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*Original Article*

# Partitioning behavior of laccase from *Lentinus polychrous* Lev in aqueous two phase systems

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**Abstract**

In the present study, an aqueous two phase system (ATPS) composed of polyethylene glycol (PEG) and potassium phosphate was employed to purify laccase produced by *Lentinus polychrous*. The assessments of system parameters such as molecular weight of the PEG, PEG concentration, phosphate salt, system pH, and neutral salt (NaCl) concentration on laccase partitioning and purification were investigated. The enzyme preferentially partitioned in the top polymer-rich phase in all tested systems. Desirable conditions for partitioning were found in the system having polyethylene glycol of intermediate molecular weight 4,000. This work revealed that the extraction efficiency was not much affected by PEG concentration. The NaCl addition decreased greatly the partition coefficient of laccase. The optimal system was obtained at pH 7.0, containing 12% w/w PEG 4,000 and 16% w/w potassium phosphate with enzyme partition coefficient of 88.30, purification factor of 3.01-fold, and 99.08% yield of enzyme activity in the top phase. Overall, the results obtained in this study revealed that ATPSs could be potentially useful technique for a first step purification of laccase from *L. polychrous*.

**Keywords:** aqueous two phase system, laccase, partitioning, *Lentinus polychrous*

## 1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multinuclear copper-containing enzymes which reduce molecular oxygen to water and simultaneously catalyze the one-electron oxidation of various phenolic substrates, such as monophenols, diphenols and aromatic amines. Moreover, laccases oxidize a wide variety of non-phenolic aromatic compounds in the presence of redox mediators. The active site of enzyme contains four copper ions of two different types: one Type I Cu, whose redox potential determines the substrates to be oxidized, and the other three Cu ions transfer electrons to the oxygen (Michniewicz *et al.*, 2008). Due to their ability to oxidize broad substrates, laccases have been extensively applied in several fields, such as decolourization of dyes (Couto, 2007; Khlifi *et al.*, 2010), degradation of xenobiotics (Saito *et al.*, 2004), pulp and paper industry (Lund and Felby, 2001), denim bleaching (PazarIIog *et al.*, 2005), food industry (Selinheimo *et al.*, 2006) and organic synthesis (Mustafa *et al.*, 2005).

Laccase can be obtained from some plants, insects, a few bacteria, and it is especially abundant in white-rot fungi (Litthauer *et al.*, 2007). Fungal laccases have been purified and characterized extensively in many studies (Fu and Viraraghavan, 2001). Wesenberg *et al.* (2003) has reviewed some properties of laccase from white-rot fungi. They reported that the enzyme is a monomeric protein with a molecular weight of 60-390 kDa and an isoelectric point of 2.6-4.5. *Lentinus polychrous* is an edible mushroom and widely cultivated in many regions of Thailand. Therefore, it is worth to

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select *L. polychrous* from the group of white-rot fungi as the source of laccase in this study.

The conventional downstream techniques for enzyme separation and purification, such as chromatography, electrophoresis, and precipitation have been widely used. However, these methods are cost intensive, providing low yields, and are not suitable for large scale production. An aqueous two phase system (ATPS) is an attractive technique for separation and purification of biological products. ATPSs have several advantages compared to the conventional techniques including biocompatibility, high water content (70-80%), low interfacial tension (Veide *et al.*, 1983), high capacity and yield, safety, ease of scale-up and operation, and the potential for polymer recycling (Naganagouda and Mulimani, 2008). ATPSs are formed by combining incompatible aqueous solutions of two polymers (such as polyethylene glycol and dextran) or from a mixing solution of polymer and salt (phosphate, sulfate, citrate, etc.) above critical concentration. ATPSs composed of polymer-salt are more popular due to high selectivity, low cost, and low viscosity (Yücekan and Önal, 2011). The partition occurred by the unvarying distribution of the salt ions in the top and bottom phases and by the difference of the electric current, which is caused from the inequality of the salt distribution (Han and Lee, 1997). In general, salt ions move to the bottom phase, which has a higher density, and then the bottom phase enriches with negative charge ions rather than the top phase. Therefore, proteins might prefer to partition to the phase which are enriched opposite their surface charge. The partition of molecules between the two phases is described by the partition coefficient (K), which is defined as the ratio of the molecules concentration in the top phase to that in the bottom phase. If the coefficient is higher than 1, the molecules prefer the top phase and if lower than 1 is in the bottom phase.

