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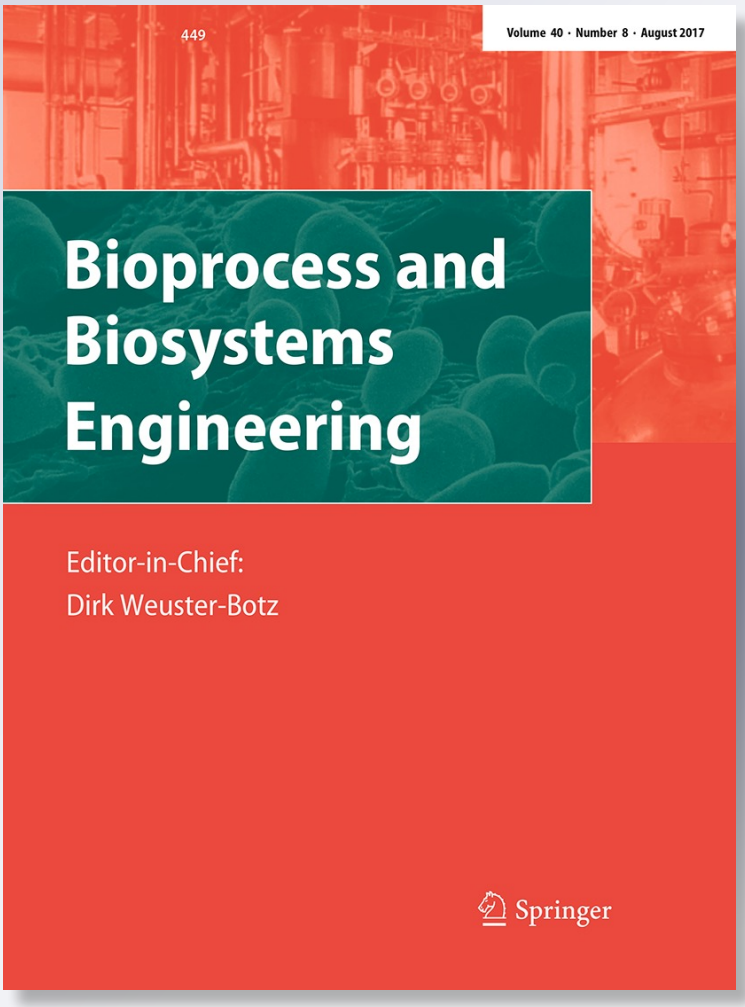

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RESEARCH PAPER

Anionic surfactant based reverse micellar extraction of L-asparaginase synthesized by *Azotobacter vinelandii*

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Abstract L-Asparaginase synthesized by *Azotobacter vinelandii* via submerged fermentation in the presence of sucrose was successfully extracted using Reverse micellar extraction. Single step enzyme purification process was developed by varying the process variables which resulted in maximum specificity and extraction of L-asparaginase. The effect of different variables, including broth pH, addition of alcohol during the forward extraction and pH of the fresh stripping aqueous phase, addition of alcohol and electrolyte during backward extraction process were studied. Lower concentration of butanol resulted in maximum activity of the enzyme during forward extraction while enzyme activity was found to increase further with the addition of higher concentrations of ammonium sulphate during backward extraction. Chromatographic analysis of L-asparaginase peak at ~7.65 min was intense for the back extracted sample confirming the maximum purity of L-asparaginase obtained. Purity of L-asparaginase was increased to about 379.68 fold.

