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Optimisation of LS54/Dx Aqueous Two Phase System Conditions for Cutinase Recovery

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Aqueous two phase system comprising Dehypon® LS 54 and K4484 Dextrin® was selected for recovery of cutinase enzyme. Parameters such as pH, system composition and type of salt as an additive, influenced the protein partitioning behaviour and optimisation of these parameters become necessary to be done in the design of primary recovery process of ATPS. The cutinase partitioning experiments were carried out with 30% of cutinase solution added to LS 54/Dx system. Results showed that cutinase enzyme preferred to partition into LS 54 rich-phase at pH 8.0 and the affinity of cutinase into top phase was observed higher with the increment of system compositions, which represented by tie line length (TLL). Furthermore, the addition of 50mM salts such as K_2SO_4 and KCl into LS 54/Dx system has led to raise partition coefficient of cutinase, k_{cut} to 2.2 and 1.95 fold, respectively. The dependence of k_{cut} on various additives such as $(NH_4)_2SO_4$, Na_2SO_4 and K_2SO_4 at the same concentration, suggested that the addition of selected ions could enhance positive electrostatic potential which could attract more cutinase to partition into LS54 rich phase. As conclusion, the best conditions obtained for cutinase partitioning were pH8.0, TLL = 23% and Na_2SO_4 = 50mM, from which the maximum k_{cut} of 2.83 with improved recovery of cutinase in top phase up to 79% can be achieved.

Keywords: Aqueous two phase, Polymer-polymer system, Optimise, Enzyme recovery, System parameters, Salt additives

INTRODUCTION

Aqueous two-phase system (abbreviated as ATPS) had been addressed among liquid-liquid extraction techniques that have capability to isolate, concentrate and stabilize the target protein from complex mixture. ATPS is usually composed of two different type of polymer solutions; or a mixture of polymer and salt solution. The common polymer could be either a 'smart' polymer such as thermo-induced kinds or other polymer such as hydroxypropyl starch. In the previous studies, ATPS has been exploited as interesting separation technique because it involves low materials cost, and simple operation since it is easy to prepare. The system can be operated at high volume and it is delicate to biological product. Similar to other liquid-liquid extraction or solvent extraction system, the basic principle of separation by ATPS is its selectivity of solutes (target component) distributed between the phases. Generally, protein solutes will be migrated into less polar top phase and the contaminants will be driven into polar bottom phase as a result of the two main factors reported in many studies that describe the partitioning behaviour of protein in ATPS. Factors that related to properties of the protein are size, isoelectric point and the strength of surface hydrophobicity. Another identified factor is the system's character which includes molecular weight of the phase components and their parameters such as concentration of polymer, pH and the addition of salt (Mazzola *et al*., 2008). By choosing the right properties of protein and the system's parameters, selective partitioning and high recovery of target protein could be expected. Although the mechanism that governs protein partitioning in ATPS is complicated, most researchers had applied practical approach to evaluate proteins' partition behaviour under different system parameters (Benavides *et al*., 2008). Meanwhile, the studied protein, cutinases are extra-cellular fungal hydrolytic enzymes that catalyses hydrolases of ester bonds of insoluble polyester cutin, a structural component of plant cuticle (Kolattukudy *et al*., 1981). Cutinases also display hydrolytic activity towards a broad variety of esters, from soluble synthetic esters into insoluble long-chain triglycerides. Cutinases have been commercially applied in laundry and dishwashing formulations as they are able to tolerate in alkaline condition, high temperature and resistance to chemical denaturation caused by surfactants (Cunha *et al*., 2000). In addition, it is also applied in the textile industry, biodegradation of plastics, as well as in synthesis of ingredients for personal care products and pharmaceuticals (Agrawal *et al*., 2008; Araújo *et al*., 2007; Calado *et al*., 2003). In term of its structure, cutinases have hydrophobic amino acids exposed on the surface which can increase the binding to the hydrophobic surface (Silva *et al*., 2007).

In this study, a new ATPS system composed of phase components of industrial ethylene oxide propylene oxide (EOPO), Dehypon® LS 54 (abbreviated as LS54) and starch derivatives K4484® Dextrin (abbreviated as Dx) was developed. The LS54 polymer possesses hydrophilic EO and hydrophobic PO with ratio 5mol of EO to 4mol of PO. The system's pH and salt additions was studied and optimized in

LS54/Dx system for high cutinase recovery.

