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# Partition of alkaline protease in aqueous two-phase systems of polyethylene glycol 1000 and potassium phosphate

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**Abstract**−This article presents a study of polyethylene glycol 1000 (PEG1000)/potassium phosphate aqueous two-phase systems (ATPSs) for *Bacillus subtilis* NS99 alkaline protease extraction. The objectives were to evaluate effects of system pH (7.5, 8.5, 9.5, and 10.5), and NaCl concentration (0, 4, 7, and 10% (w/w)) on ATPS binodal curves, effects of system pH, NaCl concentration, and tie-line length (TLL) on alkaline protease partition coefficient (K) and yield (Y%) at room temperature (30±2 °C). Casein hydrolysis was used for determination of alkaline protease activity. It was revealed that system pH had the slightest effect on locations of binodal curves (except at pH 10.5). In contrast, addition of NaCl appeared to have a significant effect on phase characteristics since binodal curves of systems with NaCl (4-10% (w/w)) shifted significantly towards the origin in comparison to the ones without NaCl. Increased NaCl concentration from 4 to 10% (w/w), however, showed trivial influence on locations of the binodal curves. Changes of system compositions due to variation in system pH, TLL, and NaCl concentrations obviously resulted in varied obtainable K and Y% of alkaline proteases. Longer TLL and higher pH generally resulted in higher K. In contrast, the lower NaCl concentration, the higher K. Since the same phase volume ration (1 : 1) was used throughout the experiments, Y% depended solely on K. The most suitable PEG1000/potassium phosphate ATPS was determined at pH 9.5, and comprised PEG1000, potassium phosphate, and NaCl 18.0, 13.0, and 0% (w/w), respectively. This system resulted in considerably high K, and Y% of 20.0, and 95.1%, respectively. Information from this study will be important for further development of an ATPS extraction unit for alkaline protease recovery.

Key words: Aqueous Two-Phase System, Enzyme Partition, PEG1000, Potassium Phosphate, Alkaline Proteases

## INTRODUCTION

Proteases are the most important industrial enzymes which account for roughly 60% of the total enzyme market [Ng and Wenealy, 1986]. Among them, alkaline proteases are of particular interest due to their broad applications in detergent, tanning, and dairy industries [Grebeskova et al., 1988; Wilson et al., 1992; Phadatara et al., 1993]. Since 50-90% of production cost resides in the purification strategy, many procedures have been developed for enzyme downstream processing [Park et al., 1997; Hong et al., 1997; Cunha et al., 2003]. One such strategy involves using aqueous two-phase systems (ATPSs), which is an economical technology with low energy consumption, low labor cost requirement, and has great potential for further process development due to its ease of scale-up and facilitation of high partition coefficients [Cunha et al., 2003].

ATPSs are formed by mixing two incompatible polymers (polyethylene glycol (PEG), dextran, etc.) or a polymer and a salt (phosphate, citrate, etc.) in aqueous condition. Partitioning of proteins between the two phases can be manipulated by changing system conditions such as pH, salt and phase compositions, etc. Although ATPSs have been applied for recovery of a wide variety of biomolecules (alpha-1-antitrypsin [Reh et al., 2002]; insulin [Haraguchi et al., 2004]; penicillin acylase [Marcos et al., 2002]; cutinase [Kepka et al., 2003]; glucose-6-phosphate dehydrogenase [Xu et al., 2002], Hexokinase [Xu et al., 2002]; pectinolytic enzymes [Lima et al., 2002], trypsin [Oliveira et al., 2002]), reports of their applications for alkaline protease extraction are still quite limited. Published articles have mainly focused on investigating effects of PEG molecular weight (PEG400, 1000, 4000, 6000), types of salts ($NaH_2PO_4$, $K_2HPO_4$, $MgSO_4$, etc.) and their concentrations [Sinha et al., 1996; Klomklao et al., 2005] on alkaline protease extraction. In this study, we decided on using PEG1000/potassium phosphate ATPSs for alkaline protease extraction based on previous suggestion that both PEG and potassium phosphate displayed stimulating effects on this particular enzyme activities [Sinha et al., 1996]. In addition, Klomklao et al. [2005] recently discovered that ATPS comprising PEG1000 (15%, w/w) and magnesium sulfate (20%, w/w) provided the best conditions for proteinase extraction from tuna spleen with a purification fold of 6.61 and yield of 69.0%.

