Winter 2012/2013 • Volume 11/Issue 4 • ISSN 1538-8786

BioProcessing JOURNAL

Trends & Developments in BioProcess Technology

A Production of BioProcess Technology Network

BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Enhanced Recovery of L-Asparaginase by the Optimization of a Three-Phase Partitioning System Using the Taguchi DOE Methodology

By SANTOSH KUMAR JHA*, DIVYA PASRIJA, HARE RAM SINGH, VINOD KUMAR NIGAM, and AMBRISH SHARAN VIDYARTHI

Abstract

L-asparaginase, produced by *Pseudomonas fluorescens,* was purified by a three-phase partitioning method of t-butanol in the presence of ammonium sulphate. Enzyme recovery was enhanced by the optimization of process parameters (*i.e.*, ammonium sulphate concentration, t-butanol ratio, temperature, and pH) using the Taguchi design of experiment (DOE) methodology. The enhanced recovery of 31.1% with 96.0% yield and purification of 10-fold was obtained.

Introduction

L-asparagine (EC 3.5.1.1) is a life-saving drug used for the treatment of acute lymphoblastic leukemia (ALL) all over the world. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonium sulphate. The principle behind the use of asparaginase as an anti-tumor agent is that it takes advantage of the fact that ALL leukemic cells are unable to synthesize the non-essential amino acid asparagines on their own.[1]

The downstream processing of therapeutic enzymes from the fermentation broth or the multi-component system is a cumbersome and expensive job. Most of the methods developed for this purpose are costly, time-consuming and need pre-treatment of broth. The three-phase partitioning (TPP) technique may be considered one alternative to these problems.[2] 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]

Throughout this investigation, the statistical method of Taguchi design of experiment was used to design and optimize the TPP system for enhanced recovery of the L-asparaginase enzyme from fermentation broth. The Taguchi experimental design examines several influential factors simultaneously and has distinctive advantages over other statistical methods since quantitative information can be extracted with minimal trial conditions.[4] Conventional optimization, based on one single factor at a time, is a shotgun approach where each parameter is considered to be insensitive to the other process variables. For most multi-variable processes in which numerous potentially influential factors are involved, determining the most important ones are not always easy. 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.[5]

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Materials and Methods

Materials

Pure L-asparaginase was purchased from Sigma-Aldrich (USA). All other chemicals were analytical grade and purchased from HiMedia (India).

Microorganism and Culture Conditions

For this investigation, the isolates of *Pseudomonas fluorescens* (MTCC 103) were obtained from BIT Metra's departmental collection. The isolates were subcultured on nutrient agar (HiMedia) slants and stored at 4 °C for further studies.

Seed culture was prepared in 100 mL of sterile medium containing glucose, 0.1% K_2HPO_4, 0.5% tryptone, and 0.5% yeast extract.[6] Medium was inoculated by transferring the loopful of cells from a slant culture. The culture flask was incubated at 37 °C for 18 hours at 200 rpm.

The batch production of L-asparaginase in this investigation was carried out in 500 mL Erlenmeyer flasks in 100 mL of the same sterile liquid medium supplemented with 1% L-asparaginase. Medium was inoculated with 10% inoculum (v/v) and incubation was carried out at 30 °C for 24 hours at 200 rpm.

Estimation of Enzymatic Activity and Amount of Protein

L-asparaginase activity in the fermentation broth and during TPP was carried out by the L-asparaginase (EC 3.5.1.1) assay method of Shirfrin *et al.* by nesslerization.[7] Protein content in the fermentation broth was estimated by the dye binding method of Bradford *et al.* by using bovine serum albumin (BSA) as a standard.[8]

Three-Phase Partitioning of L-Asparaginase

Precipitation of proteins was carried out by adding the desired level of ammonium sulphate to the fermentation broth of *Pseudomonas fluorescens*. It was mixed gently to dissolve the salt completely. Then 1:1 t-butanol was added and incubated at room temperature. After one hour of incubation, the mixture was centrifuged (2000 relative centrifugal force [rfc] for ten minutes) at 40 °C to separate the phase. Three phases were formed. The enzymatic activity was tested in interfacial precipitate and aqueous phase. Most of the enzymatic activity was retained in the aqueous phase so it was subjected to a second round of TPP at different conditions designed by the factorial method of Taguchi.[9-12]

Optimization of TPP by DOE

The purification of L-asparaginase by a three-phase partitioning system in the second round was optimized by the use of Taguchi DOE methodology. An L-16 orthogonal array (OA) was selected for this purpose. The total degree of freedom is equal to the number of trials minus one.[15] The first step of optimization was to determine which various factors should be optimized in TPP that would have critical effect on the enzyme yield. The factors like ammonium sulphate concentration, ratio of t-butanol, temperature, and pH all significantly affect the yield of desired enzyme during TPP[13] and were selected for the optimization. All four factors, at four levels (4^4) in the feasible range were considered for the design and optimization (Table 1). In total, 16 trial conditions (Table 2) were designed for the optimization of the TPP system. After the interaction studies, the proposed optimized conditions were validated in the same experimental conditions.