The experiments were carried out to establish practical strategy for the development of new liquid-liquid extraction of aqueous two phase system for enzyme recovery which the extraction protocol was developed according to Benavides & Palomares (2008).

METHODOLOGY

Materials

The industrial grade of low-foaming surfactant, Dehypon® LS 54 were purchased from Cognis Oleochemicals (Malaysia) Sdn Bhd while Dextrin K4484®, a tapioca based starch was a kind gift from N-Starch Sdn. Bhd. (Malaysia). All acids and salts were Analar grade bought from Sigma (USA). Commercial cutinase enzyme, named Novozym® 51032 (abbreviated as cut51032) was purchased from Novozymes (Denmark). Bovine serum albumin (BSA) and p-nitrophenol (pNP) and p-nitrophenyl laurate (pNPL) were obtained from Sigma-Aldrich (Malaysia).

Partitioning experiment

The LS54/Dx systems were prepared by weighing appropriate amounts of a 100%(w/w) stock solution of Dehypon LS54 and 30% (w/w) stock solution of K4484 Dextrin (diluted in buffer) in test tubes, to a final weight of 2g. Potassium phosphate was used as buffer in range of pH6.0-8.0. Meanwhile Tris-HCl buffers were used for more alkali ATPS (pH 8.6 and pH 9.0) and citrate buffers were utilized to create acidic conditions of pH4.0 and pH5.0. All systems were made up with 30% (w/w) of cutinase solution. The system without enzyme was used as reference system. The partitioning studies were conducted in 15ml test tubes and the prepared systems were thoroughly mixed in vortex shaker before being incubated in water bath at operating temperature, 25°C for the next 15min. After that, the systems were centrifuged at 4000rcf for 2min to ensure a complete phase separation. The phase volume ratios of the systems were determined and both top and bottom phases were assayed for enzyme activity.

Enzyme activity and protein content determination

Cutinase activity was measured according to Kumar et al. (2005). A reaction mixture composed of 96.5ml potassium phosphate buffer, 2.5ml of pnitrophenyl laurate (pNPL) as substrate and 1.0ml sample were prepared and allowed to react for 10min. 1 unit activity (U) of cutinase is defined as 1µmol p-nitrophenol (pNP) produced per ml per min under assay condition. The optical density (OD) readings at wavelength 405nm were taken using VersaMax microplate reader (USA).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) was used to determine the molecular weight of target cutinase on Mini-PROTEAN® Tetra Cell System (Bio-Rad, United States) . Samples were mixed with an equal volume of SDS Sample Buffer and were heated at 95°C to denature the proteins. The samples were subsequently loaded into the gel constituting of 17% resolving gel and 5% stacking gel. The gel mixture were run at

constant voltage of 180 V and finally they were stained with Coomassie Brilliant Blue.

Calculations

The cutinase distribution between the two phases can be described by partition coefficient, k_{cut} in which it can be defined as C_t/C_b, where C_t and C_b represent the concentrations in top and bottom phases, respectively. Meanwhile, percentage of the yield for the enzyme/protein in the top phase, Y_T (%) was calculated in relation to k-value$_t$ and phase volume ratio, v_R (volume top phase/volume bottom phase) according to equation below:

$$Y_T = \frac{100}{1 + \left(\frac{1}{v_R . k_{cut}}\right)} \quad (1)$$

RESULTS AND DISCUSSIONS

Molecular weight and isoelectric point (pI) of cut51032

Both molecular weight and isoelectric point of cut51032 were determined by experiments. To determine cut51032 molecular weight, a series of dilutions were prepared and were run on SDS-PAGE. According to Figure 1, a single and clear band for cut51032 was obtained at dilution factor of 1/32. The molecular weight of cut51032 was estimated to be 22kDa which was determined based on the standard protein ladder run simultaneously on the gel. The isoelectric point (pI) was determined from plot of zeta potential at different pH values as shown in Figure 2. The point of the intercept between the line and zero zeta potential is the pI value of studied cutinase (Alvarez-Silva *et al*., 2010). According to the graph, pI of cut51032 was approximately 4.3. Theoretically, at pI condition, the charge of cutinase enzyme become zero. At pH higher than pI value, this enzyme presents as a negatively charge molecule and it would become positive charge when the surrounding pH drops below its pI.