In addition to protein properties (charge, shape, size, hydrophobicity, molecular weight, etc.), a number of parameters, such as the polymer molecular weight and concentration, type of salts present in the system and the system pH, influence protein partitioning. In order to achieve an effective extraction with good yield and high purification factor of the desired protein, the composition for the ATPSs needs to be selected carefully to gain a higher amount of the desirable protein from one of the phases with minimal concentration of the interfering substances (Yang *et al.*, 2008). The aim of this study is to purify laccase produced by *Lentinus polychrous* using ATPSs composed of polyethylene glycol (PEG) and phosphate salt. The system parameters such as molecular weight of the PEG, PEG concentration, phosphate salt, system pH and neutral salt (NaCl) concentration on laccase partitioning and purification are evaluated.

## 2. Materials and Methods

### 2.1 Materials

Polyethylene glycols (MW. 1,000 4,000 and 6,000), Potassium dihydrogen phosphate (mono basic and di-basic), Potassium hydroxide and Sodium acetate were purchased from Ajax finechem, New Zealand. Trichloracetic acid was obtained from Sigma-Aldrich Co. Ltd, Germany. Bovine Serum Albumin (BSA), Coomassie brilliant Blue G250 and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Fluka, UK. All other chemicals were of analytical grade. The fungi *Lentinus polychrous* strains were kindly provided by J. Phetsom, Mahasarakham University, Thailand.

### 2.2 Cell cultivation and crude enzyme preparation

The active fungal form was cultivated under solid-state fermentation on sterile rice bran and rice husk (2:1 by weight) for 14 days at room temperature as described by Sarnthima *et al.* (2009). The solid culture was stirred with distilled water at ratio 1:3 w/v for 45 min and then filtered through a cheese cloth. The filtrate was centrifuged at 6,000 rpm for 10 min at room temperature. The obtained supernatant was referred as crude enzyme and used throughout the experiments.

### 2.3 Preparation of aqueous two phase system

Aqueous two phase systems were set up at room temperature by mixing required quantities of stock solutions of PEG in deionized water and potassium phosphate solutions at pH 7.0. Ten percentage of crude enzyme was added into the system. The total weight of the final system was 10 g. After mixing the content thoroughly, the system was allowed to separate for 10 min. Complete phase separation was achieved by centrifugation at 3,000 rpm for 10 min. After a clear separation of the two phases, the top and bottom phases were withdrawn separately with a long needle. The volumes of the two phases were then used to estimate the volume ratio ($V_r$). The aliquots of the phases were analyzed for enzyme activity and protein concentration. In order to correct for PEG and potassium phosphate interference, a blank system (without crude enzyme) was prepared and the samples were read against blank for each set of conditions.

In order to determine the effect of PEG on laccase partitioning, the concentrations (16, 18, and 20% w/w) of PEG 1,000, 4,000 and 6,000 Da were studied with 14% w/w potassium phosphate. Different concentrations of potassium phosphate (14-22% w/w) were tested with constant PEG 4,000 concentration (12% w/w). The pH of the phase system (6.5, 7.0, and 7.5) on the partitioning of the laccase was also investigated. All partition experiments were conducted in triplicates and the averages were used in the calculations.

### 2.4 Determination of phase volume ratio, partition coefficient, purification factor and yield

The partitioning parameters in ATPS were calculated as follows.

