



# Characterization of Copolymer Dehypon<sup>®</sup> LS 54 and Its Application for Aqueous Two-Phase Systems Paired with the Waxy Maize Starch for Protein Extraction

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**Abstract**— A thermo-separating aqueous two-phase system composed of Dehypon<sup>®</sup> LS 54, a polymeric surfactant and the waxy maize starch (amylopectin starch) has been used for partitioning of cutinase as a model protein. Dehypon<sup>®</sup> LS 54 were characterized by using <sup>1</sup>H NMR spectroscopy to get information regarding the chemical structure and to confirm the presence of aliphatic moiety group in this copolymer. The phase diagram obtained for these novel polymer-polymers two-phase system shows two-phases with high polymer concentration. The waxy maize starch is enriched in the bottom phase while the copolymer Dehypon<sup>®</sup> LS 54 is found in the upper phase. Since this copolymer (Dehypon<sup>®</sup> LS 54) is thermo-reactive, the upper phase can be removed and heated above the copolymer's cloud-point which resulting in the formation of a new two-phase system with a lower water phase, containing the target protein and an upper is copolymer-rich phase. Our results show that systems formed by waxy maize starch and Dehypon<sup>®</sup> LS 54 could become an alternative system to be used in large scale protein and enzyme purification due to their low cost, and also because they offer a viable solution to problems of polymer removal and recycling which makes this system more attractive.

**Keywords**— Aqueous Two-phase System, Polymeric Surfactant, Protein Partitioning, Temperature-induced.

## I. INTRODUCTION

Aqueous two-phase system is formed when two structurally different polymers are mixed above a critical concentration in water [1]. The formed two-phases are each enriched in one polymer, but the main component in both phases is water. Usually the water content is 80 – 95% and, thus, aqueous two-phase systems constitute a mild method for separation of biomaterials. Bioseparation by using two-phase systems is a fast and simple technique and is relatively easy to scale up.

The most commonly used two-polymer system is composed of polyethylene glycol (PEG) and dextran. Since dextran is a rather expensive polymer, much research effort has been devoted on finding cost-effective alternatives. Polymer types other than PEG are also studied. The examples are thermo-separating EOPO copolymer and

hydrophobic modified copolymer EOPO called HM-EOPO [2], [3]. These polymers consist of ethylene oxide (EO) and propylene oxide (PO) units. The EOPO copolymers display a lower critical solution temperature (LCST) in water [4].

In the first extraction step, a thermopolymer/polymer system is used, followed by a second extraction step where the recovered thermopolymer-rich phase from the first step is heated to a temperature above the cloud point (CP). This will give rise to the formation of two new phases, one polymer rich bottom phase and one almost pure water phase on top. The idea is that in the first extraction step the target protein is recovered in the thermopolymer-rich phase while the contaminants are collected in the polymer-rich phase. For the second extraction step it has been shown that almost all proteins are partitioned exclusively to the aqueous phase [5]. Thus, the aqueous phase including the target protein can

be processed further downstream while the concentrated polymer phase can be recycled.

As reported in previous studies, the major drawback of the most common polymer/polymer system which is composed of dextran and PEG is that the system is expensive for scaling up. Therefore, this problem may be overcome by the use of alternative polymer such as starch derivatives, maltodextrin and cellulose derivatives [6].

The polymers used in this study were amylopectin rich Waxy maize starch and the polymeric surfactant named Dehypon<sup>®</sup> LS 54. Dehypon<sup>®</sup> LS 54 is a block copolymer composed of ethylene oxide (EO)/propylene oxide (PO) with approximately 5 moles EO and 4 moles PO, which is also known as fatty alcohol polyglycol ether is a commercial surfactant that available from Cognis Corporation. The Waxy maize starch formed the heavy phase due to its higher density compared with the Dehypon<sup>®</sup> LS 54 phase. Recombinant *E.coli* cutinase-(WP)<sub>4</sub> was selected as model protein and to be partitioned in Dehypon<sup>®</sup> LS 54/waxy maize starch. In this work we also constructed phase diagram for Dehypon<sup>®</sup> LS 54/waxy maize starch.