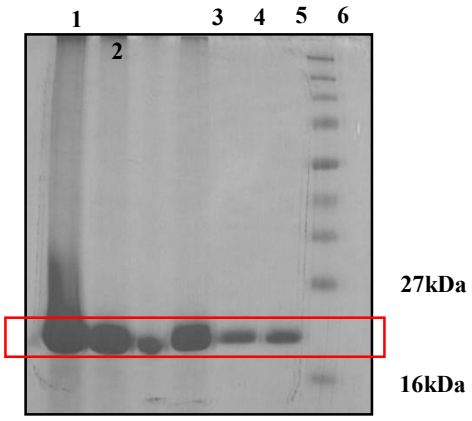

Fig. 1: SDS-PAGE analysis for cut51032. Lane 1-5: enzyme solutions at dilution factor of ½, ¼, 1/8, 1/16, 1/32, respectively. Lane 6: molecular weight marker.

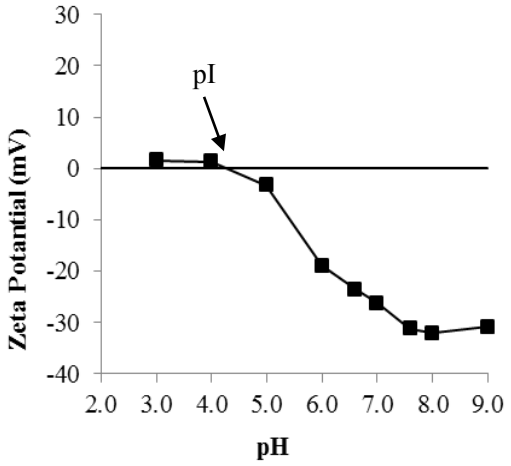

Fig. 2: Zeta potential (mV) plotted as function of pH for 0.1% cut51032 solution dispersed in 0.1M buffer solution.

Selection of pH system and TLL

The optimisation study for extraction parameters such as pH and TLL were done by identify the effective pH system for cutinase extraction. Influence of pH on cutinase partitioning behaviour using LS54/Dx system was studied using the model enzyme solutions after adjusting pH of the systems. The results were plotted in Figure 3. It was observed that by increasing k-value at higher pH, cut51032 was more likely driven into top phase.

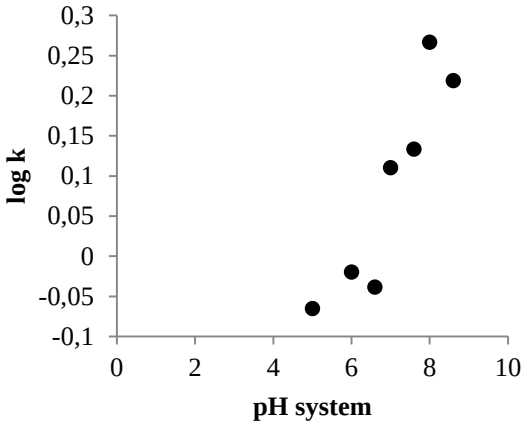

Fig. 3: Partition coefficient of cutinase, k_{cut} as a function of pH (System compositions 20%w/w LS54/ 15%w/w Dx/ 30% enzyme solution)

The change of cut51032 partitioning behaviour was observed through the net of the protein to the pH environment. Generally, the top phase is more positively charge than the bottom one. Since the pI of cut51032 is 4.3, the cutinase was more negatively charged at higher pH and as it was expected, the cutinase was attracted more into EOPO-enriched phase. The highest value of k_{cut} was obtained at pH8.0 with value of 1.85.

TLL represents the compositions of prepared system at equilibrium condition. Three systems with different TLL were prepared for cutinase partitioning experiments at pH8.0. The influences of TLL on k_{cut} at pH 8.0 were shown in Figure 4. It was observed that the partitioning of cutinase in system was altered with varying TLL. With the increased of TLL from 5.5% to 23.0%, k_{cut} increased from 0.88 to 1.25. Increasing of k_{cut} value with TLL was related to phase-polymer composition. As the phase composition shifted far from critical point, the compositional difference of the phase become more obvious (Asenjo & Andrews, 2012). Thus more extreme partitioning of enzyme in ATPS could be observed. Moreover, partial hydrophobic character offered by LS54 was another significant reason for the increment of k_{cut} with TLL as the molecular interaction could occur between LS54 and cutinase, As mentioned in previous study, polymer concentration in the phases was also related to phase hydrophobic effect of the system (Asenjo & Andrews, 2011).