This article presents extraction of *Bacillus subtilis* NS99 alkaline proteases using an aqueous two-phase system of PEG1000/potassium phosphate. The objectives were to evaluate effects of system pH, and sodium chloride concentration on ATPS binodal curves, as well as effects of system pH, sodium chloride concentration, and tie-line length (TLL) on alkaline protease partition coefficient and yield. Information from this study will be important for further development of an ATPS extraction unit for alkaline protease recovery.

## EXPERIMENTAL SECTION

### 1. Materials

Crude extract of *Bacillus subtilis* NS99 alkaline protease was

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kindly provided by Prof. Napa Siwarungson, Department of Biochemistry, Chulalongkorn University. PEG1000, $K_2HPO_4$, $KH_2PO_4$, and NaCl were obtained from Ajax, Australia. Casein from bovine milk was from Fluka, Switzerland. Trichloro acitic acid was from Merck, Germany. All chemicals used were of laboratory grade. Deionized water was used throughout the experiments.

**Table 1. Compositions (%, w/w) of the studied phase systems**

| NaCl | pH | Tie-line* | PEG1000 | $K_2HPO_4$ | $KH_2PO_4$ |
|---|---|---|---|---|---|
| 0 | 7.5 | STL | 20.70 | 10.73 | 4.56 |
| | | MTL | 27.39 | 12.62 | 6.79 |
| | | LTL | 32.31 | 13.00 | 12.48 |
| 0 | 8.5 | STL | 20.68 | 13.10 | 0.88 |
| | | MTL | 25.78 | 18.53 | 1.59 |
| | | LTL | 31.87 | 20.90 | 3.64 |
| 0 | 9.5 | STL | 18.00 | 12.99 | 0.02 |
| | | MTL | 21.86 | 14.88 | 0.07 |
| | | LTL | 22.92 | 15.61 | 0.21 |
| 0 | 10.5 | STL | 37.72 | 27.43 | 0.00 |
| | | MTL | 38.63 | 31.17 | 0.00 |
| | | LTL | 42.90 | 37.77 | 0.00 |
| 4 | 7.5 | STL | 21.00 | 15.34 | 1.71 |
| | | MTL | 26.01 | 18.01 | 2.35 |
| | | LTL | 30.69 | 20.85 | 3.61 |
| 4 | 8.5 | STL | 23.69 | 19.88 | 0.35 |
| | | MTL | 26.77 | 20.45 | 0.40 |
| | | LTL | 30.60 | 23.29 | 0.74 |
| 4 | 9.5 | STL | 22.65 | 10.52 | 0.00 |
| | | MTL | 25.35 | 11.80 | 0.00 |
| | | LTL | 25.77 | 13.40 | 0.06 |
| 4 | 10.5 | STL | 40.95 | 16.73 | 0.00 |
| | | MTL | 42.00 | 19.65 | 0.00 |
| | | LTL | 42.60 | 23.15 | 0.02 |
| 7 | 7.5 | STL | 21.02 | 9.72 | 0.70 |
| | | MTL | 30.02 | 13.50 | 1.02 |
| | | LTL | 30.80 | 15.70 | 1.49 |
| 7 | 8.5 | STL | 22.99 | 11.94 | 0.02 |
| | | MTL | 28.64 | 15.28 | 0.00 |
| | | LTL | 30.79 | 20.81 | 0.00 |
| 7 | 9.5 | STL | 22.99 | 11.73 | 0.00 |
| | | MTL | 24.46 | 12.45 | 0.00 |
| | | LTL | 26.01 | 12.77 | 0.00 |
| 7 | 10.5 | STL | 40.87 | 19.04 | 0.00 |
| | | MTL | 41.14 | 20.79 | 0.00 |
| | | LTL | 41.89 | 22.24 | 0.00 |
| 10 | 7.5 | STL | 22.38 | 9.16 | 0.62 |
| | | MTL | 27.06 | 11.40 | 0.81 |
| 10 | 8.5 | STL | 23.28 | 9.37 | 0.00 |
| | | MTL | 30.53 | 14.92 | 0.00 |
| 10 | 9.5 | STL | 25.55 | 10.06 | 0.00 |
| | | MTL | 28.15 | 13.60 | 0.00 |
| | | LTL | 33.63 | 19.17 | 0.00 |
| 10 | 10.5 | STL | 37.82 | 19.66 | 0.00 |
| | | MTL | 41.00 | 21.97 | 0.00 |
| | | LTL | 42.43 | 27.47 | 0.00 |