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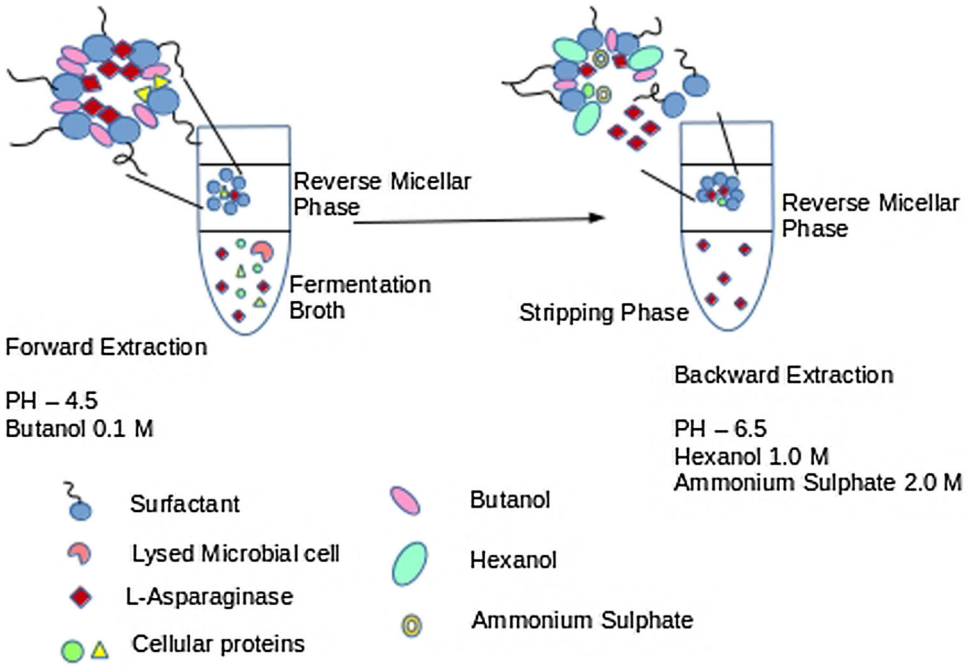
Graphical abstract



Keywords L-Asparaginase · Reverse micellar extraction · *Azotobacter vinelandii*

Introduction

L-Asparaginase (E.C. 3.5.1.1), a substrate specific anti-neoplastic enzyme has been reported to deamide asparagine into L-aspartic acid and ammonia [1, 2]. Being a non-essential amino acid in mammals, aspartate is converted into L-asparagine by L-asparaginase via biochemical pathway using ATP as energy source. Compared to normal living cells, tumor cells require an excess amount of L-asparagine for their growth and proliferation and so they depend on L-asparagine synthesized within the cell and in the serum. Administration of L-asparaginase in vivo leads to conversion of serum L-asparagine causing nutrient starved apoptosis of tumor cells [2]. L-asparaginase was first observed in the blood of guinea pig by Clementi [3] in the year 1922 and its enzyme activity was first reported by studying the inhibition of growth of lymphosarcoma in mice and rat by Kidd in the year 1953 [4]. Tsuji in the year 1957 reported the deamidation activity of L-asparaginase separated from *E. coli.* Since then characterization of L-asparaginase from different organisms and their application as an effective antitumour enzyme has been widely studied and reported [5]. L-Asparaginase has been extensively used in various fields like, as a promising acrylamide mitigating agent in food processing industries, in biosensors and as a potent chemotherapeutic agent [6].

Till date, several microbes have been reported to synthesize L-asparaginase and most of the organisms produce more than one type of the enzyme and the types are classified based on the pH optima, Km value and inactivation by inhibitors [6–9]. Two different types of L-asparaginase has been reported to be synthesized by various microbes, L-asparaginase I and L-asparaginase II, among which L-asparaginase II possess anti-leukemic activity [6]. Although, fermentative production of the enzyme via submerged and solid state fermentation mode by employing variety of microbes in pilot and industrial scale has been well documented [8], elaborative studies on purification of enzyme from fermentation broth are limited [7]. Though, chromatographic separation of L-asparaginase from different sources results in high purity [10–13], design and optimization of the process is time consuming and involves high investment and maintenance cost during large scale operation.

Reverse micellar extraction (RME), a Liquid–Liquid Extraction (LLE) technique, has been widely used in different fields ranging from separation of chemicals, pharmaceutical products and biomolecules from process stream and wastewater [14, 15]. Reverse micelles are generally referred as water-in-oil (W/O) micro-emulsions that are formed by self-assembly of surfactants in an organic solvent which entrap water and forming a hydrophilic core. The water core is surrounded by hydrophilic charged or uncharged surfactant head groups while the hydrophobic tails are aligned in the solvent to maintain thermodynamic equilibrium of the system. Hydrophilic solute from the feed