In the present studies we also characterize the copolymer Dehypon<sup>®</sup> LS 54 by using <sup>1</sup>H NMR spectroscopy with the aim to get the information regarding the chemical structure and to confirm the presence of aliphatic moiety groups on this copolymer, since this functional group has an important role on hydrophobic properties of copolymer.

As a general characterization technique for nonionic surfactant, the nuclear magnetic resonance (NMR) can be considered, due the highly recognizable chemical shift of hydrogen and carbon atoms in various organic molecules. In the case of a mixture such as block EO-PO surfactant, the NMR spectra present a high degree of complexity and information that can be extracted from NMR spectra has statistical significance and does not reflect the structure of the single and well defined molecule. The protonic NMR can also be used for the determination of the relative abundance of primary and secondary hydroxyl group in copolymers [7].

## II. MATERIALS AND METHODS

### A. Chemicals

The copolymer Dehypon<sup>®</sup> LS 54 (approx. 5 moles EO and 4 moles PO) was purchased from Cognis Oleochemicals (Malaysia). The chemical structure of Dehypon<sup>®</sup> LS 54 was characterized by NMR spectroscopy (<sup>1</sup>H NMR) to confirm the presence of lauryl alcohol moiety group. The density of copolymer Dehypon<sup>®</sup> LS 54 at 25 °C was determined by density meter DMA 4500 Anton Paar (Austria). The Waxy maize starch with molecular weight 10<sup>6</sup> – 10<sup>7</sup> g mol<sup>-1</sup> was purchased from Sigma Aldrich Company (Singapore). The Waxy maize starch is a mixture of 93 – 95% amylopectin and 5 – 7% amylose. All other chemicals used were analytical grade

### B. Protein/Cutinase Broth

*Escherichia coli* strain expressing recombinant cutinase with a genetic engineering approach at N-terminal and sequences encoding a (WP)<sub>4</sub> hydrophobic tag at the C-terminal was obtained from the previous work [8]. The

purpose of (WP)<sub>4</sub> hydrophobic tag is to increase the partitioning to the EOPO (copolymer) rich phase.

### C. Characterization of Copolymer Dehypon<sup>®</sup> LS 54 with <sup>1</sup>H NMR Spectroscopy

The NMR studies were carried out on a JEOL ECP 400 MHz instrument, operating at 400 MHz (<sup>1</sup>H). Methanol-D<sub>3</sub> was used as the solvent. The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded.

### D. Construction of The System Phase Diagram

The systems were prepared by stock solution, 60% (w/w) Dehypon<sup>®</sup> LS 54 and 8% (w/w) waxy maize starch in Tris-HCl buffer (50 mM; pH 8.0). Known masses of these solution and water were weighted into a test tube to have the desired initial overall composition. Aqueous two-phase systems were prepared with a final mass of 10 g. All samples were prepared in 15-ml graduated plastic tubes. After the phase systems had been mixed thoroughly in a closed test tube by a vortex mixer, phase separation was speeded up by centrifugation at 4500 rpm for 10 min in a Eppendorf 5804 Centrifuge. Then the phase systems were left undistributed for at least 24 h at 25.0°C in a regulator water bath Protech 631D (Malaysia), where the temperature was controlled to within ± 0.1°C. The top and bottom phases were isolated and diluted, top phase were diluted six times, and bottom phase were diluted ten times. First, the concentration of waxy maize starch was determined in both phases by polarimetry using a digital polarimeter POLAX-2L, Atago (Tokyo, Japan) by making a polarimetric standard curve for waxy maize starch. The presence of Dehypon<sup>®</sup> LS 54 had no effect on the optical rotation of waxy maize starch. The concentration of Dehypon<sup>®</sup> LS 54 in both phases was determined by measuring refractive index with a refractometer from Carl Zeiss (Ober-kochen/Württ., Germany) and by subtracting the refractive index contribution of waxy maize starch. The water contents were obtained by subtractions of copolymer and starch compositions. A few points in the phase diagram, around the critical point, were determined by titration of the two-phase system with water until the formation of a one phase system [9].