*STL=Short Tie-Line Length.
MTL=Medium Tie-Line Length.
LTL=Long Tie-Line Length.

**2. Preparation of PEG1000/Potassium Phosphate ATPSs**

ATPSs were prepared by adding predetermined amounts of potassium phosphate (10-40%, w/w; proportion of $K_2HPO_4$ and $KH_2PO_4$ was varied according to the desired pH) to stock solutions of PEG1000 (20-50%, w/w). NaCl (0, 4, 7, and 10% (w/w)) was added as required. Final pH adjustment was achieved by using either concentrated solutions of phosphoric acid or sodium chloride. The mixture was gently mixed throughout the preparation process with a magnetic stirrer (Ika Labortechnik, Germany).

**3. Determination of the Binodal Curve**

Binodal curves were determined by titrating deionized water to the prepared ATPSs. Titration was terminated once a clear solution was obtained. The amounts of water added were then used for calculations of system compositions at the binodal curves. The reported values represent the average of triplicate experiments.

**4. Determination of Partition Coefficient**

ATPSs with equal phase volume ratio were prepared according to system compositions shown in Table 1. One mL of enzyme solution was included in a total system weight of 12 g in all of the experiments. To speed up phase separation, 3,000 rpm centrifugation (Kubota5100, Japan) was used for 10 min after gentle mixing of the system components. Samples from each phase were then assayed for alkaline protease activities.

**5. Determination of Alkaline Protease Activity**

The hydrolysis of casein was used for determination of alkaline protease activity. 0.1 mL of each phase was added to 1 mL of 0.5% (w/v) casein in carbonate/bicarbonate buffer solution (pH 10.5). Another 0.9 mL of the same buffer solution was next added to make a total volume of 2 mL. The reactions were performed at 45 °C in a water bath (Labortechnik GMBH, Germany) for 20 min, and stopped by addition of 2 mL 10% (w/v) trichloro acitic acid solution. After whirly mixing, the mixtures were centrifuged at 3,000 rpm for 10 min. Filtration of the supernatant was next done by using Whatman No. 1 filter paper. The obtained clear solution was measured with a spectrophotometer (UV-VIS Jenway 6405, England) at 280 nm for tyrosine contents. One unit of alkaline protease activity (U) was defined as the amount of enzyme that liberated 1 μg of tyrosine in one min using 0.5% (w/v) casein as a substrate in a buffer solution of pH 10.5 and 45 °C. The reported values represent the average of three measurements.

**6. Calculations**

Tie-line length (TLL): is a numerical measure of compositions of the two phases and is calculated according to

$$TLL = ([\Delta C(P)^2] + [\Delta C(Q)^2])^{0.5} \quad (1)$$

where $\Delta C(P)$ and $\Delta C(Q)$ are the differences in concentrations of PEG1000 and potassium phosphate between the two phases, respectively.

