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A REVIEW ON TWO PHASE BATCH EXTRACTION OF ALKALINE PROTEASES

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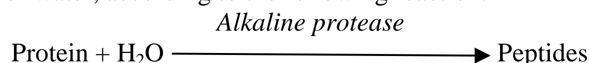
Abstract

Alkaline proteases are hydrolases that converts proteins into peptides performing better under low pH conditions. Numerous applications of alkaline proteases make them an important enzyme for industries. During conventional method of alkaline protease production, separate units are used for each phase of enzyme recovery, which is costly and time consuming. Extractive fermentation using aqueous two-phase system can reduce the time and cost for the production of alkaline proteases. Aqueous two-phase systems of interest in this review comprise polyethylene glycol (PEG) and potassium phosphate. Phase diagrams, factors and parameters affect the efficiency of two phase batch extraction of alkaline process.

Introduction

Biotechnology is advancing at a rapid speed (Ali *et al*. 2012, 2013). Alkaline proteases are very important industrial enzymes. They are widely used in detergent, cleaning stains and soils containing proteins (blood, grass, milk, gravy, tomato sauce, etc.), food, pharmaceutical, leather and film industries, as well as in waste processing industries (Hayashi *et al*. 1967). Alkaline protease acts as a biocatalyst cutting proteins into peptides chains under the presence of water, according to the following reaction:



One of the conventional methods for alkaline protease production using aqueous two-phase system comprises the following three processing steps:

A stream of medium goes into a main fermentor for enzyme production after which the fermented broth is fed to an extracting column (Fig.1) which is also fed with an aqueous solution of Polyethylene Glycol (PEG) for enzyme extraction. The top phase from the extracting column is subsequently pumped into a back-extracting column for further enzyme purification and PEG recovery. A mixing unit is used for mixing reused and fresh PEG before being fed back to the extracting column.

To produce alkaline protease, the fermentation process is carried out using some microorganisms such as alkalophilic *Bacillus pumilus*, *Spilosoma oblique, Bacillus subtilis*, *Bacillus thuringiensis* etc. (Lee and Chang, 1990; Uyar and Baysal, 2003). However, low production yields, product inhibition, and complexity of recovery system result in high production costs.

Therefore, in order to overcome the problem due to product inhibition to microorganisms, extractive fermentation a combined fermentation, and product extraction in a single unit is considered in this review for alkaline protease production. Extractive fermentation using aqueous two-phase system is a promising alternative to the conventional process, since it provides a non-denaturing natural environment for biomolecules, and stabilizes cells (Lee and Chang 1990; Hotha and Banik, 1997; Planas *et al*. 1996).

Aqueous two-phase system (ATPs) was first developed in Sweden during the mid-1950s for separation of macromolecules, cells, and organelles. As hinted by its name, aqueous two-phase is an aqueous, liquid–liquid, biphasic system that could be obtained either by mixing aqueous solution of two polymers, or aqueous solution of polymer and salt (Albertsson, 1958). This polymer-salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in an extraction step. In addition, aqueous two-phase systems offer an effective extraction process for various biomolecules. Their advantages are short process time, and possible attainment of high product yield, and purity. Moreover, extraction using aqueous two-phase system is an economical technology with low energy consumption, low labor cost requirement, and has great potential for further process development (Gupta *et al*. 2001). Scale-up processes based on aqueous two-phase systems are, furthermore, simple, and a continuous steady state is possible. Therefore, the aqueous two-phase

system was chosen in this research work as an appropriate system for extractive fermentation of alkaline protease production of which knowledge in this field is still very limited.