TABLE 1. Factors and control levels selected for optimization.

Step #	Factors	Level 1	Level 2	Level 3	Level 4
1	ammonium sulphate % (w/v)	20	30	40	50
2	t-butanol:broth (v/v)	1:0.75	1:1	1:1.15	1:1.25
3	temp (0 °C)	30	40	50	60
4	pH	5	6	7	8

TABLE 2. L-16 (4^4) orthogonal array of Taguchi DOE and levels of factors.

Trial Conditions	Levels of Various Factors				Total Enzymatic Activity (U)
	Ammonium Sulphate % (w/v)	t-butanol: broth (v/v)	Temperature (0 °C)	pH	
1	1	1	1	1	53 ± 0.2
2	1	2	2	2	49 ± 0.4
3	1	3	3	3	47 ± 0.1
4	1	4	4	4	57 ± 0.3
5	2	1	2	3	51 ± 0.6
6	2	2	1	4	48 ± 0.5
7	2	3	4	1	45 ± 0.7
8	2	4	3	2	54 ± 0.4
9	3	1	3	4	46 ± 0.5
10	3	2	4	3	43 ± 0.8
11	3	3	1	2	45 ± 0.7
12	3	4	2	1	53 ± 0.6
13	4	1	4	2	49 ± 0.5
14	4	2	3	1	51 ± 0.4
15	4	3	2	4	49 ± 0.8
16	4	4	1	3	56 ± 0.9

NOTE: Total enzymatic activity was measured in the interfacial precipitate of round 2.

Results and Discussion

The fermentation broth of *Pseudomonas fluorescens* (MTCC103), when saturated with 30% (w/v) ammonium sulphate and 1:1 (v/v) t-butanol, showed the 95% L-asparaginase activity in the aqueous phase. Therefore the aqueous phase was subjected to a second round of TPP using different trial conditions (Table 2) also designed using the Taguchi methodology for selective precipitation of L-asparaginase.

The interactions of different factors showed that the L-asparaginase purification by TPP operates through multiple effects. Hence it must be considered for the maximum yield of enzyme. This study, under designed experimental conditions, showed significant variation in the recovery of L-asparaginase activity in precipitate (Table 2). It was found that the enzyme recovery was very much dependent on the trial conditions. The main effects of the different factors at various levels affecting the recovery of enzyme were shown in Table 3. The differences 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 provide an understanding of overall process analysis. The estimated interaction severity index (SI) of the factors under study were given in Table 4. The SI value of 100% indicates a 90° angle between the lines versus 0% SI for parallel lines.[14-16] After the consideration of SI, it was found that the ammonium sulphate and t-butanol:broth ratios for both in level 3 showed the highest interaction SI (54.16%). In contrast, t-butanol and temperature had the least SI (12.50%). Other factors were intermediate SI values. Figure 1 indicates the types of interactions among the four factors.

The relative amount of ammonium sulphate and t-butanol:broth ratio was the most crucial factor (SI) for TPP of L-asparaginase.

TABLE 3. The primary effect of the selected factors at various levels on L-asparaginase production.

Step #	Factors	Level 1	Level 2	Level 3	Level 4
1	ammonium sulphate % (w/v)	53.75	46.75	52.50	54.00
2	t-butanol:broth (v/v)	55.75	50.00	46.25	55.00
3	temperature (0°C)	55.25	55.50	46.00	49.25
4	pH	56.00	53.00	52.00	45.00

TABLE 4. Estimated interaction between the factors (severity index).

Step #	Interacting Factor Pairs (order based on SI)	Columns	SI (%)	Optimum Levels
1	ammonium sulphate × t-butanol:broth	1 × 2	54.16	3, 3
2	ammonium sulphate × temperature	1 × 3	45.83	3, 1
3	t-butanol:broth × pH	2 × 4	31.25	3, 2
4	temperature × pH	3 × 4	20.83	1, 2
5	ammonium sulphate × pH	1 × 4	18.75	3, 2
6	t-butanol:broth × temperature	2 × 3	12.50	3, 1