### E. Preparation of the Aqueous Two-phase Systems

All polymer concentration were calculated as % weight/weight (w/w). The waxy maize starch were dissolved in water and added from stock solution 15%. Dehypon<sup>®</sup> LS 54 were added as pure substances. Tris-HCl buffer pH 8.0 were added from 50 mM stock solution to maintain constant pH. Aqueous two-phase systems were prepared with a final mass of 5 g. All samples were prepared in 15-mL graduated test tubes (which were calibrated for further accuracy). All experiments were performed in duplicate and the experimental data are average values. The additions of cutinase, from pre-made stock solution, were based on volume to give final concentration of 1 mg/mL. Water was then added to give final weight of 5 g. After thorough gentle mixing of the system components, the phase separation was enhanced by centrifugation 10 min at 4500 rpm. Then the phase systems were left undistributed for at least 24 h at 25°C in a regulator water bath Protech (Malaysia), where temperature was controlled to within ± 0.1°C. All systems

were made in duplicate and blank systems devoid of protein were also prepared. The volume of the top and bottom phases was estimated. Then the top and bottom phases were separated and diluted appropriately for the determination of protein content.

#### F. Determination of Cloud Point Temperature

The determination of cloud-point temperatures (CPT) were performed in a regulated water bath by immersing the co-polymer solution in a capped glass tube. The CPT was carried out by making an aqueous solution of the copolymer Dehypon® LS 54 at different concentration (from 0 to 70% w/w) and gradually raising the temperature 1 °C/min, at times until the first sign of clouding solution appeared [10]. All cloud-point measurements were repeated three times.

#### G. Temperature-induced Phase Separation

After primary separation, the top phase which containing enzyme and Dehypon® LS 54 was removed into 15 ml graduated capped test tubes and placed in regulated water bath, the temperature induced were performed to eject protein out of copolymer solution at the copolymer cloud point. To obtain a complete separation between copolymer and water phases in a reasonable time the temperature must be raised 5-10 °C higher than the cloud point [11]. The volume of the top and bottom phases of the new systems was estimated. Then the top and bottom phases were separated and diluted appropriately for the determination of protein concentration and the partition coefficient were calculated.

#### H. Protein Assay

The total protein content was determined by according to Bradford (1976) [12], using Amresco Bradford Reagent (US). The absorption was measured at 595 nm by using 10 uv ThermoSpectronic (Genesys, Madison, USA). Bovine serum albumin (BSA) was used for standard. For the determination of protein concentration, the top phase and bottom phase was diluted 10 times with Tris-HCl buffer pH 8.0. The blank reaction was prepared with the sample substituted with buffer.

In order to describe the ability of protein partition in ATPS, several parameters were determined: protein partition coefficient, yield for the top phase of the primary systems and water-rich phase after temperature-induced phase separation. The recovery of protein in the water-rich phase after temperature-induced phase separation was also determined.

#### I. Calculations

##### 1) Partition coefficient

The protein partition coefficient ( $K_p$ ) defined as the ratio between the protein concentration in top and bottom phase.

$$K_p = \frac{C_t}{C_b} \quad (1)$$

where  $C_t$  and  $C_b$  are the concentration of protein in the top phase and bottom phase, respectively. For the primary phase separation, top phase of polymer-enriched phase and bottom phase being starch-enriched phase. Meanwhile temperature induced phase separation, top phase is usually polymer

phase and heavy phase would be the protein solution (water-rich phase).

