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Full Length Research Paper

Extraction of uricase from *Candida utilis* by applying polyethylene glycol (PEG)/ $(NH_4)_2SO_4$ aqueous two-phase system

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The purification of uricase from Candida utilis was performed by applying aqueous two-phase system (ATPS). Based on polyethylene glycols (PEG)/$(NH_4)_2SO_4$ ATPSs, purification fold and recovery rate were demonstrated to influence the system parameters, such as PEG molecular mass, PEG concentration, ammonium sulfate [$(NH_4)_2SO_4$] concentration, inorganic concentration, pH and additional amount of crude enzyme. The ATPS formed by 25% (w/w) PEG2000, 9% (w/w) $(NH_4)_2SO_4$, 2% (w/w) NaCl, pH 7.5 and addition of 5% (w/w) crude enzyme showed the best separation capability. These conditions led to the 93% (w/w) recovery of uricase with 10-fold purification.

Key words: *Candida utilis*, aqueous two-phase, extraction, purification, uricase.

INTRODUCTION

Urate oxidase (uricase, E.C.1.7.3.3) catalyzes the oxidation of uric acid to a more soluble and easily excreted compound called allantoin (Nelson and Cox, 2004) and plays an important role in nitrogen metabolism. Higher primates (that is, apes and humans) lack functional uricase and excrete uric acid as the end product of purine degradation, which is a causative factor of gout (Oda et al., 2002). In patients suffering from gout, uricase can reduce the amount of uric acid, remove gouty arthritis and block the formation of tophus and uratoma. Uricase has stronger singleness and higher reaction efficiency and is one of the essential enzymes used to determine urate in clinical analysis (Duncan et al., 1982).

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Abbreviations: ATPS, Aqueous two-phase system; **PEG,** polyethylene glycols; **BSA,** bovine serum albumin.

Uricase has been found in mammalian (Wu et al., 1989), plant (Suzuki and Verma, 1991) and microbial cells (Yazdi et al., 2006; Lotfy, 2008). Microorganisms, such as bacteria, yeast and filamentous fungi are important sources of uricase. Genes encoding uricase have been cloned from *Aspergillus flavus*, *Aspergillus nidulans*, *Candida utilis*, *Arthrobacter globiformis*, *Cellulomonas flavigena,* and symbiotic of *Nilaparvata lugens* (Suzuki et al., 2004) and are expressed in *Escherichia coli* (Li et al., 2006). Uricase from *A. flavus* is expressed in the yeast *Saccharomyses cerevisiae* in soluble and active forms (Leplatois et al., 1992). However, the recombinant uricase from *E. coli* or *S. cerevisiae* still has the problem of low productivity and cost-effective separation in medical applications (Li et al., 2006).

Although uricase has been produced from many organisms and also from genetically engineered micro-organisms, its increasing importance in treatment and in clinical analysis makes it necessary to develop new methods for the isolation and purification of uricase with considerably high purity, low cost and industrial

applicability. The traditional methods involve a combination of operations, such as centrifugation, precipitation, membrane and gel filtrations, ion-exchange and affinity chromatography or dialysis, and final concentration of the product. These methods are time consuming, and some reactants are expensive, contributing to the increasing cost of the downstream processing.

Thus, partitioning in an aqueous two-phase system (ATPS) is a good alternative to separate and purify mixtures of proteins. ATPS is formed by mixing two flexible chain polymers in water or one polymer and one salt (e.g., phosphate, citrate, etc.) (Boris and Zaslavsky, 1994). Proteins are partitioned between the two phases with a partition coefficient, which can be modified by changing the experimental conditions of the medium, such as pH, salts, ionic strength and so on. ATPS have the following advan- tages over conventional methods: The partition equili- brium is reached rapidly, they can be applied in scale up, they offer the possibility of continuous state operation, the cost of separation is low and the materials are economical and recyclable. The objectives of this research are to study the shape of the uricase partition in the ATPS formed by polyethylene glycols (PEG)/ $(NH_4)_2SO_4$ and to apply these results eventually to isolate and later purify this protein from *C. utilis*.