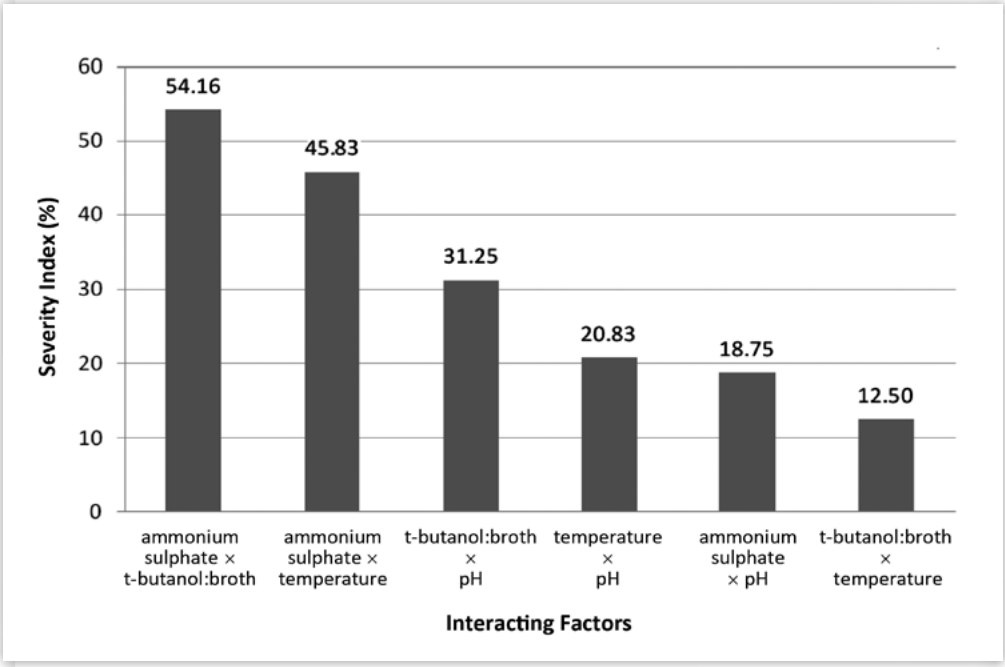

FIGURE 1. Interactions between the factors and their severity index.

The best result was obtained when the ammonium sulphate was 40% (w/v) and t-butanol to aqueous extract ratio was 1:1.15. Alternate amounts of t-butanol did not fit well with the purification process because they did not properly synergize with the ammonium sulphate. The ammonium sulphate concentration was selected so that it would be less than the concentration that would cause the salting out of any protein. The precipitation was started with a minimum salt concentration of 20% (w/v) and was optimized to obtain maximum recovery of the enzyme as interfacial precipitate.[2] The high concentration of ammonium sulphate that was required further depended upon the ratio of t-butanol. Higher concentrations of it can increase the hydrophobicity of the enzyme which in turn may be saturated by the lesser quantity of ammonium sulphate. The SI of temperature and pH was at an intermediate level (20.83%) with the favourable level of 1 and 2, respectively. The contribution of pH and temperature on the recovery of L-asparaginase was 24% and 15%, respectively (Figure 2). Lowering the pH significantly affects the activity and yield of the L-asparaginase. Low temperature (level:1) results in less yield with no significant change observed in specific activity.

The result of the OA experiments were analyzed using the analysis of variance (ANOVA) (Table 5). The F-ratio was used to determine the degree of variation contributed by each factor.[17] The factors and their respective interactions were considered in the experimental design as statistically significant effects at 95% confidence limit. By study of the main effect of each factor the general trends of the influence of the factors toward the process can be characterized. Analysis of variance has given the percentage contribution of all factors on the performance of the process. Ammonium sulphate contributed approximately 30%; t-butanol:broth ratio, 29%; pH, 24%; and temperature, 15% on the L-asparaginase purification by TPP (Figure 2).

The proposed optimized conditions by factorial design were validated by purifying L-asparaginase according to design conditions. After the study of complete interactions among all factors, the optimized conditions should give the 68.625 U/mL of enzyme yield with 32.289% enhanced recovery. The 40% (w/v) ammonium sulphate in combination with 1:1.5 ratio of t-butanol to crude at 30 °C gave the best result at pH 6 (Table 6). The L-asparaginase purified by statistically optimized TPP showed 67.500 U/mg of enzymatic activity with enhanced recovery

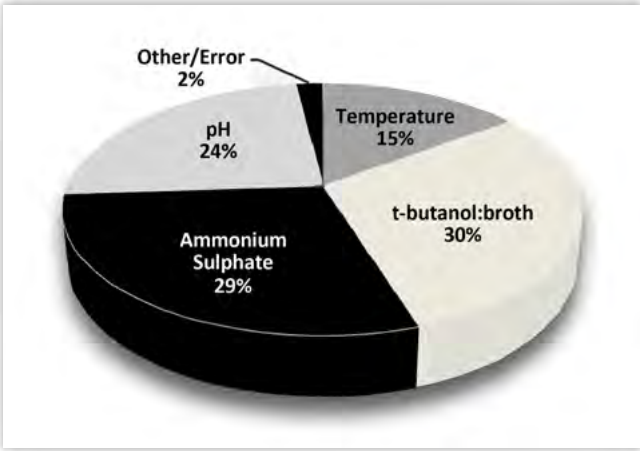

FIGURE 2. Contributing factors to the overall performance.

