Explanation of columns of the table-

- 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 i.e. ANNOVA

S. No	Factors	Sum of Squares	Variance	F-Ratio	Pure Sum	Percent
1.	Ammonium Sulphate	168.625	56.208	74.292	166.355	38.341
2.	Iso-Octane: Broth	137.625	45.875	60.634	135.355	31.196
3.	pH	93.625	31.208	41.249	91.355	21.055
4.	Temperature	19.625	6.541	8.646	17.355	4.000
Other/Error		14.375	0.7565			5.408
Total		433.875				100.000%

Table 6: Optimum condition for performance of TPP

Factor	Level Description	Optimum Level
Ammonium Sulphate	50	4
Iso-octane: Broth Ratio	1:1	3
pH	6	2
Temperature (^{o}C)	37	3

Table 7: Summary of L-Asparaginase Purification by Three Phase Partitioning

Source	Activity (IU/ml)	Protein Conc. (mg/ml)	Specific activity (IU/mg)	Purification fold
Fermentation Broth (crude)	26.062	2.9	8.98	1
Interfacial phase (after optimization)	35.5	0.35	101.42	11