MATERIALS AND METHODS

Material and reagent

C. utilis 2.281(C3) was purchased from the Institute of Microbiology and uric acid from Sigma Chem Co. (USA). Polyethylene glycols with average molecular weights of 400, 600 and 2000 (PEG400, PEG600 and PEG2000, respectively) were obtained from Merk in Germany, whereas, the yeast paste (BR) and beef extract (BR) were purchased from Beijing Aobo Xing Biotechnology Ltd. Co. (China). Peptone (503), bovine serum albumine (BSA) obtained from Chengdu Changshou Biological Preparation Ltd. Co. and the other reagents were of analytical quality.

Candida culture and crude enzyme preparation

Strains were allowed to grow at 28℃ for 24 h in an inclined culture medium and were then inoculated to the seed medium at 28℃ with shaking at 200 rpm for 23 h. Liquid seeds were added into a 250 ml triangle bottle containing 100 ml fermentation culture medium, under pro rata of 10% and incubated at 28℃ with shaking at 200 rpm for 23 h. The strains were collected by centrifugal process and the wet weight was obtained after washing. Strains were suspended in boric acid-borax buffer liquid at 50 mmol/L pH 8.5 under pro rata of 1:4 (w/v) and were crushed by ultrasonication. Broken liquid was centrifuged for 5 min (12000 rpm) at 4℃. The crude enzyme was obtained after the cell debris had being separated.

The aqueous two-phase extraction process

PEG and $(NH_4)_2SO_4$ were separately conjugated in proportion with water to a certain concentration of the solution. ATPS was prepared by mixing a suitable amount of PEG and $(NH_4)_2SO_4$ solution at room temperature. A certain crude enzyme was added into the inter- mixture. The mass was adjusted to 1.0 g with deionized water. The mixtures were vortexed together for 2 min using a standard desktop vortex mixer, maintained for 15 min and then centrifuged for 10 min (1,800 rpm) to separate the two phases. The volumes of these phases and the total volume were respectively, determined. The protein content and enzyme activity in each phase were determined by protein concentration measurements and uricase activity measurements, respectively.

Enzyme assay

Uricase activity was assayed using the method described by Klose et al., (1978). Standard reaction mixture contained 2.2 ml of 2 mmol/L uric acid dissolved in 0.1 mol/L sodium borate buffer (pH 8.5), 0.15 ml of 20 U/ml peroxidase from horseradish, 0.15 ml of 60 mmol/L 4-aminoantipyrine, 0.15 ml of 1.5% phenol, 0.15 ml of 0.1 mol/L sodium borate buffer (pH 8.5) and 0.2 ml of properly diluted enzyme solution to a final volume of 3.0 ml. The mixture was incubated at 25℃ for 20 min and the increase in absorbance at 505 nm was spectrophotometrically measured. One unit of enzyme is defined as the amount of enzyme that produces 1.0 µmol of H_2O_2 per minute under standard assay conditions.

Total protein concentration determination

Protein concentration was determined according to Bradford (1976) with BSA as standard. Phases of the same two-phase systems without proteins were used as references.

Determination of partition coefficients, phase volume ratio, percentage enzyme activity recovery and purification factor

The partition coefficient of uricase and protein (Ke, Kp) were calculated as the ratio of activities or concentrations in the top and bottom phases.

$$Ke = A_T / A_B$$

$$Kp = P_T / P_B$$

Where, A_T, A_B and P_T, P_B represent the activities of uricase (in U/mL) and protein concentration (in mg/mL) in the top and bottom phases, respectively.

The phase volume ratio (R) is defined as the ratio of the volumes of the top and bottom phases.

$$R = V_T / V_B$$

Where, V_T and V_B are the volumes of the top and bottom phases, respectively.

Activity recovery is determined as the ratio of activity of uricase in the top phase to that of the initial extract using the following equation:

$$\text{Recovery}\% = \frac{A_T \times V_T}{A_I \times V_I} \times 100$$

Where, A_I and V_I are the volume and uricase activity of the crude extract, respectively. The purification factor of uricase is defined as the ratio of specific activity of uricase in the top phase to that of the crude extract. It can be calculated using the following equation:

Table 1. Influence of PEG molecular mass on uricase partitioning.