Aqueous two-phase systems of interest in this research work comprise polyethylene glycol (PEG) and potassium phosphate. Although previous works of other groups concentrated on extractive cultivation of alkaline protease in ATPs containing PEG and dextran T500 (Lee and Chang 1990), but the exorbitant price of dextran T500 limits its use (Hotha and Banik, 1997). PEG is a macromolecule with chemical formula of $H(OCH_2CH_2)_nOH$. Due to its nontoxic character, this chemical is commonly used in cosmetics, food, and pharmaceutical products. The biocompatible character of PEG explains success of this polymer in biotechnology applications. In addition, it is also commonly used for liquid-liquid partitioning and precipitation of biomacromolecules (Albertsson, 1986). More importantly, research work in our group previously revealed that partition coefficient of alkaline protease determined at the value of 49.1 at pH 7.5 in PEG 1000/ PO^{3-}_4 system is relatively high (Chuayyok, 2001). Therefore, it should be a suitable for extractive fermentation in this research work.

The cells are cultivated in an extractive fermentor with ATPs of PEG and phosphate. After fermentation, both phases will be pumped to separating unit for phase separation (Fig.2). The top phase will then be moved to back-extracting column for enzyme purification and PEG recovery. The reused PEG is refreshed with fresh PEG in mixing unit before taken back to extractive fermentor. In this process, an extracting column and a fermentor are combined to a single unit of extractive fermentor. The process is considered simpler than the process shown in Fig.1.

Aqueous two-phase system: An aqueous two-phase system, a system of two immiscible aqueous phases, occurs in aqueous mixtures of different water-soluble polymers such as dextran, PEG, or a single polymer and specific salt (e.g., PEG and ammonium sulfate). Aqueous two-phase system contains mainly water, with the first polymer predominating in one phase and the second polymer (or salt) predominating in the other phase. When a mixture of, for example, enzymes, is added to an aqueous two-phase system, each enzyme distributes uniquely between the two phases. Enzyme partitioning depends on specific features of an enzyme itself, and partition conditions (compositions of the system, pH, etc.). Under appropriate conditions the target enzyme will be concentrated in the upper phase, while all the others partition into the lower phase resulting in target enzyme isolation (Zaslavsky, 1995).

Phase diagrams: The composition of aqueous two-phase system can be presented on many kinds of phase diagram, such as equilateral triangular diagram, right triangular diagram, and rectangular diagram, etc. In our study, to record the concentrations of PEG and phosphate in both phases, a single rectangular phase diagram that was simple diagrams. The effect of concentration of the phase constituents on the phase diagram shows the state of the system with its characteristic binodal curve. All points in the homogeneous region (to the left of the phase diagram) will be in one phase, while any mixtures in the heterogeneous region (to the right of the phase diagram) will separate into two phases. In Fig.3, point M represents a two-phase mixture consisting of a top phase at composition T and a bottom phase at composition B. Tie-lines connect phase compositions on the phase diagram that are in equilibrium with one another. Points on the same tie line give rise to systems with identical top and bottom phase compositions but with differing mass of phase.

The phase mass ratio is given by: $$\frac{V_T \rho_T}{V_B \rho_B} = \frac{MB}{MT} \quad \text{(Eq.1)}$$

The phase volume ratio is given by: $$\frac{V_T}{V_B} = R_v \quad \text{(Eq. 2)}$$

The plait point or critical point, P, corresponds to the theoretical case which the compositions and volume of two-phase are equal.

1. Phase separation: Velocity of phase separation can be considered as a function of the height of interphase change varies with time:

$$\left(\frac{dh}{dt}\right) = f_2\left(\Delta_\rho, \sigma, \mu_B, \mu_T, d_d\right) = \left(\mathrm{Re}, We\right) \quad \text{(Eq.3)}$$

The mean droplet diameter is a function of the hydrodynamic condition in the mixer:

$$d_d = f_3(\mathrm{Re}, \mathrm{We}, t_m) \quad \text{(Eq. 4)}$$

With an increase in phase concentration, interfacial tension increases which results in an increase of droplet diameter, which in turn, decreases fractional dispersed phase hold-up and the mass transfer coefficient. Moreover, the PEG-rich phase viscosity increases with phase concentration. In conclusion, fractional dispersed phase hold-up as well as the mass transfer coefficient decrease with an increase in system composition (Parwa *et al*. 1993).

