CHAPTER 2

THEORY AND LITERATURE REVIEW

A. Theoretical review

2.1. 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).

2.1.1 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 figure 2.1, 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:

$$\frac{V_T \rho_T}{V_B \rho_B} = \frac{MB}{MT} \qquad \text{(Eq.2.1)}$$

The phase volume ratio is given by:

$$\frac{V_T}{V_B} = R_v \qquad \text{(Eq.2.2)}$$

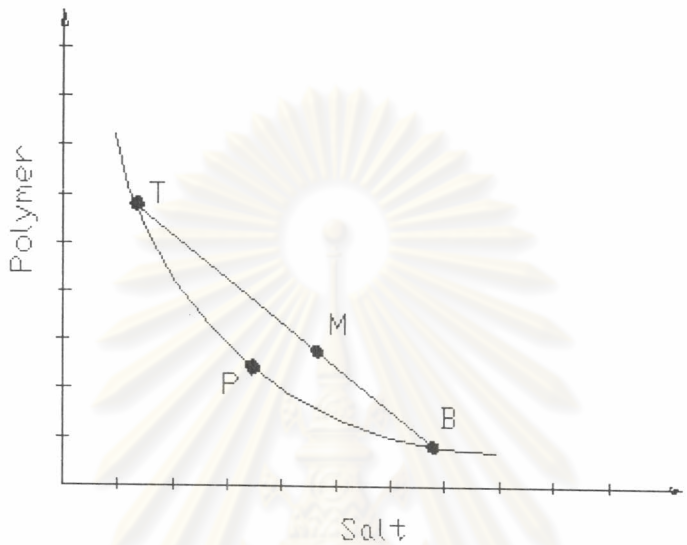

Figure 2.1: Rectangular phase diagram

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

2.1.2 Phase separation

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

$$\left(\frac{dh}{dt}\right) = f_2\left(\Delta_\rho, \sigma, \mu_B, \mu_T, d_d\right) = (Re, We) \qquad \text{(Eq.2.3)}$$

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

$$d_d = f_3(\, Re,\, We,\, t_m) \qquad (\, Eq.2.4)$$

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, 1993).

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:

$$t_{sj} = f_4(\text{Re}, \text{We}) \qquad (\text{Eq.2.5})$$

2.1.3 Partition coefficients

The partition coefficient of component *i* is defined as:

$$K_i = \frac{x_i^{(1)}}{x_i^{(2)}} \qquad (\text{Eq.2.6})$$

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:

$$\beta_{ij} = \frac{K_i}{K_j} \qquad (\text{Eq.2.7})$$

2.1.4 Extraction factor

For solute B (Seader et al, 1998) the extraction factor was defined as:

$$E_B = \frac{K_B V_S}{V_C} \qquad (\text{Eq.2.8})$$

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

2.2. *Fermentation, extraction*

2.2.1 Fermentation in batch reactor with well mixed assumption

For constant volume batch reactors, the mass balance may be calculated as:

- For cells: $\frac{dX}{dt} = \mu X \qquad (\text{Eq.2.9})$

Or $$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{(Eq.2.10)}$$

- For substrate: $$\frac{dC_S}{dt} = -q_S X \quad \text{(Eq.2.11)}$$

Or $$q_S = \frac{-1}{X}\frac{dC_{St}}{dt} \quad \text{(Eq.2.12)}$$

- For product: $$\frac{dC_P}{dt} = q_P X \quad \text{(Eq.2.13)}$$

Or $$q_P = \frac{1}{X}\frac{dC_{Pt}}{dt} \quad \text{(Eq.2.14)}$$

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

$$\ln X(t_i) = \mu[t_i - t_{io}] + \ln X(t_o) \quad \text{(Eq.2.15)}$$

$$C_S(t_i) = -q_S \int_{t_o}^{t_i} X(t)dt + C_S(t_o) \quad \text{(Eq.2.16)}$$

$$C_P(t_i) = q_P \int_{t_o}^{t_i} X(t)dt + C_P(t_o) \quad \text{(Eq.2.17)}$$

The apparent specific growth rate can be found by plotting $\{\ln X(t_i); \Delta t\}$, 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.

2.2.2 *Batch extraction*

Mass balance of solute: $V_M = V_S + V_C$ (Eq.2.18)

Or $x_i^M . V_M = x_i^S . V_S + x_i^C . V_C$ (Eq.2.19)

B. Literature review

2.3 *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 , 1981).

Sebastiao (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 (Ananthapandmanabhan et al, 1986) a higher salt concentration will be required for phase formation (Huddleston, et al, 1991) and the interfacial potential difference will be reduced (Zaslavsky, et al, 1982). 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).

2.4 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:

$$\ln K = \ln K_0 + \gamma Z \qquad \text{(Eq.2.20)}$$

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.

2.5 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.

2.5.1 Extractive fermentation for other products

Extractive fermentation of non-alkaline protease production was studied by many authors. Umakoshi.H 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

2.5.2 Extractive fermentation for alkaline protease production

Lee et al (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 et al (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 results 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, the lower the enzyme production yield.