Parameters	Values		
PEG molecular weight	400	600	2000
R	2.0 ± 0.1	2.1 ± 0.15	2.8 ± 0.1
Recovery (%)	96.3 ± 0.7	70.5 ± 0.6	92.1 ± 0.5
Purification factor	1.07 ± 0.08	1.35 ± 0.05	7.8 ± 0.05

Phase system: 25% PEG/12% $(NH_4)_2SO_4$ and 10% crude enzyme liquid at 25°C ± 2; R, phase volume ratio. Data are the means ± SDs from three different experiments.

$$\text{Purification factor} = \frac{\text{specific activity}_{\text{top phase}}}{\text{specific activity}_{\text{crude extract}}}$$

Selection of extraction conditions

Following the example in previous research (Shang et al., 2004), the variables chosen for evaluation were PEG molecular mass, PEG concentration, $(NH_4)_2SO_4$ concentration, pH, inorganic concentration and addition of crude enzyme.

RESULTS AND DISCUSSION

Influence of polyethylene glycols molecular mass on uricase partitioning

The differences in solutes in the two-phase system mainly depend on various interactions between the solutes and the components in the system. The most important differences are electrostatic interaction, hydrophobic interaction and affinity interaction. In the two-phase system consisting of polymers and salts, the salt phase has stronger electrostatic interaction, whereas, the polymer phase has stronger hydrophobic and affinity interactions (Wu, 2006). We selected PEG/$(NH_4)_2SO_4$ ATPS as the extraction system to extract and separate uricase. Under nearly identical circumstances, we initially selected 12% $(NH_4)_2SO_4$, 25% PEG and 10% crude enzyme liquid and then fixed the influences of the various PEG molecular masses on the purification fold of uricase. The results are shown in Table 1.

For ATPS, phase ratio R is not an independent variable but a dependent one. It can be influenced by components and their concentrations and it is different from ordinary extraction. If comfortable conditions are selected, increasing the phase volume ratio is advantageous for enzyme extraction. The heavier the PEG molecular weight, the higher its viscosity and the stronger its hydrophobicity, causing the demixing time to be longer and the phase ratio R to be larger. Therefore, a heavier PEG molecular weight is not desirable because it will make the phase separation difficult and the hydrophobic interaction stronger. Neither can we select lighter PEG molecular weight because it will reduce the phase volume ratio and influence the recovery rate of uricase.

Compared with the specific activity of crude enzyme liquid, a higher purification fold and a reasonable recovery rate could be obtained when PEG2000 for extracting uricase was selected (Table 1). On the other hand, although the recovery rate was higher, the purification fold was lower, which is nearly similar to the traditional $(NH_4)_2SO_4$ fractionation when PEG400 was selected. Hence, PEG2000 can be used for optimum PEG to extract uricase.

Effects of polyethylene glycols concentration on uricase partitioning

To set PEG2000 as the ingredient of ATPS to extract uricase, we selected 12% concentration of $(NH_4)_2SO_4$, changed the concentration of PEG2000, and formed a series of PEG2000 concentration gradients for the two-phase system. When the concentration of PEG2000 was 20%, the specific activity of uricase was maximum, which means that the purification fold was optimized. However, the partition coefficient was minimum, which means that uricase nearly reached the lower phase. As uricase accounts for the lower ratio, its recovery rate in the upper phase was low (Figure 1). For a certain PEG molecular weight, if its concentration is increased, its hydrophilic groups and phase ratio also increase. Thus, both the partition coefficient of the enzyme and its recovery rate increase. The phenomenon is a good factor for separation. However, if the concentration is too high, the increased phase ratio and increased volume of the upper phase are undesirable for producing concentrated enzyme, which will result in an increase in phase material amounts and total production cost. Considering the production cost and recovery rate, we can conclude that the optimum PEG2000 concentration of extracting uricase is 25%.

