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Research Article

A Heuristic Approach for the Enhanced Recovery of Anti-leukemic L-Asparaginase from *Bacillus licheniformis* by the Statistical Optimization of Three-Phase Partitioning System

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ABSTRACT

L-Asparaginase has been shown to possess anti-leukemic characteristic, mainly against Acute Lymphoblastic Leukaemia. The three phase partitioning (TPP) method was used to purify the L-asparaginase produced by *Bacillus licheniformis.* The partitioning was carried out by using iso-octane and ammonium sulphate fractionation. The recovery of enzyme was enhanced by optimizing the factors like ammonium sulphate concentration, ratio of iso-octane to fermentation broth, temperature and pH by Taguchi orthogonal array. Under the optimized condition 37.93 % enhanced recovery of enzyme with fold purification of 10 was achieved.

Key words: Three Phase Partitioning, Iso-Octane, L-Asparaginase, Taguchi DOE

INTRODUCTION

The application of microbial L-asparaginase has mde a revolutionary impact on the treatment of acute lymphoblastic leukemia and lymphosarcoma. The use of this enzyme as the first line of treatment increased the overall servival rate upto 93.7% in children suffuring from the disease. The advances in the L-asparaginase based therapy may ledtoimprove the long-term survival rate in pediatric and adult patients. L-asparagine is an amino acid which is synthesized by the normal cells with the help of an enzyme asparagine synthetase. Most of the cancer cells lack this enzyme and hence fulfil their nutritional requirement from the circulating blood. This forms the basis for the use of L-Asparaginase as a drug which deaminates the asparagine present in the circulating plasma, thus, depriving the cancerous cells from this important amino acid essential for their protein synthesis. So a huge economical demand of the drug reflects the need to develop a more efficient bioprocess thechnology for the production of L-asparaginase[1, 2].

The bioseparation techniques most commonly used in the industries are very tedious and cumbersome process. It takes lot of time along with money[3]. The three phase partitioning may be a method of choice to overcome these disadvantages. TPP is based on the basic principles of salting out, co-solvent precipitation, iso-ionic precipitation etc.

The present investigation exploited the potentials of three phase partitioning along with Taguchi design of experiment (DOE) method to get the enhanced recovery of L-asparaginase from the fermentation broth of *Bacillus licheniformis*. The optimization was carried out by orthogonal array method of DOE. The large number of experimental situation can be described by orthogonal arrays to reduce experimental errors and to enhance their efficiency and reproducibility. 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 ratio, pH and temperature were selected for optimization.

MATERIALS AND METHOD

Microorganism and Culture conditions

The culture of *Bacillus licheniformis* (MTCC 1483) was used as the producer of L-asparaginase. The cultures were maintained by sub-culturing on the slants of Luria-Bertani agar (NaCl 10 g/L, Tryptone 10 g/L, Yeast extract 5 g/L, pH 7.2±0.2). The media were sterilized by autoclaving at 121^0C at 15 lb $inch^{-1}$ for 15 minutes. The L-asparaginase producing potential of microorganism was confirmed by qualitative and quantitative screening by the rapid plate assay method[4] and batch production 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 *Bacillus licheniformis* from LB agar slants. The fermentation process was carried out for 24 h at 37^0C and 180 rpm agitation.

Estimation of enzymatic activity and protein concentration

L-Asparaginase assay has been performed by the Worthington manual protocol, but few modifications were done to optimize it in the laboratory conditions. The 200 µL of enzyme samples were added in a reaction

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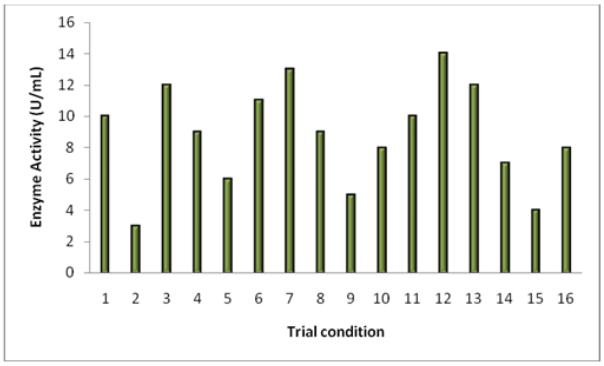