The effect of pH and TLL on cutinase partitioning behaviour is summarized in Figure 5. The plots show that the combination of pH and TLL with k_{cut} more than 1.1. k_{cut} values higher than 1.0 describes the preference of cutinase to partition into the top phase and it can be observed that cutinase could be driven more toward the top phase by system with TLL in range of 15-25% and pH system of 8.0±0.5. Thus, the extraction of cutinase by LS54/Dx system is recommended to be conducted within these parameters values.

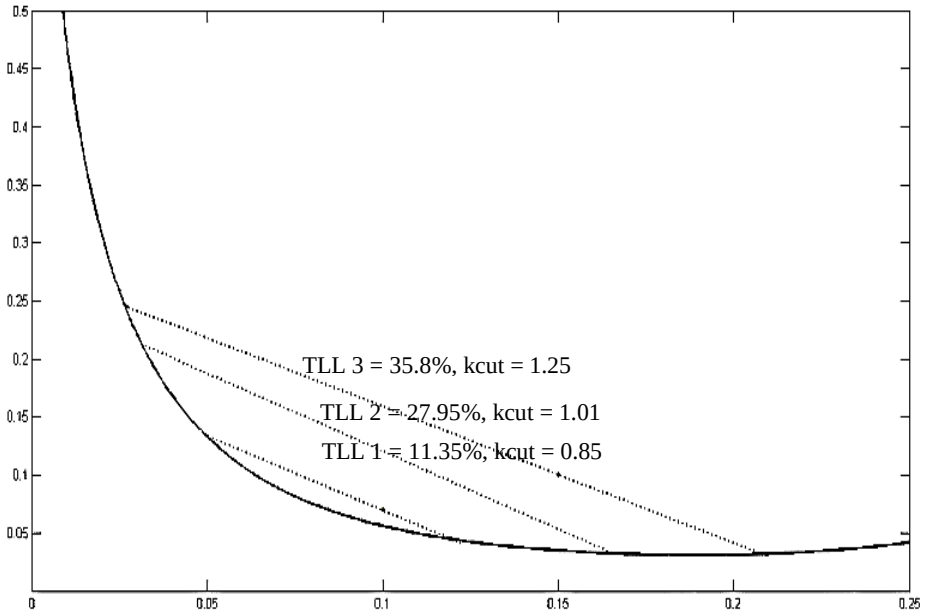

Fig. 4: Partition coefficient of cutinase, k_{cut} as function of TTL (System compositions 20%w/w LS54/15%w/w Dx/30% enzyme solution)

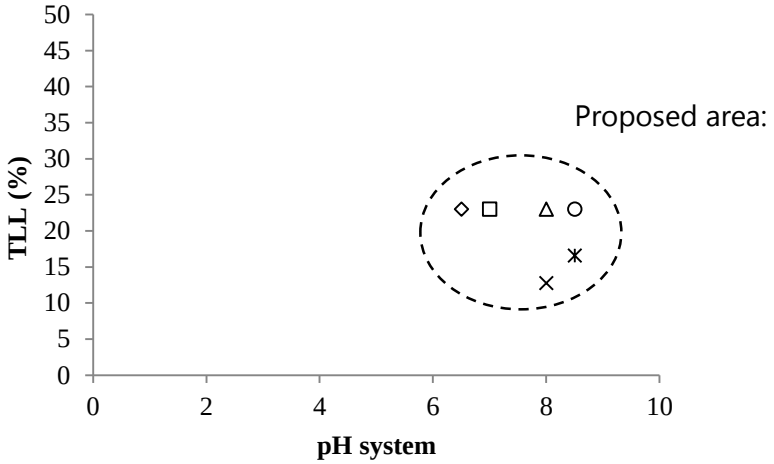

Fig. 5: Propose pH and TTL for cutinase partitioning in LS54/Dx system. k_{cut} values obtained for each plots were as follow: (◊) = 1.29, (□) = 1.36, (Δ) = 1.85, (o) = 1.65, (∗) = 1.16, (×) = 1.27