are solubilized in the water core while hydrophobic solutes interact with the surfactant tail and remain solubilized in the organic solvent [16]. Solubility of hydrophilic solutes can be enhanced by increasing the water content of a reverse micelle, which can be attained by varying the system pH, addition of alcohol and electrolytes [16, 17]. Reverse micellar extraction is considered advantageous over other separation processes as it can be tailored easily, low operational and maintenance cost, ability to reuse surfactants and simple design and operation, which can be scaled up to continuous separation process. RME has been successfully employed towards purification of several enzymes and the effect of different process parameters on the enzymatic activity and purification fold have been published earlier [18–23]. This research article is first of its kind involving RME towards purification of commercially important enzyme L-asparaginase synthesized by *Azotobacter vinelandii* [24] present in the crude fermentation broth. Significance of reverse micellar extraction on the purification of L-asparaginase from a pool of other enzymes/biomolecules and optimization of different process variables to increase the specific activity was studied by employing a simple, efficient and well explored anionic surfactant based reverse micellar system. The present article elaborates AOT/Isooctane based reverse micellar extraction of L-Asparaginase and the effect of different process variables such as pH, addition of alcohol and electrolytes on RME efficiency.

Materials and methods

Azotobacter vinelandii MTCC 14 was procured from microbial type culture collection centre MTCC, IMTECH Chandigarh, India. Dioctyl sulfosuccinate sodium salt 96% (AOT) was procured from Sigma-aldrich, India. Isooctane, sucrose, potassium dihydrogen phosphate (KH_2PO4), dipotassium hydrogen phosphate (K_2HPO4), sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$), sodium sulphate (Na_2SO_4), ammonium sulphate ($(NH_4)_2SO_4$), Acetonitrile (HPLC grade), Methanol (HPLC grade), Trifluoroacetic acid (TFA) and dilute hydrochloric acid (dil. HCl) (37%) were obtained from Merck, India. Magnesium sulphate ($Mg_2SO_4{\cdot}7H_2O$), calcium chloride ($CaCl_2{\cdot}2H_2O$), ferric chloride ($FeCl_3{\cdot}6H_2O$), sodium chloride (NaCl), ammonium chloride (NH_4Cl) and sodium hydroxide (NaOH) were procured from Nice chemicals, India. Butanol, pentanol, hexanol, heptanol and octanol were obtained from Loba chemicals, India. Deionized water was used and room temperature was maintained unless and otherwise stated. LABINDIA analytical UV 3000 + UV/Vis spectrophotometer and Shimadzu HPLC LC 20 A series were used for UV spectral and chromatographic analysis respectively.

Submerged fermentative production of L-asparaginase

Seed culture was prepared by inoculating *Azotobacter vinelandii* in sterilized nutrient broth, which was incubated in an incubator shaker maintained at 30 °C and 150 RPM for 24 h. Production medium was prepared by dissolving (per liter) Sucrose-20 g; K_2HPO_4-0.2 g, KH_2PO_4-0.8 g; $Mg_2So_4{\cdot}7H_2O$-0.2 g: $CaCl_2{\cdot}2H_2O$-0.2 g; $FeCl_3{\cdot}6H_2O$-5 mg and $Na_2MoO_4{\cdot}2H_2O$-3 mg in deionized water. $Mg_2So_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeCl_3{\cdot}6H_2O$ were added separately via filter sterilization (0.2 μM) to the sterilized medium containing the other ingredients. A known volume of the inoculum was added to the production medium and the flask was incubated for 48 h in an incubator shaker maintained at 30 °C with a rotational speed of 150 rpm. After incubation, the broth was subjected to homogenization for 10 min and the homogenized broth was stored at 4 °C until further use.