##### 2) Recovery after thermo-separation step

The percent protein recovery ( $R_p$ ) in the water phase (heavy phase) after temperature-induced polymer separation steps are calculated according to equation below.

$$R (\%) = \frac{C_{water} \times V_{water}}{C_i \times V_i} \times 100 \quad (2)$$

where  $C_{water}$  and  $V_{water}$  are the protein concentration and the water phase volume in the water-rich phase after thermo-separation, and  $C_i$  and  $V_i$  are the initial protein concentration and the system volume, respectively.

3) The yield (%) of the target protein in the top phase of the primary systems ( $Y_{Pp}$ ) was calculated by:

$$Y_{Pp} = \frac{100}{1 + \frac{V_b}{V_t} \times \frac{1}{K_p}} \quad (3)$$

Subscript  $P, p$  refers to the primary system target protein. And  $V_t$  and  $V_b$  are volume of the top and bottom phase, respectively.

4) The yield (%) of the target protein in the water-rich phase after thermo-separation ( $Y_{Tp}$ ) was calculated by:

$$Y_{Tp} = \frac{100}{1 + \frac{V_{copolymer}}{V_{water}} \times K_{Tp}} \quad (4)$$

where subscript  $T$  refer to the water-rich phase after thermo-separating step. And  $V_{copolymer}$  and  $V_{water}$  are the copolymer-rich phase volume and the water-rich phase volume, respectively [9], [13].

##### 5) The tie line length (TLL) measurement

The tie line length (TLL) is defined as follows:

$$TLL (\%) = \sqrt{(W_1^T - W_1^B)^2 + (W_2^T - W_2^B)^2} \quad (5)$$

where  $W_i^T$ ,  $W_i^B$  represent the weight percentages of phase forming component  $i$  in top and bottom phases, respectively

### III. RESULTS AND DISCUSSION

#### A. Characterization of Copolymer Dehypon® LS 54 with <sup>1</sup>H NMR Spectroscopy

Dehypon® LS 54 is a lauryl alcohol ethoxylates, contains 5 ethylene oxide units and 4 propylene oxide units with a chemical formula  $C_{12}H_{25}(OC_2H_4)_5(OC_3H_6)_4OH$  (US Patent, 2003) [14]. The <sup>1</sup>H NMR spectra of Dehypon® LS 54 is shown in Fig. 1. Based on this chemical formula, the resonances at  $\delta_c$  0.9 and 1.1 are ascribed to  $CH_3$  protons from methyl groups of the lauryl alcohol moiety unit and from methyl protons of PO groups respectively. The resonances of the methylene groups and hydroxyl group of the lauryl alcohol moiety unit were observed at  $\delta_c$  1.3 - 1.54 and 1.56 respectively. The signals within  $\delta_c$  3.3-3.9 are ascribed to backbone protons, CHO and  $CH_2O$  in PO and EO units. In

this  $^1\text{H}$  NMR spectrum, the intensities of the resonances of methylene PEO block are higher than the methyl groups of the PPO block, thus, this copolymer has a higher percentage of PEO block than PPO block.

The chemical structure of Dehypon<sup>®</sup> LS 54 gained from the interpretation of  $^1\text{H}$  NMR spectra are agree with the chemical structure of Dehypon<sup>®</sup> LS 54 as reported previously by US patent [14]. US Patent had reported the chemical formula of Dehypon<sup>®</sup> LS 54 is  $\text{C}_{12}\text{H}_{25}(\text{OC}_2\text{H}_4)_5(\text{OC}_3\text{H}_6)_4\text{OH}$  and based on this chemical formula and  $^1\text{H}$  NMR spectra interpretation, Dehypon<sup>®</sup> LS 54 was identified as a block copolymer that composed of approximately 5 moles ethylene oxide (EO) and 4 moles propylene oxide (PO) with lauryl alcohol as a functionalize group.