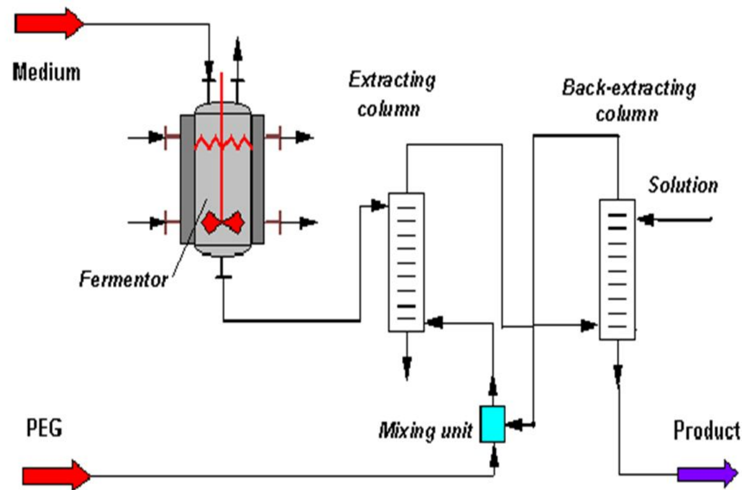


Fig. 1. Traditional fermentation process using aqueous two-phase system.

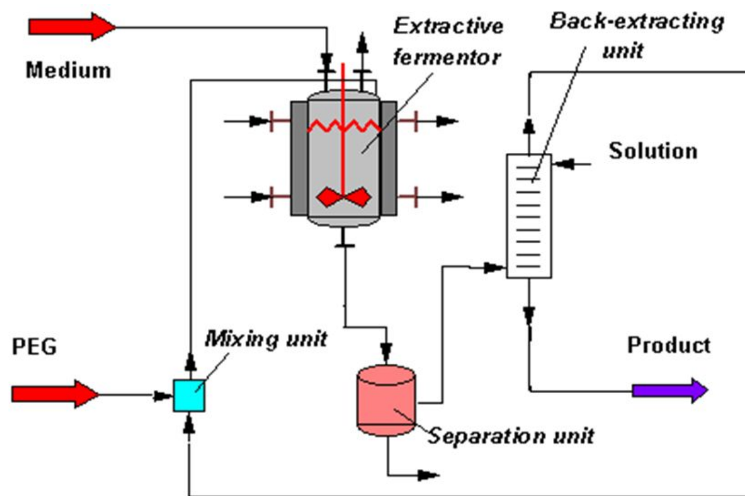


Fig. 2. Extractive fermentation process.

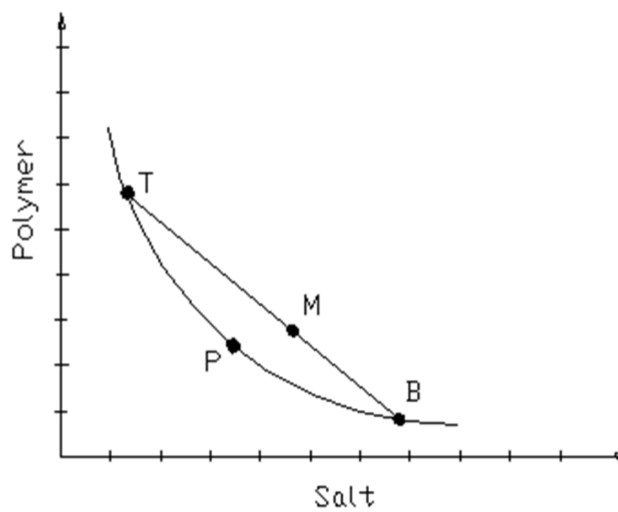


Fig. 3. Rectangular phase diagram.