Total protein content of the homogenized sample was determined through Bradford assay [25]. The standard asparaginase assay protocol was used to measure the enzyme activity. The enzyme activity was calculated using the Eq. (1), and the results obtained were compared to a standard curve constructed using ammonium sulphate.

$$\text{Activity}\left(\frac{\text{Units}}{\text{ml}}\right) = \frac{(\mu \text{ moles of } NH_3\text{liberated} \times 2.2)}{(0.2 \times 30 \times 0.1)} \quad (1)$$

2.2—volume of the first step of activity assay (mL).
0.2—volume from the first step of activity assay (mL).
30—Time of assay in minutes.
0.1—volume of enzyme used (mL).
Specific activity was calculated using the Eq. (2)

$$\text{Specific activity}\left(\frac{\text{Units}}{\text{mg}}\right) = \frac{\text{Enzyme activity}}{\text{Total protein content}} \quad (2)$$

Reverse micellar extraction of L-asparaginase

0.01 M AOT solubilized in Isooctane was used as the organic phase during reverse micellar extraction protocol. Equal volumes of organic phase and homogenized broth (aqueous phase) were added to centrifuge tubes and the mixture was centrifuged at 10,000 rpm for 5 min. After centrifugation, total protein content and L-asparaginase enzyme activity in the top and bottom phases were estimated by withdrawing appropriate volume of the samples from the tubes. Purification fold was calculated using the Eq. (3)

$$\text{Purification factor} = \frac{\text{Specific activity of enzyme extracted}}{\text{Specific activity of feed}} \times 100 \quad (3)$$

Effect of pH was studied by varying the pH of the homogenized broth adjusted between 3.5 and 7.5 using dilute HCl or 0.1 M NaOH solution and pH adjusted samples were used as aqueous phase for the RME protocol. The activity and specific activity of the enzyme in both the top and bottom phases were estimated and purification fold was calculated. Effect of increasing chain length of alcohol and their varying concentration on the reverse micellar extraction of the enzyme was studied by considering butanol, pentanol, hexanol, heptanol, octanol at varying concentration of 0.05, 0.1 and 0.5 M.

Forward extraction was performed in bulk while maintaining the process variables which gave maximum activity of the enzyme. Samples were withdrawn from the bulk top phase and was used as reverse micelle phase during backward extraction. Phosphate buffer with a pH range of 5.8–8.0 was used as stripping phase during backward extraction. Effect of stripping phase pH was studied by adjusting the pH of the buffer between 4.5 and 7.5 using dilute HCl or 0.1 M NaOH solution. Purification fold for the backward extraction process was calculated using the Eq. (4).

$$\text{Purification factor} = \frac{\text{Specific activity of enzyme in stripping phase}}{\text{Specific activity of enzyme in bulk reverse micellar phase}} \times 100 \quad (4)$$

Effect of increasing chain length of alcohol and their varying concentrations on the back extraction of enzyme was studied by considering butanol, pentanol, hexanol, heptanol and octanol of concentrations—0.5, 1 and 1.5 M. Effect of electrolytes on the back extraction efficiency was studied by considering different salts from hofmeister series like Na_2SO_4, $(NH_4)_2SO_4$, NaCl and NH_4Cl with varying concentrations of 1, 1.5 and 2 M.

Chromatographic analysis of reverse micelle extracted L-asparaginase

Reverse phase high performance liquid chromatography (RP HPLC) analysis of the sample with maximum activity of L-asparaginase obtained during forward extraction and backward extraction steps was performed with a reverse phase column—packcell MGII from Shimadzu, Japan. Column oven temperature of 30 °C and PDA detector temperature of 40 °C was maintained during the analysis. Binary gradient elution mode was maintained by considering the following mobile phases: 99% acetonitrile +0.1% TFA was used as mobile phase A; 0.5% acetonitrile +0.1% TFA containing solution was used as mobile phase B. 20 µl of the sample was loaded to the column and the peaks obtained were compared for their respective intensity and retention time.

Results and discussion

Though a number of microbes have been reported to synthesize L-asparaginase via both solid and submerged fermentation [6–8], there exist very limited literature on the production and purification of L-Asparaginase from *Azotobacter vinelandii* [24]. *Azotobacter vinelandii* is potentially used for the production of polyhydroxybutyrate (PHB) as an intracellular product, while disposal of large volumes of supernatant after PHB removal impose waste disposal threat. L-Asparaginase, medically acclaimed enzyme present in the supernatant was separated and purified as a secondary product through reverse micellar extraction which aids supernatant as useful byproduct. Though, optimum active pH of L-Asparaginase from other sources have been reported in literature [6–8], the active pH and isoelectric point (pI) of L-asparaginase from *Azotobacter vinelandii* are in paucity. Hence, 'Expasy pI computing software' was used to calculate the pI of type II Asparaginase from *Azotobacter vinelandii*, 362 amino acids representing the structure of the enzyme was obtained from 'UniProt' protein database and was used for the calculation. Isoelectric point (pI) of the Asparaginase type II from *Azotobacter vinelandii* was found to be 5.78 and the approximate molecular weight of the protein was calculated as 38 kDa.