Partition coefficient (K): is defined as alkaline protease activity in the top phase ($A_T$) divided by the correspondent value in the bottom phase ($A_B$).

$$K = \frac{A_T}{A_B} \quad (2)$$

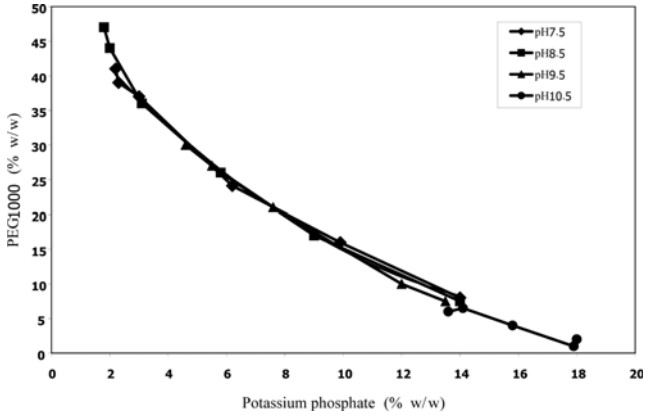


**Fig. 1. Binodal curves for PEG1000/potassium phosphate aqueous two-phase systems at 30±2 °C and varied pH.**

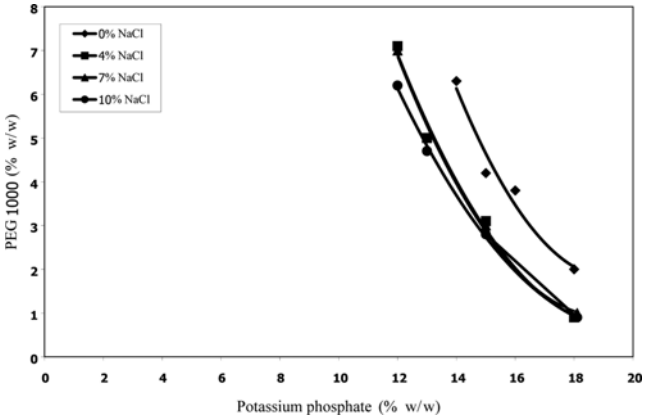


**Fig. 2. Binodal curves for PEG1000/potassium phosphate aqueous two-phase systems at 30±2 °C, pH 10.5 containing varied amounts of NaCl (in %w/w).**

Yield (Y%): is defined as

$$Y(\%) = \frac{100}{1+\left(\frac{V_T}{V_B}\right)K} \quad (3)$$

where $V_T$ and $V_B$ are the top and bottom phase volumes, respectively.

## RESULTS AND DISCUSSION

### 1. Effects of System pH and NaCl Concentration on Phase Separation

Information on binodal curves of ATPSs under study was necessary because it indicated areas where phase separation occurred and where enzyme extraction could be carried out. Fig. 1 demonstrates effects of system pH on positions of binodal curves. Apart from system pH 10.5, all of the curves appear to lie in pretty much the same position. Higher phosphate and lower PEG concentrations were needed for phase separation at pH 10.5. Phase separation, as suggested by Zaslavsky et al. [1989], is governed by water structure of the two phases which can be perturbed by many factors such as temperature, system compositions, and additives. The two phases will separate once the difference between the water structures induced by varying system conditions exceeds a critical level. In our study, variation of system pH was achieved by using different weight ratios of $K_2HPO_4$ and $KH_2PO_4$, which also meant changes of system compositions. Negligible effects of system pH on phase separation in cases of pH 7.5-9.5 indicated that $K_2HPO_4$ and $KH_2PO_4$ in the applied ratios exerted comparable effects on water structure of the two phases. Therefore, in these cases phase separation did not depend on weight ratios of the two salts, but on weight fractions of total potassium phosphate in the system. An Effect of $K_2HPO_4/KH_2PO_4$ proportions on phase separation was noticed when $K_2HPO_4$ was solely utilized to constitute ATPSs of pH 10.5.