Effects of $(NH_4)_2SO_4$ concentration on enzyme extraction

Based on the fixed PEG concentration, the next step was to select the optimum concentration of $(NH_4)_2SO_4$. As shown in Figure 2, with the increasing concentration of $(NH_4)_2SO_4$, the salting-out effect was enhanced, and uricase approached to the upper phase. However, if the concentration was too high, parts of the enzyme precipitated. When the concentration of $(NH_4)_2SO_4$ was 9%, both

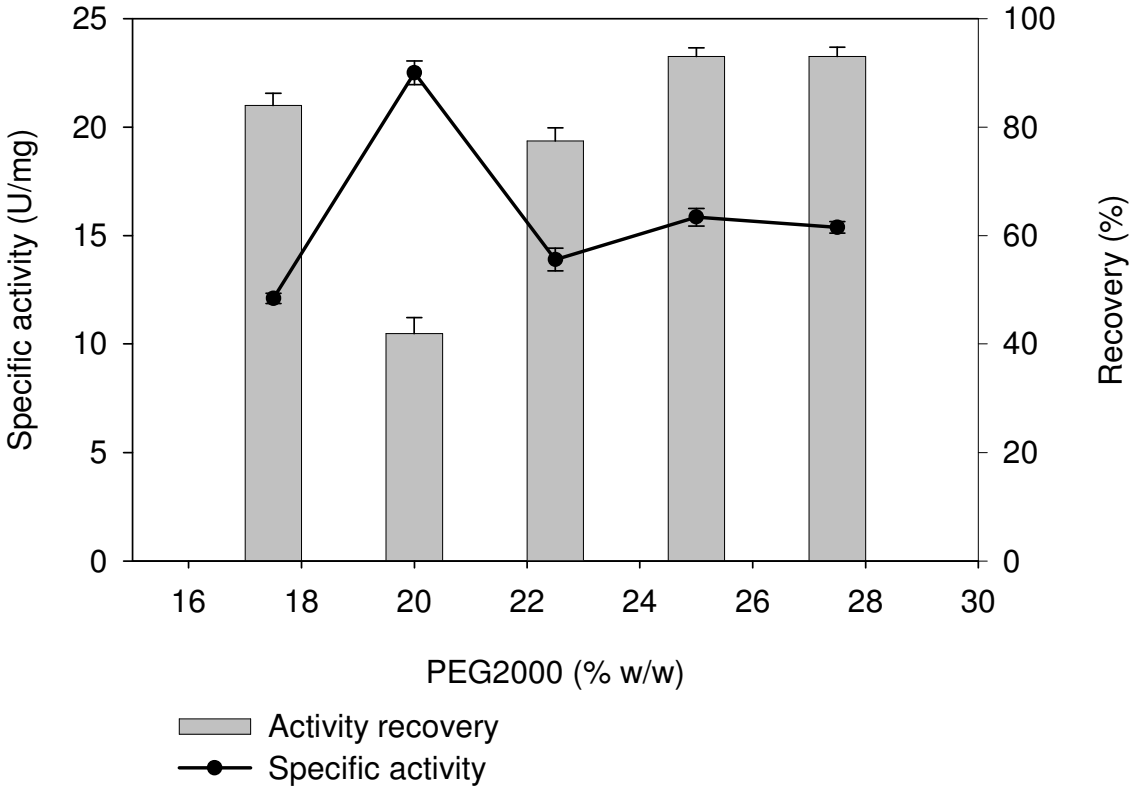

Figure 1. Effects of PEG 2000 concentrations on the partition of uricase, concentrations of $(NH_4)_2SO_4$ (w/w) were 12%. Data are the means ± SDs from three different experiments.