Integration of equation 3 gives the profile of inter-phase height as a function of time and from it the time required for a complete separation t_{sj} can be estimated as:

$$t_{sj} = f_4(Re, We) \quad \text{(Eq. 5)}$$

2. Partition coefficients: The partition coefficient of component *i* is defined as:

$$K_i = \frac{x_i^{(1)}}{x_i^{(2)}} \quad \text{(Eq. 6)}$$

In a particular aqueous two-phase system, the partition coefficient of a protein will be constant over a range of protein concentration as long as the protein concentration is below its saturation range (Albertsson, 1986).
The relative selectivity is defined as:

$$\beta_{ij} = \frac{K_i}{K_j} \quad \text{(Eq. 7)}$$

3. Extraction factor: For solute B (Seader and Earnest, 1998) the extraction factor was defined as:

$$E_B = \frac{K_B V_S}{V_C} \quad \text{(Eq. 8)}$$

Large values of E_B result from large values of the partition coefficient K_B, or large ratios of solvent to carrier.

Fermentation, extraction

1. Fermentation in batch reactor with well mixed assumption: For constant volume batch reactors, the mass balance may be calculated as:

- For cells: $$\frac{dX}{dt} = \mu X \quad \text{(Eq. 9)}$$

Or $$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{(Eq. 10)}$$

- For substrate: $$\frac{dC_S}{dt} = -q_S X \quad \text{(Eq. 11)}$$

Or $$q_S = \frac{-1}{X}\frac{dC_{S\,t}}{dt} \quad \text{(Eq. 12)}$$

- For product: $$\frac{dC_P}{dt} = q_P X \quad \text{(Eq. 13)}$$

Or $$q_P = \frac{1}{X}\frac{dC_{Pt}}{dt} \quad \text{(Eq. 14)}$$

Let us assume at this point that the specific rates are constant during the interval $[t_o,t_i]$. Under this assumption, integrating equations 10, 12 and 14 yield

$$\ln X(t_i) = \mu[t_i - t_{io}] + \ln X(t_o) \quad \text{(Eq. 15)}$$

$$C_S(t_i) = -q_S \int_{t_o}^{t_i} X\ (t)dt + C_S(t_o) \quad \text{(Eq. 16)}$$

$$C_P(t_i) = q_P \int_{t_o}^{t_i} X(t)dt + C_P(t_o) \quad \text{(Eq. 17)}$$

The apparent specific growth rate can be found by plotting {lnX(t_i); Δt }, the slope of that curve represents μ .

Similarly, q_S, q_P can be obtained as a negative and positive slop of curves {$C_S(t_i)$; $\int_{t_o}^{t_i} X\ (t)dt$ } and {$C_P(t_i)$; $\int_{t_o}^{t_i} X\ (t)dt$ }, respectively.

2. Batch extraction

Mass balance of solute: $V_M = V_S + V_C$ (Eq. 18)

Or $$x_i^M . V_M = x_i^S . V_S + x_i^C . V_C \quad \text{(Eq. 19)}$$

Parameters affecting phase diagram characteristics: Since we are interested in applying an aqueous two-phase system for extractive fermentation, knowledge on phase diagram and its phase separation are very important. It was found that the position of phase diagram depends on many factors such as pH, temperature, molecular weight of polymer, type of salt, etc.

Albertson, (1986) indicated that phase separation in an aqueous mixture of a polymer with a given salt depends on polymer type more than on polymer size. In addition, Zaslavsky, (1995) reported that with the same type of polymer, the higher molecular weight results in shifting the binodal curve towards the original point [(0,0) co-ordinate]. However, the slope of the binodal curve was found to be altered. Similarly to increasing the molecular weight, an increase in temperature relocates the binodal curve nearer to the original point (Zaslavsky, 1995).

Introduction of an additive into an aqueous polymer solution may change the (clouding) temperature at which phase separation in a given polymer solution occurs at fixed polymer concentration. The effectiveness of anions to depress the cloud point temperature clearly exceeds that of cations and follows the order: $I^- < Br^- < Cl^- < F^- < OH^- < SO_4^{2-} < CO_3^{2-} < PO_4^{3-}$. Among alkali metal cations K^+ and Rb^+ appear to be most effective while Li^+ is the least effective (Zaslavsky, 1995).