Forward extraction

Effect of fermentation broth pH

Effect of pH on forward extraction of L-Asparaginase from homogenized broth into the reverse micelle phase was studied by varying the broth pH between 4.5 and 7.5 and the results obtained are represented as Fig. 1. Maximum activity of 4.19 (Units/mL) and specific activity of 36.76 (Units/mg) was achieved at an acidic pH of 4.5. As the pH of the broth is adjusted below the pI of the enzyme, the enzyme attains net positive charge and interacts electrostatically with the negatively charged surfactant head group (AOT) while solubilized in the water core [26, 27] resulting in higher enzyme activity in the top phase. However, change in broth pH has an effect on the partitioning of other cellular proteins, which affects the overall activity and specific activity of L-asparaginase. With increasing pH, enzyme activity was found to decrease but increased

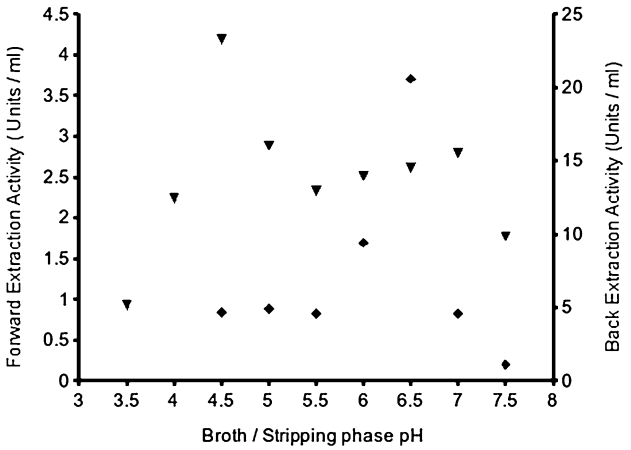


Fig. 1 Effect of broth pH on L-Asparaginase activity during forward—*inverted filled triangle* and stripping phase pH during back extraction—*filled diamond*

slightly at neutral pH owing to nullification of overall cellular protein charge. Purification fold of L-asparaginase was found to be 7.81 at pH of 4.5. However, the effect of other cellular proteins in the homogenized broth will also experience the pI effect and lower the overall purification fold of the enzyme at higher pH. Similar results have been reported by Alves et al. [28] towards extraction of penicillin acylase using AOT/Isooctane reverse micelles.

Effect of alcohol

Broth pH of 4.5 was maintained to study the effect of alcohol for its increasing chain length and varying concentrations on the enzyme purification. From Fig. 2, it can be inferred that the increase in the chain length of alcohol decreases the purification fold, while an increase in concentration till 0.1 M lead to increases in purification fold. On addition of alcohol to reverse micelle, individual alcohol molecules align amidst the surfactant head group, thereby altering the reverse micelle size. This alteration in size enhances or disrupts the water core within the reverse micelles, as a result of which the number of protein molecules solubilized within the water core increases or decreases. Lower chain length alcohols with maximum solubility in water tends to increase the water core by getting incorporated between the surfactant head groups while addition of higher chain length alcohols (heptanol, octanol) disrupts the reverse micelles and more often behave like co-surfactants, thereby replacing the water core [29, 30]. Thus, the activity of the enzyme was found to decrease by incorporating alcohol from butanol to octanol. Reverse micelle size increases with increasing concentration of alcohols due to the incorporation of alcohol within the reverse micelles and results in increased enzyme activity. However, the purification fold starts declining when the alcohol concentration is increased beyond 0.1 M. At higher alcohol concentration, the reverse micelle gets disrupted and reform secondary micelles. Butanol at a concentration of 0.05 M gave maximum enzyme activity of 8.01 (Units/mL) and specific activity of 95.36 (Units/mg). The purification fold was found to increase about 2.35 times by adding butanol as co-solvent and reached a maximum of 24.57.