Figure 1: Variability within the enzymatic activity in trial conditions

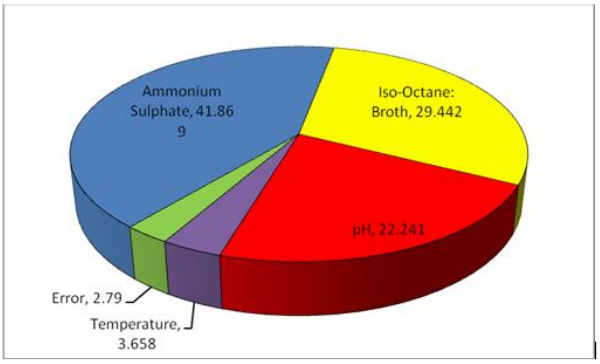

Figure 2: Prcentage influence of significant factor on l-asparaginase purification by TPP

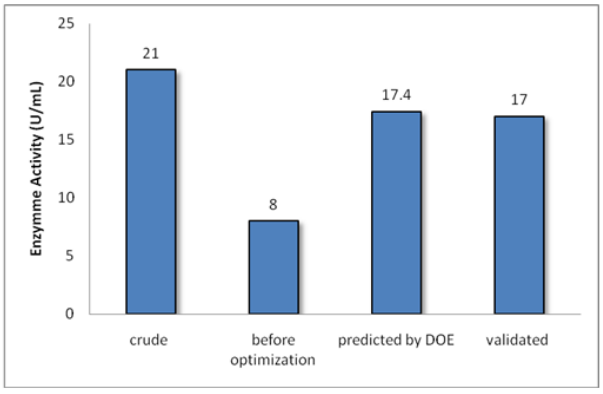

Figure 3: Comparison of the enzyme activity before and after the optimization of TPP

mixture having 10 mM L-asparagine dissolved in 50 mM TrisHCl (pH 8.6). The assay mixtures were incubated at 37°C for 10 minutes, after which the reaction was stopped by addition of 200µl of 1.5M TCA. The amount of ammonia released was determined by Nessler's reagent using an ammonium sulphate solution as standard. The enzymatic activity was estimated in terms ammonia evolved during enzymatic reaction. The rate of hydrolysis of asparagine was 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 [5]. Protein content in the fermentation broth was estimated by Lowry's method of protein estimation using BSA (Bovine Serum Albumin) as standard[6].

Three Phase Partitioning (TPP) of L-Asparaginase

The three phase partitioning was started with the crude extract obtained after the centrifugation of fermentation broth at 10000 rpm and 4^0C. The supernatant was taken as crude extract. The crude extract was subjected to ammonium sulphate fractionation with proper adjustment of pH. The calculated amount of iso-octane was added to get the particular ratio with crude extract (fermentation broth). The reaction mixture was incubated at appropriate temperature for 1 hour and then centrifuged (2000 rpm for 10 minutes) at 4^0C in order to separate the phases. The enzymatic activity was measured in all the three phases, i.e. aqueous phase (bottom), interfacial precipitate (middle) and organic phase (upper). The steps were repeated to concentrate the L-asparaginase at middle phase[7].

Optimization by Taguchi Design of Experiment (DOE)

Taguchi orthogonal array based design of experiment methodology was adopted for the optimization of significant factors involved in the TPP. Four important factors viz. ammonium sulphate concentration, iso-octane to broth ratio, pH and Incubation temperature were optimized using the L-16 orthogonal array. Sharma *et al.* (2001)[8] reported that the factors like ammonium sulphate concentration, ratio of Iso-octane to broth, pH and temperature were significantly affect the yield of desire enzyme during three phase partitioning. The selected factors and their levels are shown in Table 1. On the basis of selected orthogonal array total 16 trial conditions were designed (Table-2). The result obtained in the trial conditions were further analysed statically to predict the optimum level of each factors. The result was validated by performing the experiment under the designed optimum conditions.

RESULTS AND DISCUSSION

The L-asparaginase producing potential of the *Bacillus licheniformis* was confirmed by formation of pink colouration during the plate assay. The quantitative confirmation by batch production has given the 21 U/mL of L-asparaginase activity in fermentation broth. After the partitioning of enzyme by TPP, the most of the activity was still found in aqueous phase, so second round was partitioning was carried out under designed trial conditions.

The enzyme actvity obtained at the interface during the 16 trial conditions having broad range of variation from 3 to 14 U/mL. The variability within the activity in different trial conditions are shown in figure-1. These variability within the enzyme avtivity proved that the number of factors are interaction with each other and influencing the partitioning of enzyme. The comparative interation of all selected factors were represented in terms of severity index (SI). It may provide an insight into the understanding of overall process. The SI value of 100% indicates 90 degree angle between the lines while, 0% SI for parallel lines.[9,10,11]. The maximum severity index of 29.31% was found for the interacting pair of ammonium sulphate and temperature. The higher value indicates the significant interaction between these factors during the TPP. Ammonium sulphate and pH having least severity

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	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 (°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 (°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: Estimated interaction between the factors i.e. Severity Index