Sebastiao *et al.* (1996) showed that PEG concentration in the top phase and phosphate concentration in the bottom phase increased with pH while the polymer concentration in the bottom phase and the phosphate in the top phase decreased. According to the results, the pH of a phase system can influence its tie-line length; which can be explained by the displacement of the binodals of PEG-sodium or potassium phosphate systems towards lower polymer and salt concentrations as a consequence of increasing pH. The tie-line length increased in the following order: system with sodium phosphate at pH 4.5 < system with potassium phosphate at pH 6.0 < system with potassium phosphate at pH 7.5 < system with potassium phosphate at pH 9 < system with sodium phosphate at pH 6. The pH difference between coexisting phases may result in cutinase and total protein partition dependence on pH (the presented pH values refer to the initial mixtures before phase separation). Maintaining the pH and type of cation constant, the tie-line length increased with increasing PEG 1000 concentration.

Changing pH introduces variations on both charge of protein ionic groups and ion composition of aqueous two-phase systems. When pH is lowered from 9 to 6, cutinase becomes less negative while at the same time the ratio $H_2PO_4^-/ HPO_4^{2-}$ increases. As the mono-valent anion is less effective in salting out the PEG a higher salt concentration will be required for phase formation and the interfacial potential difference will be reduced (Zaslavsky, 1995). Because of the increased phosphate concentration in the lower phase, the water molecules available for solute solvation decrease and protein reach their solubility limit (Sebastiao *et al.* 1996).

Parameters affecting partition coefficient: Many factors affect partitioning of a solute in aqueous two-phase systems. These factors include type, molecular weight and concentration of phase polymers, type and concentration of additives, pH, temperature, etc. However, it is difficult to choose appropriate partition conditions for a particular mixture as most of these variables are mutually dependent and their influences on solute partitioning is not well understood.

The pH values in the aqueous two-phase systems are, in particular, commonly used to steer partitioning of biopolymers or proteins. Most of the studies on pH-effect on protein partitioning were performed in two-phase systems of a fixed polymer composition with or without various salt additions. The protein with zero net charge at isoelectric (pI) distributes between two phases with the partition coefficient of the solute at the pH corresponding to the solute isoelectric point (K_0) independent of the salt composition of the system. Zaslavsky, (1995) reported that solute partitioning in aqueous two-phase system (represents in terms of the *ln*K value) is linear dependent upon the difference between concentrations of phase polymers in the two phases. The author also indicated that the effect of molecular weight of phase polymers on the solute partitioning is realized through the influence on the phase diagram and is completely taken into account once the phase diagram is determined. The partition coefficient could also be calculated as:

$$\ln K = \ln K_0 + \gamma Z \quad \text{(Eq. 20)}$$

For good separation and purification, the product should have a high K value and the contaminant should have a very low K value (Zaslavsky, 1995). In addition, Zaslavsky, (1995) and Albertsson, (1986) reported that partition coefficient (K) and K_0 decreased with increase of solute molecular weight (MW). The effect of MW of a solute on its partition behavior while clearly observed for some solutes seems to be counterbalanced by some other factors or nonexistence for the other solute.

Extractive fermentation using aqueous two-phase system: To our knowledge, up to date, there are only two published papers regarding to extractive fermentation for alkaline protease production. To give a broader ideal, we therefore, include some information on extractive fermentation of other products in this report.

1. Extractive fermentation for other products: Extractive fermentation of non-alkaline protease production was studied by many authors. Umakoshi *et al*. (1996) used PEG/dextran aqueous two phase system for an extractive fermentation to produce, release, and separate heat shock proteins (HSPs; GroEL and GroES). The results shown that with 0.1 M potassium phosphate salts (KPi) added, the productivity of HSPs increased while keeping the relatively high growth rate of *E. coli*. Partition coefficients of HSPs were improved to greater values when phosphate salts were added at a concentration of more than 0.1 M. Therefore, to improve productivity and partition coefficients of enzyme, addition of other salts may be considered.