The volume ratio ($V_r$) was defined as the ratio of volume in the top phase ($V_T$) to that in the bottom phase ($V_B$).

$$V_r = \frac{V_T}{V_B} \quad (1)$$

The enzyme partition coefficient, $K_{E,}$ was defined as the ratio of enzyme concentration in the top phase ($E_T$) to the one in the bottom phase ($E_B$).

$$K_E = \frac{E_T}{E_B} \quad (2)$$

The protein partition coefficient, $K_{P,}$ was defined as the ratio of the protein concentration in the top phase ($P_T$) to the one in the bottom phase ($P_B$).

$$K_E = \frac{P_T}{P_B} \quad (3)$$

The purification factor, PF, was calculated by the ratio of the specific activity in the top phase ($SA_T$) to the specific activity in the crude extract ($SA_i$).

$$PF = \frac{SA_T}{SA_i} \quad (4)$$

The laccase yield was defined as:

$$Yield(\%) = \frac{100}{1 + \frac{1}{V_r K_E}} \quad (5)$$

### 2.5 Determination of enzyme activity

Laccase activity was determined following the change in optical density at 420 nm using 2, 2'-Azinobis (3-ethyl-benzothiazoline-6-sulfonate) diammonium salt (ABTS) as a substrate ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). The modified assay was previously described by Sarnthima and Khammuang (2007). Briefly, the assay mixture consisted of 0.1 M acetate buffer pH 4.5, 10 mM ABTS and enzyme extract sample. The mixture was incubated at 32°C for 10 min and the reaction was stopped with 50% w/v trichloroacetic acid (TCA). The activity of the enzyme was expressed as enzyme unit (U). One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol ABTS per minute.

### 2.6 Determination of protein concentration

Protein concentration was measured according to the Coomassie Blue G-250 method described by Bradford (1976). Different concentrations of bovine serum albumin (BSA) were prepared as a standard protein. The protein content was measured at the absorbance 595 nm.

## 3. Results and Discussion

### 3.1 Effect of PEG molecular weight on laccase partitioning

Enzyme partitioning and extraction depended on the polymer molecular weight in ATPS (Madhusudhan *et al.*, 2008; Mohamadi *et al.*, 2007; Naganagouda and Mulimani, 2008). To obtain the optimal enzyme extraction, the selection of the appropriate molecular weight of PEG is critical. The ATPS containing different molecular weights of PEG (MW. 1,000, 4,000 and 6,000) and concentrations of 16-20% w/w on laccase partitioning was investigated. Other parameters such as potassium phosphate concentration (14% w/w), pH of the system (pH 7.0), and amount of crude enzyme were kept constant. The effect of PEG molecular weight on the partition coefficient of enzyme ($K_E$), the partition coefficient of protein ($K_P$), volume ratio ($V_r$), purification factor (PF) and percent yield are shown in Table 1. The results showed that the volume ratio of the system ranged from 1.00 to 1.56. The

Table 1. Partitioning of laccase in different molecular weights of PEG.

| PEG molecular weight | PEG concentration (% w/w) | $K_E$ | $K_P$ | $V_r$ | PF | Yield (%) |
|---|---|---|---|---|---|---|
| 1,000 | 16 | 30.80±1.21 | 1.28±0.08 | 1.50±0.02 | 1.43±0.04 | 97.88±0.08 |
| | 18 | 17.52±0.85 | 1.91±0.04 | 1.00±0.23 | 0.69±0.09 | 94.60±1.39 |
| | 20 | 24.64±4.01 | 0.84±0.07 | 1.50±0.06 | 1.19±0.06 | 97.37±0.47 |
| 4,000 | 16 | 66.93±1.82 | 1.54±0.05 | 1.50±0.03 | 1.55±0.10 | 99.01±0.02 |
| | 18 | 66.23±1.55 | 1.39±0.08 | 1.50±0.06 | 1.61±0.06 | 99.00±0.06 |
| | 20 | 63.37±1.18 | 1.06±0.09 | 1.56±0.07 | 1.98±0.05 | 99.00±0.02 |
| 6,000 | 16 | 44.96±1.62 | 1.29±0.11 | 1.50±0.10 | 1.28±0.09 | 98.54±0.14 |
| | 18 | 26.64±2.44 | 1.09±0.09 | 1.50±0.04 | 1.43±0.07 | 97.56±0.18 |
| | 20 | 26.67±2.21 | 0.71±0.20 | 1.50±0.07 | 1.41±0.07 | 97.56±0.29 |

Phase system: PEG/14% w/w potassium phosphate, pH at 7.0. $K_E$: enzyme partition coefficient; $K_P$: protein partition coefficient; $V_r$: volume ratio; PF: purification factor (fold); Yield: enzyme recovery (%). *Means±S.D. from triplicate determinations.