Fig. 2 shows effects of low molecular weight additive (NaCl) on phase separation of ATPSs at pH 10.5. Inclusion of NaCl markedly resulted in shifting of binodal curves towards the origin. However, varying its contents from 4-10% (w/w) showed insignificant effect on positions of the binodal curves. Conway [1981] suggested that addition of salts to ATPSs leads to ionic hydration or ion-water interactions. The water molecules located in the vicinity of an ion tend to orient to the negative oxygen end of the molecule inward or outward according to the sign of the charged on the ion [Zaslavsky, 1995]. This alters the structure of water in both hydration sphere of ions and bulk water in the solution [Krestov, 1984]. Therefore, phase separation was induced by NaCl addition. Increased NaCl concentrations decreased the mean distance of separation between hydration spheres around each ion. NaCl of 4-10% (w/w) used in this study might not be concentrated enough to induce overlapping of hydration spheres around the ions; therefore, significant effects on phase separation due to increased NaCl concentrations were not observed. Similar effects of NaCl concentration on phase separation were also observed in systems with pH 7.5, 8.5, and 9.5 (data not shown).

### 2. Effects of Tie-line Length, System pH, and NaCl Concentration on Alkaline Protease Partitioning

After information on phase separation was known, PEG1000/potassium phosphate ATPSs were tested for *B. subtilis* NS99 alkaline protease extraction. Partition coefficient (K) and yield (Y%) were used as criteria for system evaluation. Partition behavior of solutes is known to be influenced by many factors which affect solvent features of the two phases and/or important solute properties involved in solute interactions with solvent in both phases [Zaslavsky, 1995]. According to Albertsson's classical model for protein (or enzyme) partitioning in ATPSs, protein partition coefficient is separately influenced by short range (van der Waals) and long range (electrostatic) molecular interactions as follows [Albertsson, 1986]:

$$\ln K_p = \ln K_0 + (Z_p F/RT)\Delta\varphi \quad (4)$$

where $K_p$ and $K_0$ denote protein partition coefficients at a given pH and at the protein isoelectric point, respectively. The term $(Z_pF/RT)\Delta\varphi$ is the electrostatic interaction which is a product of protein surface charge $Z_p$, the Faraday constant F, and electrostatic potential difference between the two phases. Therefore, the three factors--TLL, pH, and NaCl concentration--investigated in this study obviously affected both terms on the right handed-side of Eq. (4).

Fig. 3 illustrates effects of both TLL and NaCl concentration on K at system pH 10.5. Since TLL is a numerical measure of compositions of the two phases, a longer TLL indicates larger differ-

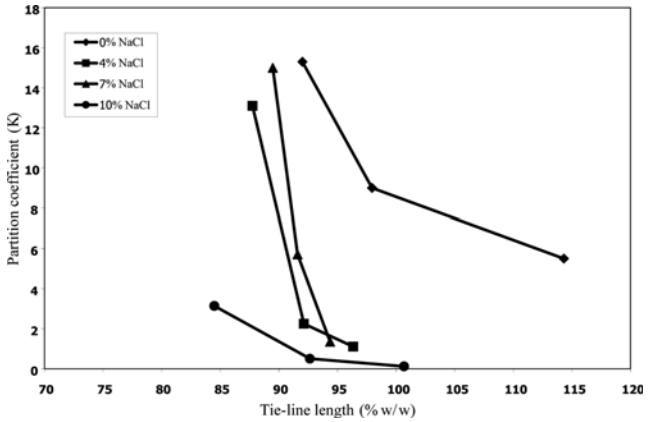


**Fig. 3. Effects of tie-line length on alkaline protease partition coefficient (K) in PEG1000/potassium phosphate aqueous two-phase systems at 30±2 °C, pH 10.5 with varied amounts of NaCl (in %w/w).**

ences in phase compositions and characteristics of the two phases. It was found, however, that the longer the TLL, the smaller K was obtained with every salt concentration tested. This was in accordance with Hotha and Banik [1997] who investigated partitioning of alkaline protease in PEG(4000, 6000, or 9000)/potassium phosphate ATPSs. Oliveira et al. [2002] also reported a decrease of trypsin partition coefficient in PEG/cashew-nut gum tree ATPS with increased TLL. Increased PEG concentrations with TLL probably caused molecular exclusion of the enzyme from the top to the bottom phase [Oliveira et al., 2002]. As a result, K was found to decrease with increasing TLL. Our results also showed sensitive changes of K upon varying TLL.