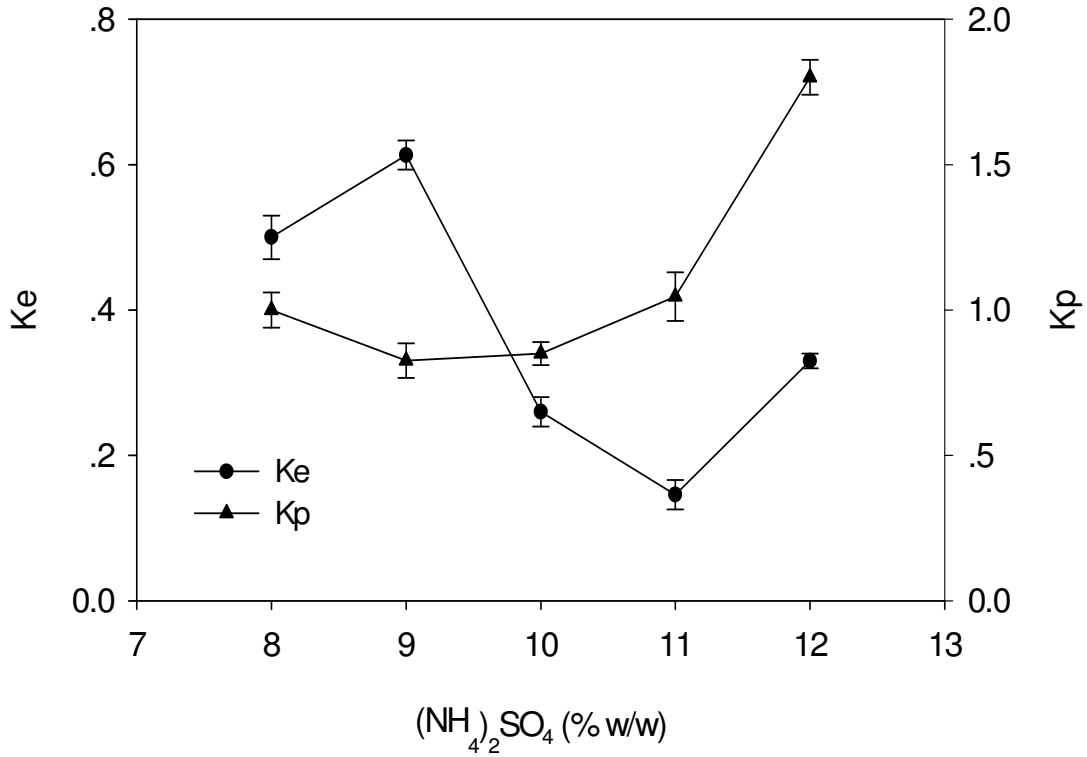

Figure 2. Effects of $(NH_4)_2SO_4$ concentrations on the distribution coefficient of uricase, concentrations of PEG 2000 (w/w) were 25%. Data are the means ± SDs from three different experiments.