Sl. No.	Interacting Factor Pairs (Order based on SI)	Columns	SI %	Opt.
1.	Ammonium Sulphate x Temperature	1 x 4	29.31	[3,4]
2.	Iso-octane : Broth x pH	2 x 3	22.41	[2,3]
3.	pH x Temperature	3 x 4	18.96	[3,4]
4.	Ammonium Sulphate x Iso-octane : Broth	1 x 2	14.56	[3,2]
5.	Iso-octane : Broth x Temperature	2 x 4	14.41	[2,4]
6.	Ammonium Sulphate x pH	1 x 3	10.22	[3,3]

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 4: Analysis of Variance i.e. ANNOVA

Sl.No	Factors	DOF	Sum Of Squares	Variance	F-Ratio	Pure Sum	Percent
1.	Ammonium Sulphate	3	180.25	60.083	95.131	178.355	41.869
2.	Iso-Octane: Broth	3	126.75	42.25	66.895	124.855	29.442
3.	pH	3	95.75	31.916	50.534	93.855	22.241
4.	Temperature	3	15.75	5.25	8.312	13.855	3.658
Other/Error		19	12	0.631	-	-	2.79
Total		31	430.5	-	-	-	100.00%

index of 10.22%. (Table-3). These factors play very important role in the salting out or kosmotropic precipitation of the enzyme during three phase partitioning. The protein precipitation ability of ammonium sulpahte is a complex phenomena associated with the interplay between solute-solvent interaction and water purturbation effects[9]. The precipitation of any protein at interface depends upon the concentration of ammonium sulphate. The concentration of salt should be less that the concentration causes the salting out of the protein[10].

The interation between the pairs of factors were further used to analyse the significant effect of individual factors on the purification of enzyme. The analysis of variance (ANOVA) was used to estimate the percentage contribution of each factors. The analysis of variance is most suitable method to identify the influence of factors in terms of sum of square value, F-ratio and percentage contribution. It can be easily use to analyse the complex data sets[11]. Table-4 represents the ANOVA of selected orthogonal array. The analysis were carried out at 95% confidence limit with three degree of freedom for each factor. The ammonium sulphate having largest contribution of 41.869% in total sum of squares, iso-octane: broth ratio having second largest contribution of 29.442%. pH and temperature having 3.658% and 2.79% of contribution respectively. The percentage contribution to total sum of square relects their ability to influnce the purification of protein by the TPP. Similar trend was recorded by the F-ratio. The highest F-ration of 95.131 was found for the ammonium sulphate. The other factors, viz. iso-octane: broth, pH and temperature having the value 66.895, 50.534 and 8.312 respectively. The F-ratio can used to determine the degree of variation contributed

Table 5: Predicted optimum condition for TPP

Factor	Level Description	Optimum Level
Ammonium Sulphate	40	3
Iso-octane: Broth Ratio	0.9:1	2
pH	7	3
Temperature (^{o}C)	30	2
Predicted enzyme activity at optimized condition		17.5 U/mL
Validated enzyme activity		17 U/mL

Table 6: Summary of L-Asparaginase Purification by Three Phase Partitioning

Steps	Activity (U/ml)	Protein Conc. (mg/ml)	Specific activity (U/mg)	Purification fold
Crude Sample	21	2.814	7.46	1
Interfacial phase (after optimization)	17	0.24	70.83	10

by each factors as mentioned [12, 13].

The analysis of variance and severity index based statistical analysis was used to proposed the optimum level of each factors for the second round TPP. The ammonium sulphate at level- 3 (40% w/v), iso-octane : broth at level-2 (0.9:1), pH at level-3 (7) and temperature at level-2 (30), was found to be optimum for the enhanced recovery of L-asparaginase by TPP. The orthogonal based design of experiment predicted the 17.5 U/mL of enzymatic activity under optimized condition for *Bacillus licheniformis* L-asparaginase. After the validation of result by performing the experiment under the optimized condition has given the 17 U/mL of activity (Figure-3). Table-6 show the overall summary of purification of L-asparaginase from *Bacillus licheniformis.* The optimization of second round of TPP has given the 81% partitioning of enzyme with specific activity of 70.83 U/mg and fold purification of 10. The overall 47% enhanced recover of enzyme (17 U/mL) was observed in comparison to the activity observed before optimization (i.e. 8 U/mL).

CONCLUSION

The purification of proteins by TPP may be a better alternative to reduce the overall process cost. It was estimated that 70-80% cost of the process is due to cumbersome and time consuming downstream processing. The suitability of TPP based method can be justified by this study. The optimization of TPP has given 47 % enhanced recovery of enzyme with the fold purification of 10. Along with all these advantages, the process is easily scalable.

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