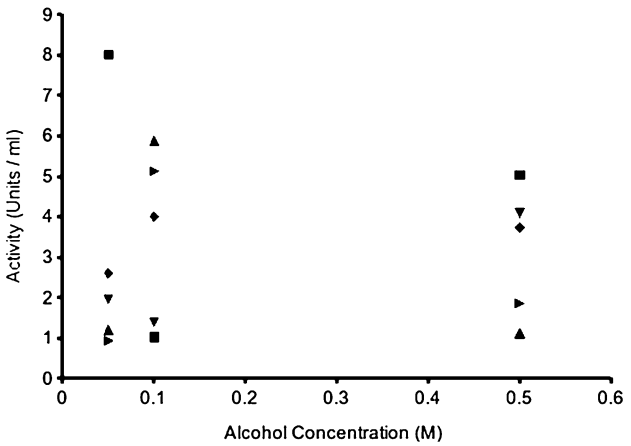


Fig. 2 Effect of different chain length of alcohol and its varying concentrations on L-Asparaginase activity during forward extraction. *Empty square* Butanol, *filled diamond* pentanol, *inverted filled triangle* hexanol, *filled triangle* heptanol, *filled arrowhead* octanol

Back extraction

Bulk forward extraction was performed by adding 0.05 M butanol to the reverse micelles and pH adjusted broth (pH 4.5). Top phase of the bulk RME sample was considered for backward extraction protocol. Phosphate buffer was used as stripping phase by considering the working pH range of the extraction that is similar to cellular fluids and its negligible change in pH with respect to temperature.

Effect of stripping phase pH

Stripping phase pH plays a vital role on the electrostatic force on the surface of the protein that in turn disrupts/enhances the enzyme-surfactant head group interaction and aids in the release of enzyme into fresh stripping phase [31]. Effect of stripping phase pH was studied by varying the pH of phosphate buffer between 4.5 and 7.5 and its effect on enzyme activity is represented as Fig. 1. L-Asparaginase activity was found to increase with increasing stripping phase and reached a maximum of 20.59 (Units/mL) at pH 6.5. Purification fold of the enzyme was found to increase 2.83 times than the bulk forward extraction and reached a maximum of 69.720 with a specific activity of 245.05 (Units/mg). Stripping phase pH of 6.5, enhanced the

back extraction of enzyme from the reverse micelle into the phosphate buffer while composing the electrostatic interaction of other cellular proteins with the reverse micelle head group. However, enzyme activity was found to decline with further increase in buffer pH (>6.5). At higher pH, electrostatic interaction of cellular proteins attached to surfactant head groups of the reverse micelles are altered and partition into the fresh stripping phase, which decreases the purity of L-asparaginase enzyme being extracted.

Effect of alcohol concentration

Effect of alcohol, considering increase in alcohol chain length and varying concentrations on the back extraction was studied by considering the same alcohols (Butanol, pentanol, hexanol, heptanol and octanol at concentration of 0.5, 1 and 1.5 M) that were used during forward extraction. As shown in Table 1, maximum activity of 29.44 (Units/mL) and specific activity of 2452.78 (Units/mg) was achieved by adding hexanol at a concentration of 1 M, enzyme purity increased 2.5 times and reached a maximum of 174.63 purification fold. It was observed that as the chain length of alcohol increased, back extraction of the enzyme as well found to increase from butanol to hexanol and further increase in chain length lead to decline in enzyme partitioning into the stripping phase. Mathew and Juang [32] had reported that the addition of higher chain length alcohols disrupts the reverse micelle structure, thereby leading to release of protein from the water core to the stripping phase. However, presence of alcohols such as heptanol and octanol lead to protein precipitation which ultimately leads to low activity and purity of the enzyme being back extracted. Similar effect has been reported for increasing concentration of a particular alcohol. However, with the presence of butanol in the bulk reverse micelle phase, addition of alcohol during back extraction process leads to increased breakage of reverse micelle structure and release of encapsulated solutes into the stripping phase.