The hydrophobicity of the copolymer increases with the increasing of PO content. Although this copolymer has a lower EO content, the presence of lauryl alcohol as a functional group makes this copolymer as a “hydrophobic” character. Furthermore, the hydrophobic properties of this copolymer play an important role in partitioning behavior of model protein and it could be described by the influence of percentage PO groups and aliphatic moiety groups (as hydrophobic groups) that make more protein attracted to the hydrophobic groups during the partitions.

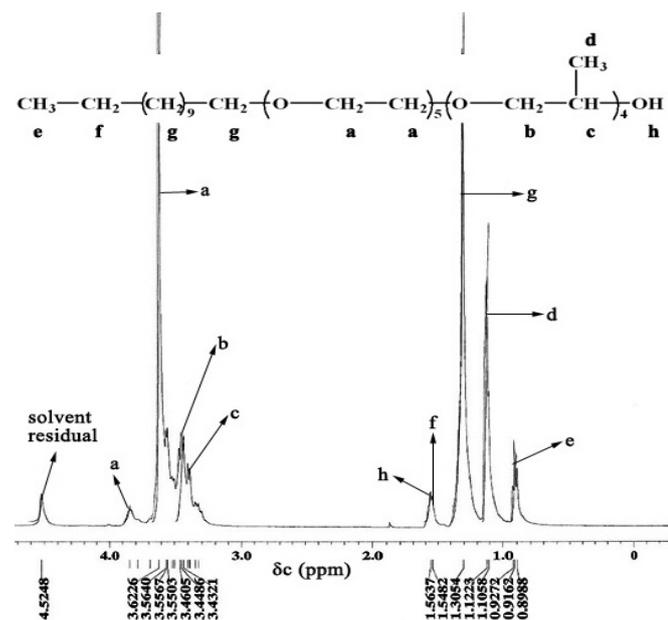


Fig. 1  $^1\text{H}$  NMR spectrum of Dehypon<sup>®</sup> LS 54 copolymer

### B. Phase Diagram for the Primary Phase Systems

The phase diagram determined for Dehypon<sup>®</sup> LS 54, the waxy maize starch (amylopectin starch) and water (Fig. 2) at  $27^\circ\text{C}$  have close similarity to earlier determined phase diagrams by Kepka et al. [13] for  $\text{EO}_{50}\text{PO}_{50}$  and waxy barley starch systems. Both systems were also composed of a thermo-separating EOPO copolymer and a low cost starch polymer system. The phase diagram for Dehypon<sup>®</sup> LS 54/waxy maize starch shows that the binodial has been shifted towards lower polymer concentration compared with the Waxy barley starch system. This is accordance with the higher molecular mass of waxy maize starch (1000 – 10.000

$\text{kg mol}^{-1}$ ) relative to the Waxy barley starch ( $500 \text{ kg mol}^{-1}$ ). Thus, one advantage of using Waxy maize starch with less molecular mass could reduce the consumption of both polymers in a large-scale process [13]. The phase diagram was determined without neither biomass nor salts. However, their presence could be expected to displace the binodial towards lower polymer concentration [9]. The tie lines for each mixture were constructed by plotting the best line that could fit the points of initial composition, between the top and the bottom phases. The compositions of the phases for the Dehypon<sup>®</sup> LS 54-waxy maize starch-water system at  $25^\circ\text{C}$  are listed in Table 1.