partition coefficient of laccase for all systems was significantly above 1, indicating that laccase preferentially partitioned to the top phase. This partition behavior shows that laccase is relatively hydrophobic and interacts well with PEG. Similar observation was reported by Mayolo-Deloisa *et al.* (2009). In the case of $K_P$ values, there was a negligible change as the molecular weight of PEG increased. To achieve an effective purification factor, the contaminant proteins should not be partitioned in the same phase as the enzyme. Therefore, for a high value of $K_E$ a low $K_P$ value is desirable. From Table 1, the system consisted of PEG 4,000 provided better results for the $K_E$ value (in range of 63.37-66.93), purification factor (1.55-1.98-fold), and percent yield (up to 99%) than those of PEG 1,000 and 6,000 comparing the data at the same concentration. This indicated that the intermediate molecular weight of PEG was preferable for laccase purification and was selected for further experiments.

In fact, the molecular weight of PEG influences protein partitioning by changing the number of hydrophobic interactions between PEG and the hydrophobic area of protein (Mohamadi *et al.*, 2007; Yücekan and Önal, 2011). In general (Hachem *et al.*, 1996) for a high MW polymer the interactions between PEG and hydrophobic enzyme decrease. This leads to a decrease in $K_E$. For example, Bassani *et al.* (2007) have studied the partitioning of procine pancreatic lipase by using PEG-phosphate salt system and found that the high $K_E$ obtained in the system of low MW PEG (2,000-4,000) while increasing the PEG MW from 6,000 to 10,000 gave low $K_E$. Similarly, Ketnawa *et al.* (2010) have studied the bromelain partitioning in PEG-$MgSO_4$ system and observed that PEG 2,000 provided high $K_E$ and enzyme recovery compared with that of PEG 4,000 and 6,000. The reason of this phenomenon is probably because an increase in MW of PEG results in an increase in the chain length of the polymer and an exclusion effect, which lead to the reduction in the free volume. Thus, a polymer acquires a more compact conformation with intramolecular hydrophobic bonds and hinders the partition of protein into the top phase. However, the mentioned rule cannot be used to explain the partition behavior in all cases of ATPSs. Mohamadi *et al.* (2007) reported that the use of low MW polymer is probably unsuitable for a good efficiency of enzyme extraction because the interfacial tension between the phase decreases and as a result the polymer can attract all the desired and contaminated proteins to the top phase. Therefore, the selection of intermediate MW of PEG is appropriate in some extraction systems, for example, the extraction of invertase in PEG-$Na_2SO_4$ system (Yücekan and Önal, 2011), lipase in PEG-$Na_2HPO_4$ system (Nandini and Rastogi, 2011) and xylanase in PEG-$(NH_4)_2SO_4$ system (Yang et al., 2008).

### 3.2 Effect of PEG concentration on laccase partitioning

The effect of PEG 4,000 concentration from 12-20% w/w on enzyme partitioning at a constant potassium phosphate concentration (14% w/w) and pH system at 7.0 was investigated. Increasing the concentration of PEG influences the partition coefficient of enzyme ($K_E$) and protein ($K_P$) as can be seen in Figure 1. The $K_E$ increased from 38.43 to 66.93 when the concentration of PEG changed from 12% (w/w) to 16% (w/w), respectively. Increasing the PEG concentration enhances the hydrophobic interaction between PEG and the surface of protein (Bassani *et al.*, 2007). The greater interaction also depends on the chemical structure of protein. Mayolo-Deloisa *et al.* (2009) found that laccase from *A. bisporus* is a hydrophobic protein. Therefore, the high presence of hydrophobic areas of laccase interact with more PEG. The enzyme was then preferable to the top phase. However, the $K_E$ slightly decreased above 16% w/w PEG concentration. This would be explained according to the partitioning theory that high PEG concentration in the system enhances the viscosity and the interfacial tension between the phase. Then the partition of the enzyme molecules to the top phase is more difficult and this results in decrease in partition coefficient of enzyme. The trend is that the $K_P$ values decreased with the increase of the PEG concentration. This implied that the protein more favorably partitioned to the bottom phase. The purification factor slightly increased from 1.16 to 1.98 (data not shown).