TABLE 5. ANOVA.

Step #	Factors	Sum of Squares	Variance	F-Ratio	Pure Sum	Yield (%)
1	ammonium sulphate concentration	260.000	86.666	104.000	257.500	29.394
2	t-butanol:broth	261.500	87.177	104.600	259.000	29.566
3	temperature (0°C)	135.500	45.166	54.199	133.000	15.182
4	pH	216.500	72.166	86.600	214.000	24.429
	Other/error	**2.500**	**0.833**			**1.429**
	TOTAL	**876.000**				**100.000**

TABLE 6. Optimum conditions and performance.

Step #	Factors	Level	Level Description	Contribution
1	ammonium sulphate concentration	3	40	5.125
2	t-butanol:broth	3	1:1.15	3.875
3	temperature (0°C)	1	30	3.375
4	pH	2	6	4.375
	Total contribution from all factors			**16.750**
	Current grand average of performance			**51.875**
	Expected result at optimum condition			**68.625**
	% increase in performance			**32.289**

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of 31.12% (Figure 3). The yield of the enzyme after the optimization was 95% with 10-fold purification. Table 7 shows the overall purification summary of L-asparaginase in terms of specific activity, yield, and fold purification in the interfacial precipitate of round 1 (before optimization) and round 2 (after optimization).

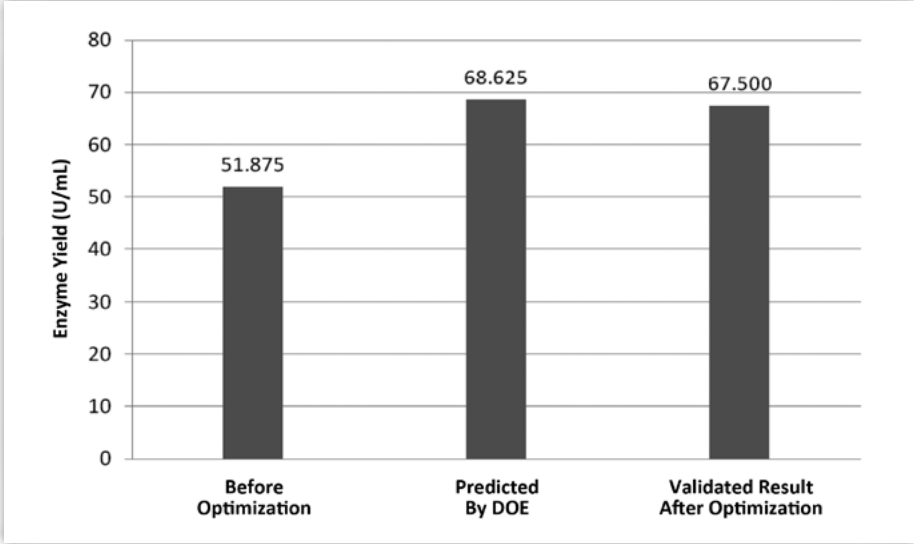

FIGURE 3. Comparison of the enzyme yield before and after TPP optimization Taguchi DOE.

TABLE 7. Summary of L-asparaginase purification by three-phase partitioning.

Steps	Activity (U)	Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Fermentation broth (crude)	64.40	9.30	6.90	100	1
Interfacial precipitate – round 1	4.60	3.35	1.40	7	–
Interfacial precipitate – round 2 (under optimized conditions)	61.00	0.90	67.50	95	10
Aqueous phase – round 2	1.23	0.60	2.05	2	–

NOTE: The first round of precipitate was obtained by the 30% saturation, 1:1 t-butanol, and fermentation broth ratio. The second round was performed under different trial conditions (optimized). Results reported in this table represent the fully optimized process obtained by the validation of the result. Each experiment was carried out in duplicate.

Concluding Remarks

The TPP system may be an economical and easily scalable method of enzyme purification, especially the therapeutic enzymes where 70–80% of the process is contributed by downstream processing. The application of statistical principles (*i.e.*, Taguchi DOE) can further improve the purification process in terms of enhanced recovery. In this investigation, suitability of DOE was justified by the 31.12% enhanced recovery of the L-asparaginase enzyme with 10-fold purification and yield of 95%. These facts indicate that TPP, in combination with Taguchi DOE, may be a useful approach for separation of the enzymes from crude fermentation broth. TPP may be highly helpful in reducing the number of steps in downstream processing of therapeutic proteins by reducing the unwanted proteins in the final interfacial precipitate of three-phase.

ACKNOWLEDGEMENT

The authors are highly thankful to the Government of India, University Grant Commission for financial support.

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