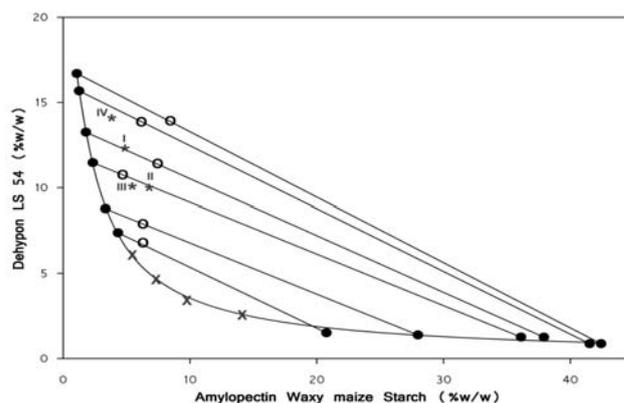


Fig. 2 The phase diagram with binodial and tie-lines for the Dehypon<sup>®</sup> LS54 and Waxy maize starch at room temperature (approximately  $27^\circ\text{C}$ ). Experimental values determined by analyzed two-phase systems (•), and by titration (X) is shown. (o): Phase compositions used for diagram determination, (\*): Phase compositions used in the primary extraction step

### C. Partitioning of Protein Dehypon<sup>®</sup> LS 54/Waxy maize primary phase systems

The partitioning of protein in aqueous two-phase system strongly affected by the composition of the phase system: where the most important variables are type of polymer, polymer concentration, salt composition and pH. The effects of different polymer concentrations were studied at four tie-line lengths.

Protein partitioning can be affected by altering the polymer concentration in the system. It has previously been shown that with increasing polymer concentrations, i.e. increased tie-line length, more extreme partitioning towards one of the two phases will be observed [9], [15], [16]. However, in this case a slightly stronger partitioning to the Dehypon<sup>®</sup> LS 54 phase was observed with decreased tie-line length (Table 2, system II). The partition coefficient values (K) higher than 1 were observed for all the ATPSs assayed, thus indicating that the partition equilibrium is displaced to the Dehypon<sup>®</sup> LS 54-enriched phase. A higher yield in the top phase was achieved for protein in the Dehypon<sup>®</sup> LS 54/waxy maize starch system when tie-line length was increased (Table 2). The enhancement of yield was due to the increased partition coefficient, but also with increased volume ratio [13]. The protein partitioning results showed that the copolymer concentration and top phase volume were the most important phase system parameter.

TABLE I  
Composition of the Phases in the Waxy Maize Starch-Dehypon® LS 54-Water System at 25°C

Total System			Top Phase			Bottom Phase		
Waxy maize	Dehypon® LS 54	H <sub>2</sub> O	Waxy maize	Dehypon® LS 54	H <sub>2</sub> O	Waxy maize	Dehypon® LS 54	H <sub>2</sub> O
(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)
8.00	14.00	78.00	1.05	16.64	82.31	42.30	0.84	56.86
6.00	14.00	80.00	1.19	15.57	83.24	41.82	0.89	57.29
7.00	11.50	81.50	1.62	13.29	85.09	36.61	1.13	62.26
4.00	11.00	85.00	2.18	11.36	86.46	34.74	1.20	64.06
6.00	8.00	86.00	3.14	8.76	88.10	26.52	1.32	72.16
6.00	6.50	87.50	4.11	7.20	88.69	18.53	1.27	80.20

TABLE III  
Composition Partitioning (K), Yield (Y), Volume Ratio (V<sub>t</sub>/V<sub>b</sub>) and Tie-Line Length in Aqueous Two-Phase Systems with Different Composition of Dehypon® LS 54 and Waxy Maize Starch. All Systems Contained a 50 mM Tris-HCl Buffer pH 8.0

No.	Total system composition (%w/w)		Volume Ratio V <sub>t</sub> /V <sub>b</sub>	Partition Coefficient (K)	Yield % (Y <sub>p</sub> )	Tie-line length
	Waxy maize starch	Dehypon® LS 54				
I	5.00	12.00	1.52	1.56	29.66	37.04
II	6.00	10.00	1.19	1.89	30.78	34.11
III	5.00	10.50	1.24	2.85	22.06	34.10
IV	4.00	14.50	1.55	1.16	35.74	43.20

#### D. Cloud-point Diagram

The cloud-point depends on the structure of the copolymer and on its concentration. An increased temperature will result in the separation of copolymer solutions into a water-rich phase and a copolymer-rich phase. The cloud-point of a copolymer solution can be affected by addition of salt, by different the ratio of ethylene oxide to propylene oxide or by changing copolymer concentration. In this experiment, the cloud-point temperatures used have an accuracy of 0.1°C. The gellification was observed in some conditions, especially with high copolymer concentration of 40-50%.