Effect of electrolyte concentration

Electrolytes from hofmeister series were added to the stripping phase to study the effect of chaotropic and cosmotropic salts on the back extraction of L-asparaginase from the reverse micelle phase. Na_2SO_4, $(NH_4)_2SO_4$, NaCl and NH_4Cl were added in varying concentrations and the results are graphically represented as Fig. 3. Chaotropic anions are employed in the extraction of proteins owing to their high and stable interaction, even though they impose denaturing properties [33]. Addition of chaotropic anions such as ammonium on interaction with reverse micelles, cause strong electrostatic repulsion of negatively charged surfactant head groups, which in turns leads to expansion of reverse micelle and back extraction of the solubilized enzyme in the water core, while the comparative effect of cosmotropic anions like sodium are less on the electrostatic repulsion of proteins from reverse micelles [34, 35].

From data obtained, it is concluded that enzyme activity of L-asparaginase partitioned in the stripping phase was higher with the addition of stronger cation (ammonium sulphate) compare to that of a weaker cation (sodium chloride). As ammonium has higher ionic radius in comparison to sodium which adds up to the repulsive effect of surfactant head groups. Similarly, considering the ionic radius of sulphate to that of chloride, sulphate leads to higher back extraction rather than chloride. Similar results were reported by Gaikaiwari et al. [36] towards purification of lipase in the presence of chaotropic salt, the authors reported that increase in the salt concentration lead to reduction of reverse micelle size that in turn caused expulsion of water core and the solubilized protein into the stripping phase. Thus, combination of ammonium and sulphate strongly enhance enzyme partitioning into the stripping phase than that of sodium chloride. Ammonium sulphate at a concentration of 2 M gave maximum enzyme activity of 42.11 (Units/mL) and specific activity of

Table 1 Effect of increasing chain length of alcohols and its varying concentrations on enzyme activity during back extraction of L-asparaginase

Alcohol	Concentration (M)		
	0.5	1.0	1.5
	Activity (Units/mL)		
Butanol	1.58	2.14	2.04
Pentanol	1.30	3.82	2.51
Hexanol	6.33	29.44	8.1
Heptanol	5.49	2.79	1.77
Octanol	4.19	1.39	0.93

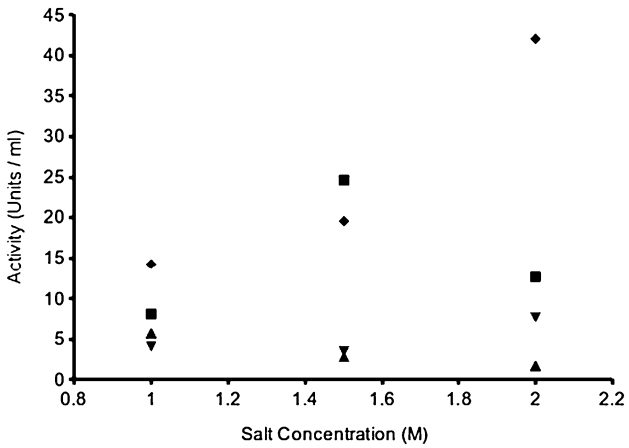


Fig. 3 Effect of electrolytes and its varying concentrations on enzyme activity during back extraction of L-asparaginase. *Empty square* ammonium chloride, *filled diamond* ammonium sulphate, *inverted filled triangle* sodium chloride, *filled triangle* sodium sulphate

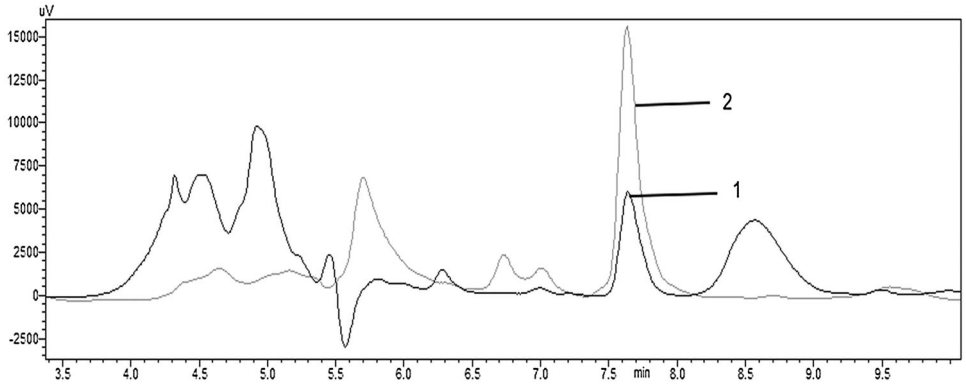


Fig. 4 Chromatographic analysis of peaks corresponding to L-Asparaginase in forward (*1*) and back (*2*) extracted samples