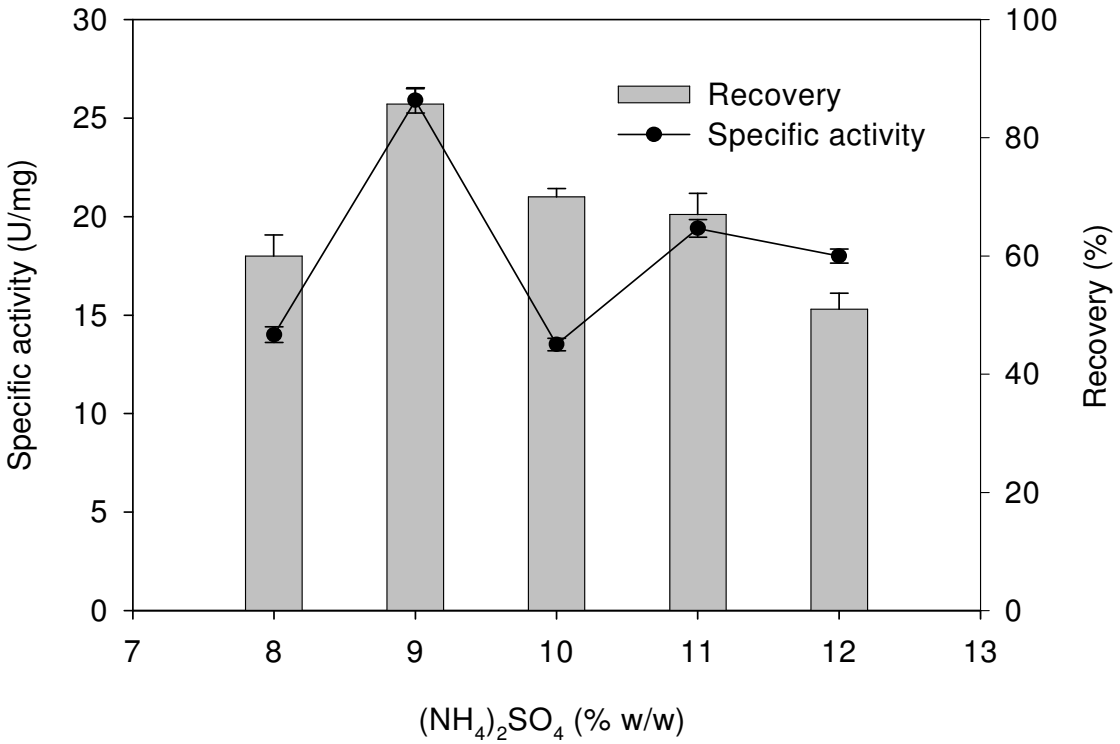

Figure 3. Effect of $(NH_4)_2SO_4$ concentration on the specific activity and activity recovery of uricase, concentrations of PEG 2000 (w/w) were 25%. Data are the means ± SDs from three different experiments.

the partition coefficient of enzyme in the upper phase and the specific activity were maximum and the recovery rate of enzyme was also high (Figure 3). Thus, it is desirable to fix the 9% $(NH_4)_2SO_4$ concentration.

Effects of pH on enzyme extraction

Results showed that in an ATPS, pH had a great effect on the distribution of extraction. The pH of a solution influences the degree of dissociation of protein and enzyme in a solution, thus changing the electrical charge of protein and enzyme. On the other hand, changed pH can also alter the electrical charge of the molecules of the phase components and phase interface. These factors can alter the molecular interaction among the extractions, phase components and partition coefficient. Therefore, in the process of ATPS extraction, adjusting the pH to improve the partition co- efficient of separated protein or enzyme can always increase separation efficiency. As shown in Figures 4 and 5, in the ATPS used in this experiment, when pH value was 7.5, the partition coefficient of uricase and the recovery rate and purification fold of enzyme were optimized.

Effects of salt concentration on uricase extraction

According to the results in previous research, the next step was to fix the effects of neutral salt, NaCl, and its concentration in the recovery rate and purification fold of enzyme. As shown in Table 2, the partition coefficient of uricase and the purification fold and recovery rate of enzyme in the upper phase increased with the addition of inorganic salts. However, if the concentration of NaCl was beyond 2% (w/w), the aforementioned three figures would be reduced. This may be attributed to two conditions: a) the increased concentration of NaCl can increase the salting-out effect, and parts of the enzyme are precipitated on the interphase. The most undesirable result is the loss of enzyme activity; b) parts of Na^+ are in the upper phase, increasing the positive electrical property of the upper phase. When pH value is 7.5, uricase is positive, which is undesirable in reaching for the upper phase.

Effects of crude enzyme addition on enzyme extraction

Under identical conditions of the ATPS, 25% PEG2000, 9% $(NH_4)_2SO_4$, and 2% NaCl, we respectively, added various amounts of crude enzyme and compared their extraction results. According to the results shown in Table 3, the larger the crude enzyme liquids are added, the lower the values for both partition coefficient and recovery rate of uricase. Moreover, when the concentration of the crude enzyme is 5%, enzyme in the upper phase is