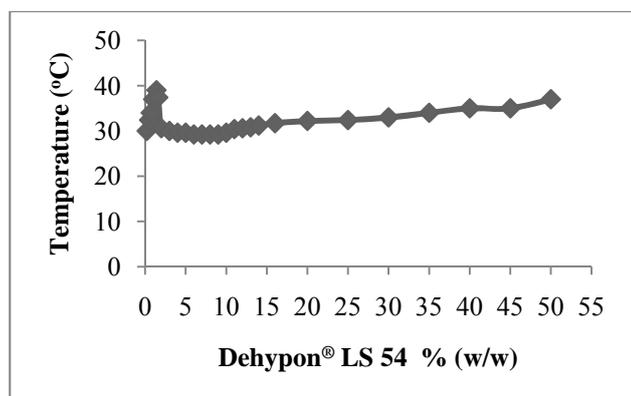


Fig.3 Cloud-point diagram for the system Dehypon® LS 54 / water

The cloud-point diagram for the binary system of Dehypon® LS 54 in water solution is shown in Figure 3. The curve shape is very specific for this copolymer. The critical point was 30 °C at Dehypon® LS 54 concentration between 3 and

10% (w/w). It should be noted that the presence of salts and a second polymer will affect the cloud-point and, therefore, this diagram will be used as a guideline to the range for working cloud point in the future [17].

#### E. Protein Partitioning in Temperature-induced Two-phase Systems

After primary phase separation in the primary system, the copolymer-rich phases were isolated in the separated test tube and the temperature-induced phase separation at 36°C was performed overnight to ensure complete separation phases is achieved. Generally, upon heating, water solution of thermo-separating copolymer will form a turbid solution. At cloud-point temperature (CPT), every part of the copolymer will start to form macroscopic aqueous droplets which would disperse in the water phase and when higher temperature was applied the copolymer droplets will aggregate and form one top aqueous copolymer phase in equilibrium with water phase in the bottom [13]. However, in this study the top rich-copolymer phase was at the bottom while the water phase at the top. This could be due to lower density of Dehypon® LS 54 being 0.933-0.938 g/cm<sup>3</sup>, as compared to the density of water-rich phase. As a consequence, the target protein will be located at the top phase. After phase separation, the bottom phase is composed more than 50% of water and the upper phase is copolymer-rich phase. The protein concentration was measured in both water-rich and polymer-rich phases the partition coefficient of protein was also calculated.

In the temperature-induced two-phase systems, protein was migrated into water-rich bottom phase (Table 3), that results in  $K_p$  values within the range 0.4-0.9. The high affinity of the protein for the lower water phase is due to

both strong interaction between charged groups in the protein and the polar water molecule without taking into account the volume effect in the copolymer-enriched phase.

When separated the bottom phase is composed of approximately more than 50% of water and the upper phase is Dehypon®LS54-enriched. The protein partition coefficient (Table 3) shows that proteins were more evenly partitioned in the bottom phase (water-enriched phase). The percent protein recoveries (*R*) in the bottom water phase after thermo-separation is shown in Fig. 4. A significant *R* values between 60 and 80% is observed in systems with total composition 12% (w/w) Dehypon® LS 54, 5 % (w/w) waxy maize starch and 14.4% (w/w) Dehypon® LS 54, 4% (w/w) waxy maize starch, respectively.