Table 2 Extraction efficiency as an effect of individual process parameters during forward and back extraction of L-asparaginase synthesized by *Azotobacter vinelandii*

Process variable	Activity (Units/mL)	Specific activity (Units/mg)	Purification fold (Standard deviation)
Forward extraction			
Broth pH—4.5	4.19	36.767	7.81 (±0.633)
Alcohol – Butanol, 0.1 M	8.01	95.36	24.57 (±0.615)
Back extraction			
Stripping phase pH – Phosphate buffer, pH 6.5	20.59	245.05	69.72 (±1.243)
Alcohol – Hexanol, 1 M	29.44	2452.78	174.63 (±2.056)
Electrolyte – Ammonium sulphate, 2 M	42.11	1754.20	379.68 (±2.630)

1754.20 (Units/mg). Specific activity of the enzyme was found to decrease compared to the back extraction effect of alcohol owing to the fact that addition of salt in the presence of alcohol would lead to denaturation of protein. However, purification fold of 379.68 was obtained with the addition of 2 M ammonium sulphate. Table 1 summaries the increase in activity, specific activity and purification fold during step by step forward and backward extraction of L-asparaginase synthesized by *Azotobacter vinelandii.*

Chromatographic analysis of reverse micelle purified L-Asparaginase

RP HPLC analysis of forward extracted sample (broth pH of 4.5 and 0.1 M butanol) and back extracted sample (stripping phase pH of 6.5, 1 M hexanol and 2 M $(NH_4)_2SO_4$ concentration which successfully gave maximum purification of L-asparaginase (Table 2) was performed as individual chromatographic runs. Chromatograms obtained were compared for the peaks obtained at 254 nm as shown in Fig. 4, it is inferred from figure that a sharp intense peak was obtained at ~7.65 in both samples that corresponds to L-Asparaginase, peak area % of backward extracted sample (2) as shown in Fig. 4 was 5.5 times than that of peak area % of forward extracted sample (1) confirming the increase in purity during forward to backward extraction step. It can also be observed that a number of peaks that appear in forward extracted sample (1) are evicted in backward extracted sample (2).

Conclusion

Although cloud point extraction of L-Asparaginase from *E. coli* employing Triton X 100 and dipotassium hydrogen phosphate has been successfully studied [37], reverse micellar extraction of L-Asparaginase from crude broth is first of its kind. By varying the process variables during RME, L-Asparaginase was successfully separated with an overall purification fold of about 379.86, activity of 42.11 (Units/mL) and specific activity of 1754.20 (Units/mg), which are on par with the literature reported activity of 36.260 (IU) and specific activity of 1250.54 (IU/mg) and purification fold of 44.63 obtained by performing ammonium sulphate precipitation followed by DEAE cellulose chromatographic purification of L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 [38]. Hence, the authors believe that L-asparaginase with the maximum purity obtained through RME, will satisfy the required purity factor of an enzyme for its application in medical and pharmaceutical sector. However, RME purified L-asparaginase may be tested extensively for its efficacy and

virulent effects by performing in vitro studies. Though, usage of organic solvents are non-sustainable, reverse micellar extraction of L-asparaginase or any other cellular protein can be performed with maximum solute specificity and higher extraction efficiency from the crude broth as a single step extraction process. Considering the ease in scale up and simple operation with reproducibility of the purification folds, this batch process can be scaled up to continuous reverse micellar extraction that also includes the recycling of reverse micellar phase and reuse.

Compliance with ethical standards

Conflict of interest All the authors of this manuscript express no conflict of interest towards the article submitted.

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