Effect of the presence of salt on cutinase recovery

An attempt to isolate more cutinase in top phase can be performed by adding salt. The effect was evaluated on cutinase partitioning behaviour at the selected pH8.0 and 23.3%TLL. Systems with added salt and a control (without salt) was compared and shown in Figure 6 against cutinase partitioning behaviour. The results indicated that k_{cut} value increase and more cutinase were attracted into the top phase when the salt was added. The presence of salt often helped in directing protein to any

particular phase because it creates chemical potential different between the phases. Furthermore, ionic effects of the salt may also interact target proteins and subsequently changes the partitioning behaviour of protein.

The influence of salt ions on cutinase partitioning behaviour in LS54/Dx system occurs when the charge of cut51032 become negative in pH 8.0. Figure 6 shows that addition of 50mM K_2SO_4 had resulted higher k_{cut}-value and increased cutinase recovery in top phase as compared to system with KCl. This could be due to higher preference of SO_4^{2-} to migrate into Dx phase compared to Cl^- (Johansson *et al*., 1998). As a result, it would cause the accumulation of anions in bottom phase that would repel the negative charge protein and transfer it to the top phase.

Furthermore, by applying another two sulphate salts of different type of cations such as Na^{2+} and NH_4^+, both k_{cut} and cutinase recovery in top phase were increased (Figure 6). Addition of salts in the system had also increased volume of top phase, especially for system with Na_2SO_4. Yield of cutinase at the other hand depends on volume of phase-forming. Therefore, the highest cutinase recovery obtained in top phase was 79% and it was attained with Na_2SO_4 in the system.

CONCLUSIONS

The optimum conditions of LS54/Dx system for cutinase recovery were obtained at pH8.0 and 23% TLL. The k_{cut} achieved at this optimum pH and TLL was 1.85. It is suggested that k_{cut} with more than 1.1 could be obtained with pH value 8.0 ± 0.5 and TLL in range of 15-25%. With addition of salt, more cutinase could be driven to partition into the top EOPO-enriched phase. The highest recovery of cutinase that able to be achieved by using LS54/Dx system was attained with addition of 50mM Na_2SO_4, wherein 79% of cutinase was recovered in the top phase which is equal to k_{cut} of 2.43.

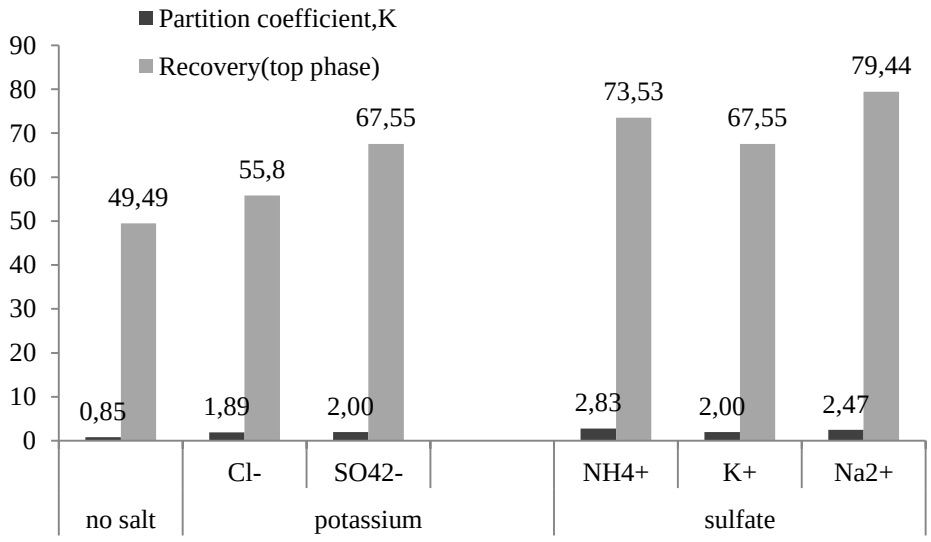

Fig. 6: Influence of different type of ion on cutinase partitioning in LS54/Dx system. (System compositions: 23%w/wLS54/11%w/w Dx/ 50mM salt/ 30% enzyme solution)

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