Fig. 3 also demonstrates that alkaline protease partition coefficient decreases with increasing NaCl concentration. This is in agreement with results found from other proteins [Lima et al., 2002; Capezio et al., 2005]. In our study, reduction of NaCl concentrations from 10 to 0% (w/w) at identical TLL and pH 10.5 resulted in an increase in K between 1.6-45.8 folds. This suggested marked effects of salt additive on target enzyme partitioning. The presence of a salt can create electrical potential differences between the phases [Johansson et al., 1995], hence influences enzyme partitioning. Since addition of NaCl might also induce structural changes in alkaline proteases, marked reduction of K in the case of 10% (w/w) NaCl (especially at short TLL) compared to those of lower concentrations might suggest specific changes in enzyme conformation induced by the enzyme-salt interactions [Zaslavsky, 1995].

Fig. 4 shows effects of system pH at various NaCl concentrations and TLL on alkaline protease partitioning. In most cases, K was found to increase with pH. Changing pH introduces variations on both charge of protein ionic groups and ion composition of ATPSs [Sebastiao et al., 1993], thus causes variation in K. According to Isable and Otero [2003], negatively charged proteins generally prefer the upper phase in PEG-salt systems. Higher K values obtained at system pH of 10.5 in our study therefore are in accordance with them since alkaline proteases (pI around 9 [Owen, 1983]) were negatively charged at this pH. Interestingly, opposite results were observed when similar ATPSs were used for alkaline protease extraction from fermentation broth [Chouyyok, 2005]. Chouyyok et al.

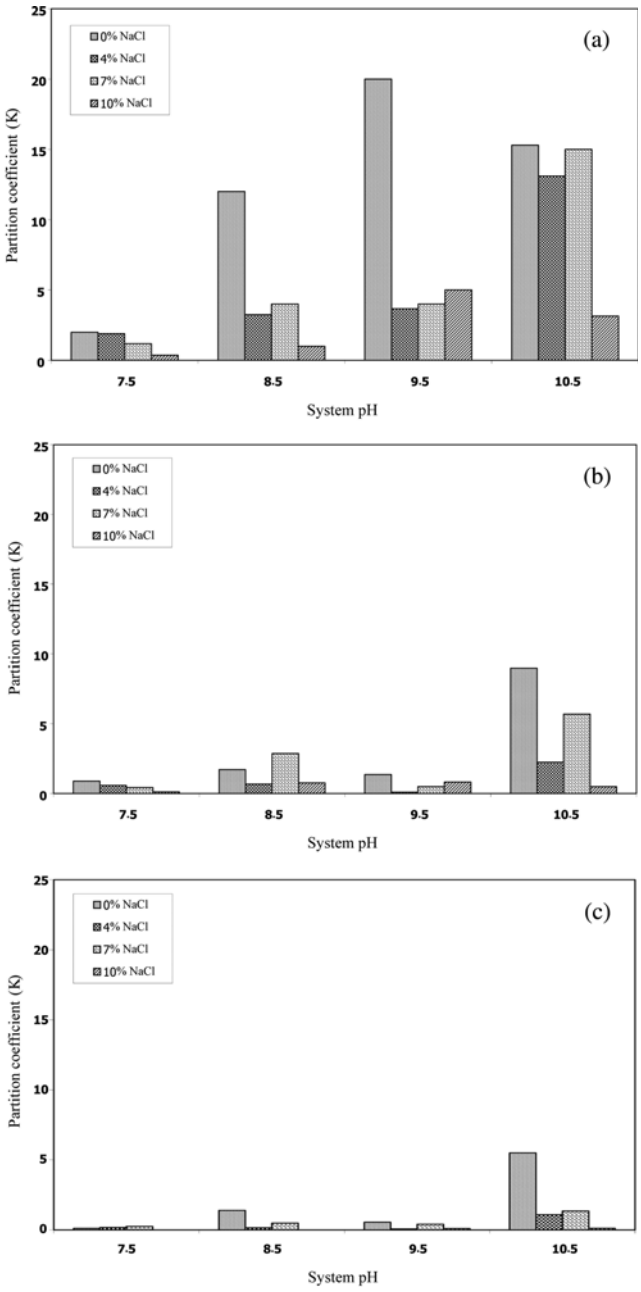


**Fig. 4. Effects of system pH and NaCl contents (%w/w) on alkaline protease partition coefficient (K) in PEG1000/potassium phosphate aqueous two-phase systems at 30±2 °C: (a) Short Tie-Line Length; (b) Medium Tie-Line Length; (c) Long Tie-Line Length.**