TABLE III  
PARTITIONING (*K*), YIELD (*Y*) AND VOLUME RATIO ( $V_t/V_b$ ) IN TEMPERATURE-INDUCED TWO-PHASE SYSTEMS WITH DIFFERENT COMPOSITION OF DEHYPON® LS 54 AND WAXY MAIZE STARCH. LETTERS I, II, III AND IV REPRESENT THE DIFFERENT TOTAL SYSTEM COMPOSITION ACCORDING TO TABLE 2

Total system composition (%w/w)	Volume Ratio ( $V_t/V_b$ )	Partition Coefficient ( <i>K</i> )	Yield (%) ( <i>Y</i> )
I	0.47	0.79	72.90
II	0.67	0.92	61.98
III	0.50	0.39	83.68
IV	0.39	0.42	85.96

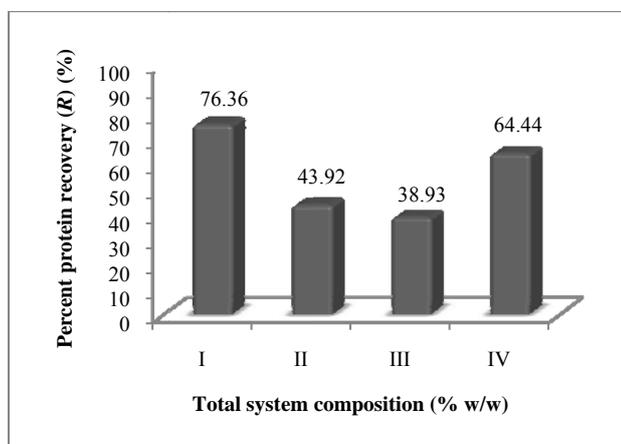


Fig. 4 Percent protein recoveries (*R*) for cutinase in the water enriched phase of a temperature-induced two-phase systems obtained after heating the top phase of a Dehypon® LS 54/ Waxy maize systems with different compositions (I, II, III and IV) on x-axis represent the different total system composition according to Table 2.

#### IV. CONCLUSIONS

An aqueous two-phase extraction based on Dehypon® LS 54-The waxy maize starch has been used for the partitioning of recombinant cutinase (WP)<sub>4</sub> as a model protein and enzyme. The chemical structure of Dehypon® LS 54 gained from the interpretation of <sup>1</sup>H NMR spectra are agree with the chemical structure of Dehypon® LS 54 as reported previously by US patent [14]. The hydrophobicity of the

copolymer increases with the increasing of PO content. Although this copolymer has a lower EO content, the presence of lauryl alcohol as a functional group makes this copolymer as a “hydrophobic” character. Furthermore, the hydrophobic properties of this copolymer play an important role in partitioning behavior of model protein and it could be described by the influence of percentage PO groups and aliphatic moiety groups that make more protein attracted to the hydrophobic groups during the partitions. In this work, the separation properties of a novel two-phase system were evaluated. The top phase has high EOPO concentration, while the bottom is waxy maize starch-enriched. The tie line of the phase diagram are practically parallel, thus allowing us to determined the top and bottom compositions for any given total polymer composition. The partitioning of the total protein in the primary phase system shows that the partition equilibrium is displaced to the Dehypon® LS 54-enriched phase, whereas, the partitioning of cutinase enzyme indicating that the enzyme was precipitated together with other protein in the bottom phase (starch-rich phase). A temperature increase of the copolymer phase, above the copolymer cloud point, results in a second temperature induced two-phase system with a copolymer-enriched upper phase and a water-enriched lower phase, containing most of initial protein. Although the aqueous two-phase systems composed of Dehypon® LS 54-The waxy maize starch have a relative low level in recovery percentage, the high value of the yield percentage of total protein after temperature-induced phase separation step makes this system potential to be used in protein and enzyme purification. In addition, the copolymer Dehypon® LS 54 is also advantageous because of low cloud point temperature, which reduces the risk for denaturation effects to the enzymes in the temperature-induced phase separation. In the future study, with the aim to enhance more cutinase recovery and purification factor, more factors (such as addition of salt and detergent) should be taken into consideration and apart from that it is important to evaluate the physical properties of the phases.

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