Bärbel *et al*. (1998) reported one application of an aqueous two phase system for the production of ethanol from lignocellulosic materials with semi-continuous and batch process, which increased the amount of recoverable enzyme activity and improved amount of ethanol that was produced from five-carbon sugars.

An extractive fermentation for animal cell to produce IgG and hybridoma was reported by Zijlstra, (1998). The report showed that with increasing PEG-dye-ligand concentration up to 100% did increase the partition coefficient, but was not effective in concentrating the IgG in the top phase of the ATPs culture medium at a pH of 7.8. Furthermore, addition of the PEG-dye-ligand to ATPs culture medium changed the hybridoma cell partitioning from the bottom phase to the interface.

Kulkarni *et al*. (1999) indicated that an increase in the polymer concentrations from 6 to 20% in the polyethylene glycol phosphate aqueous two-phase system resulted in an increase in the phase volume ratio with a concomitant decrease in the partition coefficient (K) and recovery of xylanase in the top phase. However, varying phosphate concentrations from 8 to 16% decreased both the phase volume ratio and the partition coefficient of xylanase. The polyethylene glycol (6%) and phosphate (12%) system was found to be optimum for extracellular cultivation of *E. coli*, where extracellular xylanase was selectively partitioned to the top phase giving a purification ratio of above 1.

Shinha *et al*. (2001) recommended an aqueous two phase system that made by 6.5% (w/w) dextran and 7.5% (w/w) polyethylene glycol 6000 for production of endoglucanase. With the partition coefficient of 1.31 for endoglucanase from an intergeneric fusant of *T. reesei* (WT), the fusant produced 0.43U of endoglucanase (overall production: 0.34 U) in the top phase of an aqueous two-phase system (ATPs) for *T. reesei*, compared to 0.3 U in control system. The authors also explained one of the important problems in the aqueous two-phase system that may cause low overall enzyme production by *T. reesei* in the two-phase system. This is a limitation in oxygen transfer to the dispersed phase where the enzyme is produced.

Another successful application of the aqueous two phase system PEG/ phosphate was reported by Ryoichi, (1994) when the author produced and separated subtilin from *Bacillus subtilis* ATCC 6633, with enhanced stability, release of subtilisin recovered from the top phase was found to be 1.6 fold higher than that in Spizizen minimal salts single-phase medium. Ryoichi, (1994) asserted that application of ATPs for extractive bio-conversion is found to be possible in PEG/KPi systems which have high selectivity for the separation of the hydrophobic bioproducts.

2. Extractive fermentation for alkaline protease production: Lee and Chang, (1990) reported that the PEG/dextran T500 aqueous two-phase system is suitable for extractive fermentation of alkaline protease. The results shown that with 5 % (w/w) of PEG 6000 and 5 % dextran T500, after 50 h of fermentation, the total enzyme activity reached 1.3 times of that of the control culture. In order to improve the productivity of protease, repeated batch cultivation were successfully carried out four times by optimizing the top phase composition of freshly added media, which resulted in 13.8, 35.9, 27.8 and 34.7 units.$h^{-1}ml^{-1}$ of protease based on the amounts of replaced top phase, respectively.

Hotha and Banik, (1997) suggested that PEG X (X = 9000, 6000, 4000) and potassium phosphate is also suitable for extractive fermentation of alkaline protease, and that these systems have more advantages than PEG/ dextran systems due to lower chemical cost, higher total protease production, shorter time required for the total production in the aqueous two-phase system compared to that in a control system. The above discussion showed that alkaline protease produced during fermentation, partitioned into the upper phase (about 80 %) and total protease produced were about 2.8 and 2.26 times higher than that of homogeneous fermentation when the fermentation were carried out in aqueous two phase system from the beginning and made after 45 h of inoculation, respectively. The authors also suggested that the higher molecular weight of PEG, lower the enzyme production yield.

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