Similar enzyme partitioning behaviors were observed for xylanase in PEG-$(NH_4)_2SO_4$ system (Yang *et al.*, 2008), procine pancreatic lipase in PEG-phosphate system (Bassani *et al.*, 2007) and bromelain in PEG-$MgSO_4$ system (Ketnawa *et al.*, 2010). The opposite partitioning behavior found in polyphenol oxidase in PEG-phosphate system (Babu *et al.*, 2008). In overall, the results in this study did not provide a significant difference in purification factor after increasing the PEG concentration. Moreover, a high viscosity from a high PEG concentration might impact further the purification process (Ratanapongleka, 2010). Therefore, a higher quantity of PEG consumption with the additional costs in the extraction system needs to be considered.

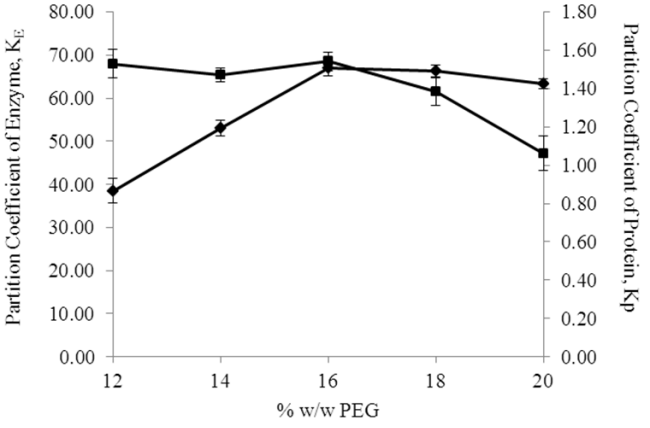


Figure 1. Effect of PEG concentration on the partition coefficient of enzyme (♦) and the partition coefficient of protein (■) in PEG 4,000-phosphate system.

### 3.3 Effect of potassium phosphate concentration on laccase partitioning

The phase compositions influence the partitioning of enzyme, protein and the purification factor. As Mayolo-Deloisa *et al.* (2009) detected that the partitioning behavior of laccase from *A. bisporus* in ATPSs composed of a PEG-phosphate system provided much better enzyme recovery than that of a PEG-sulphate system. Moreover, the PEG-phosphate system is the most used and characterized system near neutral pH in many studies (Rito-Palomares, 2004). The potassium phosphate was then selected in this study. In order to reduce the use of PEG and to improve the enzyme partitioning to the top phase, the influence of phosphate concentration was determined. The constant concentration of PEG 4,000 (12% w/w) was chosen based on the binodal curves by considering the minimum concentration for the formation of a two-phase system with 14-22% (w/w) potassium phosphate. The system pH was at 7.0. Figure 2 shows the effect of phosphate concentration (14-22% w/w) on the partition coefficient of laccase and protein. The increase in potassium phosphate concentration from 14% w/w to 16% w/w resulted in an increase in $K_E$ and $K_P$. This phenomenon could be due to the effect of salting out, which resulted in an increased partitioning of laccase and undesired proteins to the top phase. The highest $K_E$ value (88.30) and $K_P$ value (5.17) were observed at 16% w/w phosphate concentration. The results showed no regular relation between laccase partitioning and salt concentrations at above 16% w/w. The trends of both partition coefficients decreased at a phosphate concentration of 18-22% w/w.

The addition of salts to the aqueous PEG solution induces an arrangement of ordered water molecules around the PEG molecules. The formation of water around the cation (PEG molecules) resulted in a more compact structure and the reduction in volume of PEG molecule (Farruggia *et al.*, 2004). Then increase in salt concentration provided the higher proportion of the bottom phase (Ketnawa *et al.*, 2010; Nalinanon *et al.*, 2009). This leads to a reduction in volume ratio. This mechanism agrees with the result of volume ratio shown in Figure 3. The increase in phosphate concentration from 14% w/w to 22% w/w was found to reduce the volume ratio from 1.56 to 0.54 and the percent yield from 98.36 to 82.74 (data not shown). The highest purification factor was 3.01-fold at 16% w/w phosphate salt.