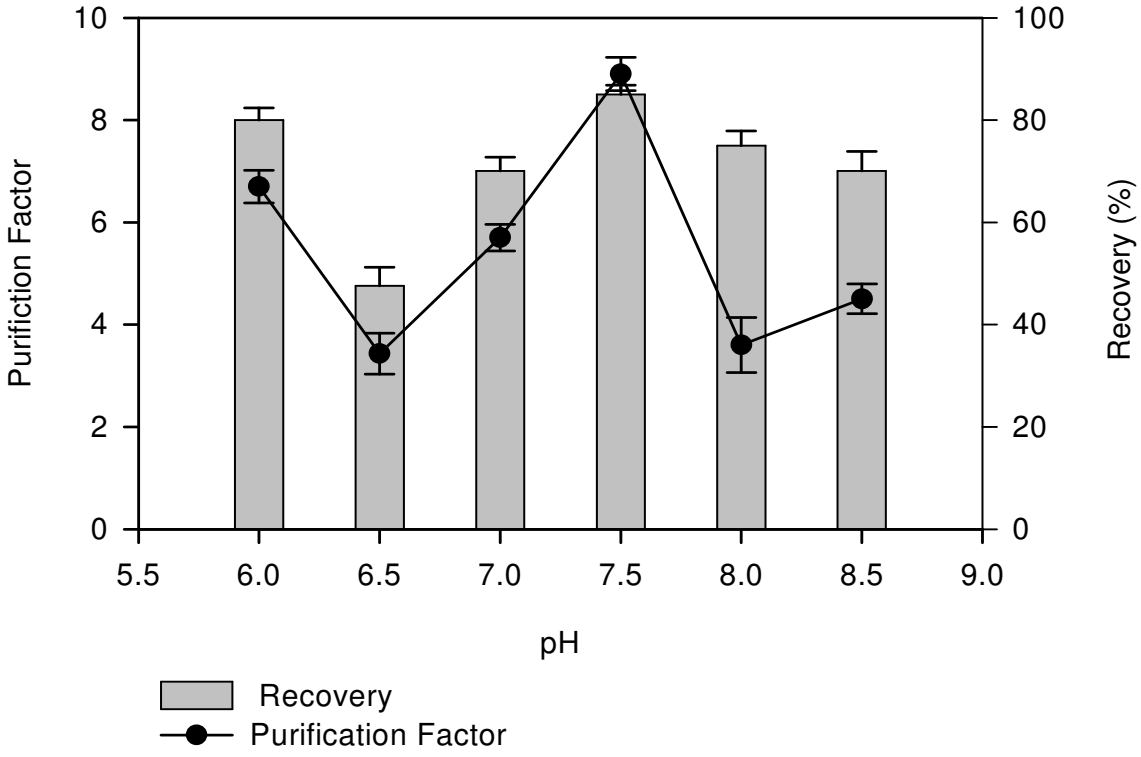

Figure 4. Effect of pH on the purification multiple and activity recovery of uricase, concentrations of PEG 2000 (w/w) were 25%, whereas concentrations of $(NH_4)_2SO_4$ were 9 % (w/w). Data are the means ± SDs from three different experiments.