[2005] reported that alkaline protease partition coefficient decreased with increasing system pH. Contradictory results may be due to different phase compositions and sources of enzymes of the two cases. Fermentation broths are known to comprise various other compounds which could largely affect enzyme partitioning.

Since phase volume ratio was kept constant at 1 : 1 in all of the experiments, extraction yields of alkaline proteases determined in this study were solely dependent on K. In other words, yield increased with K. Therefore, Y% was found to decrease with increased TLL (see Fig. 5). At pH 10.5 with changing NaCl concentrations from

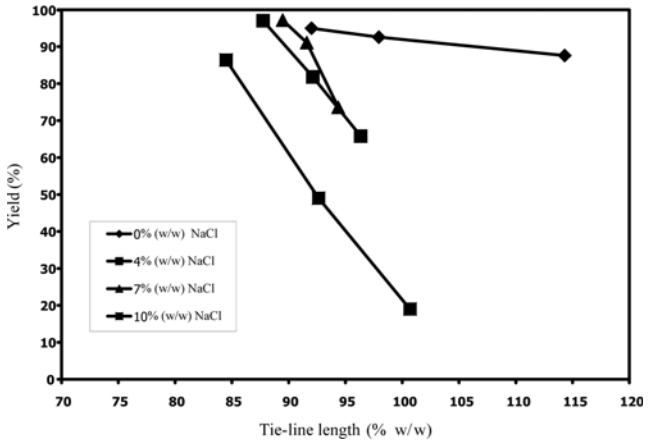


**Fig. 5. Effects of tie-line length on alkaline protease yield (Y%) in PEG1000/potassium phosphate aqueous two-phase systems at 30±2 °C, pH 10.5 with varied amounts of NaCl (in %w/w).**

0-10% (w/w), Y% was found to be noticeably varied from 19.0 (at 10%w/w NaCl) to 97.2% (at 7%w/w NaCl). Since addition of NaCl might have deteriorating effects on enzyme stability and its addition negligibly improved extraction yield, we opted for PEG1000/potassium phosphate ATPSs without additives. Therefore, the most suitable phase compositions for *B. subtilis* NS99 alkaline protease extraction were PEG1000 of 18% (w/w), potassium phosphate of 13.01% (w/w) under pH9.5, 30±2 °C and without NaCl addition. Partition coefficient and yield obtained were 20, and 95.1%, respectively. This achieved value of K is approximately 4 to 14 folds higher than those reported by Hotha and Banik [1997] for *Bacillus thuringiensis* H14 alkaline protease extraction in PEG(4000, 6000, 9000)/potassium phosphate ATPSs.

## CONCLUSIONS

PEG1000/potassium phosphate ATPSs were selected for investigation of *B. subtilis* NS99 alkaline protease extraction. It was found that system pH did not have significant effects on phase separation (except at pH 10.5), but addition of NaCl did. Binodal curves were found to noticeably move towards the origin when NaCl was included. However, notable effects on phase separation due to increased NaCl concentrations from 4 to 10% (w/w) were not observed.

Changes of system compositions due to variation in system pH, TLL, and NaCl concentrations obviously resulted in varied obtainable K and Y% of alkaline proteases. The longer TLL and the higher pH generally resulted in the higher K. In contrast, the lower NaCl, the higher K. Y% in this study depended solely on K since the same phase volume ration of 1:1 was used throughout. The most suitable system compositions determined were PEG1000 of 18% (w/w), potassium phosphate of 13.01% (w/w) under pH 9.5, 30±2 °C, and without NaCl addition. Partition coefficient and yield obtained were 20, and 95.1%, respectively.

## ACKNOWLEDGMENT

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