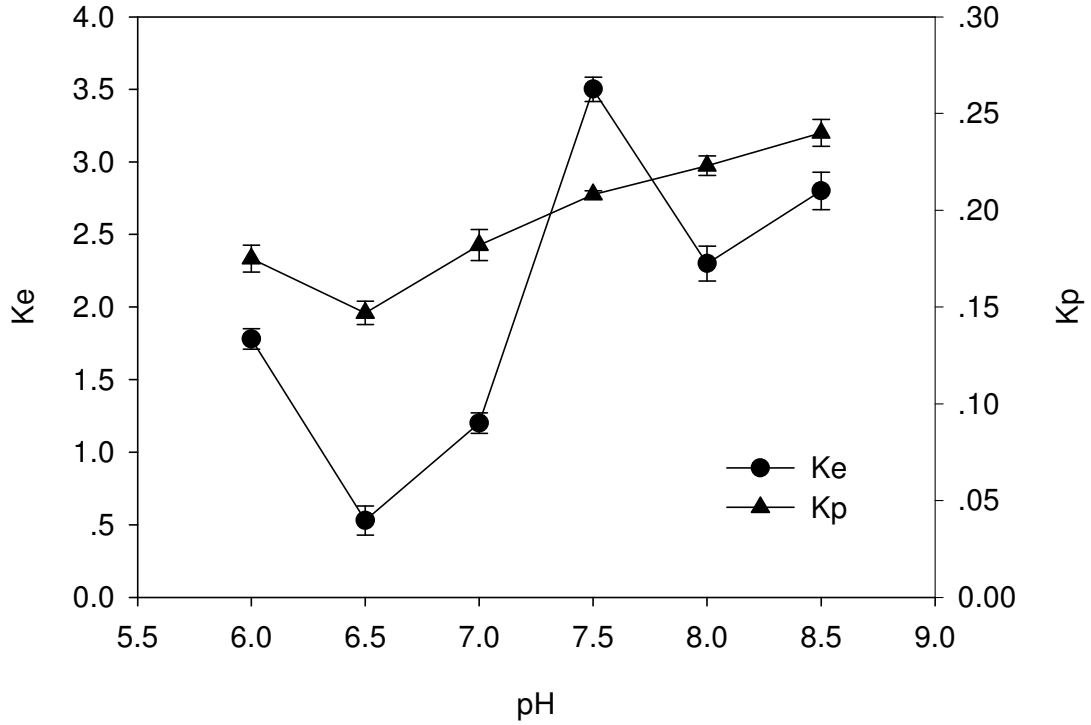

Figure 5. Effect of pH on the distribution coefficient of uricase, concentrations of PEG 2000 (w/w) were 25%, whereas concentrations of $(NH_4)_2SO_4$ were 9 % (w/w). Data are the means ± SDs from three different experiments.

Table 2. Effects of salt concentration on uricase partitioning.

Parameters	Values			
NaCl (% w/w)	0	2	4	6
Ke	1.57 ± 0.05	8.68 ± 0.18	4.33 ± 0.12	1.2 ± 0.1
Purification Factor	0.95 ± 0.05	10 ± 0.18	2.25 ± 0.15	0.59 ± 0.06
Recovery (%)	77.6 ± 0.5	92.5 ± 0.7	87.9 ± 0.3	60.8 ± 0.8

Phase system: 25% PEG/9% $(NH_4)_2SO_4$, pH 7.5 and 10% crude enzyme liquid at 25°C ± 2; Ke, partition coefficient of uricase. Data are the means ± SDs from three different experiments.

Table 3. Effect of biomass concentration on the extraction process parameters.

Parameters	Values					
Crude enzyme addition (%)	5	10	15	20	25	30
Ke	9.88 ± 0.24	8.0 ± 0.31	7.4 ± 0.37	5.5 ± 0.26	5.1 ± 0.19	6.24 ± 0.31
Recovery (%)	93.5 ± 0.8	88 ± 0.7	90 ± 0.5	84 ± 0.8	83.6 ± 0.4	83.7 ± 0.3
Purification Factor	10.17 ± 0.33	6.69 ± 0.11	5.7 ± 0.2	2.48 ± 0.15	6.5 ± 0.31	8.0 ± 0.42

Phase system: 25% PEG/9% $(NH_4)_2SO_4$, pH 7.5 and 2% NaCl at 25°C ± 2; Ke, partition coefficient of uricase. Data are the means ± SDs from three different experiments.

optimized as well as the recovery rate.

Conclusions

Uricase is an important enzyme for medical application. Uricase from *C. utilis*, *A. flavus,* and *Bacillus* sp, and recombinant uricase from *E. coli* or *S. cerevisiae* are now commercially available, but their production procedures still have the problems with low productivity, complicated isolation and purification for clinical applications (Li et al., 2006; Lotfy, 2008). In this study, the *C. utilis* uricase was successfully extracted applying PEG/ $(NH_4)_2SO_4$ ATPS for the first time.

This paper primarily analyzed the purification process of an ATPS consisting of PEG/ $(NH_4)_2SO_4$. The results showed that the system, under selected experimental conditions containing 25% PEG2000, 9% $(NH_4)_2SO_4$, 2% NaCl, pH 7.5 and addition of 5% crude enzyme, could produce the most desirable results. Moreover, the partition coefficient of uricase was 9.88, purification fold was approximately 10 times and recovery rate was 93%. All these parameters are superior to those in traditional purification. Thus, the elements that can influence uricase distribution behavior in PEG/ $(NH_4)_2SO_4$ system are more difficult and complex and they may be investigated in future experiments.

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