### 3.4 Effect of system pH on laccase partitioning

The effect of system pH on enzyme partitioning has been studied extensively (Naganagouda and Mulimani, 2008; Shang *et al.*, 2004; Vaidya *et al.*, 2006; Yang *et al.*, 2008). In general, the top phase (PEG rich phase) carries a positive charge while the bottom phase (phosphate rich phase) has a negative charge. Consequently, positively charged proteins are favorable to the bottom phase, while negatively charged proteins move directly to the top phase. The partitioning is then related to the surface properties of protein. The proteins are charged or can be charged due to modifications of the overall molecule at different pH conditions. At the isoelectric point of the protein (pI), the sum of all charges on the protein is zero. For all other pH values the protein has a net charge (Shang *et al.*, 2004). Thus, partitioning of a protein in a two phase system frequently depends on the net biomolecule charge, which is a function of the solution. For pH values above pI, the protein surface charge becomes negative and partitioned to the top phase and as a result there is an increase in the partition coefficient.

In order to study the influence of pH on laccase partitioning, the selected phase system (12% w/w PEG 4,000-16% w/w phosphate) was tested at the pH 6.5, 7.0, and 7.5. The volume of top and bottom phase changed slightly with the

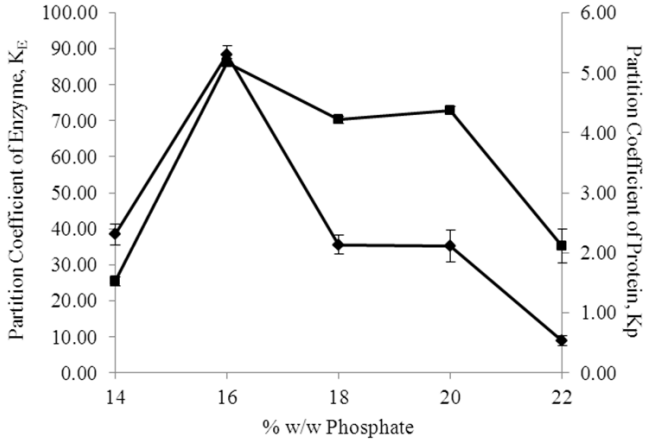


Figure 2. Effect of phosphate concentration on the partition coefficient of laccase (♦) and protein (■) in the system containing 12% w/w PEG 4,000 and pH 7.0.

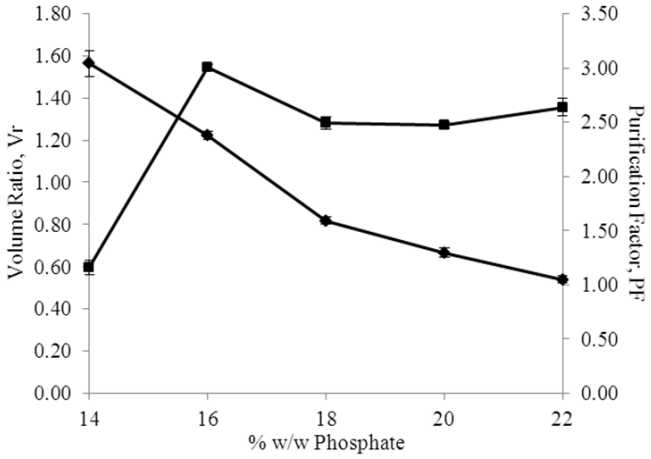


Figure 3. Effect of phosphate concentration on the volume ratio (♦) and purification factor (■) in the system containing 12% w/w PEG 4,000 and pH 7.0.

change in the system pH. The results are shown in Figure 4. An increase in the system pH from 6.5 to 7.0 was found to increase $K_E$ from 8.87 to 88.30, purification factor from 0.84 to 3.01, and yield from 91.06 to 99.08% (data not shown). This indicated that the increase in pH (higher than pI) resulted in an increased laccase partitioning to the top phase since the enzyme become more negatively charged. These results were in agreement with a previous study (Mayolo-Deloisa *et al.*, 2009). Conversely, these parameters ($K_E$, PF, %-yield) decreased when the system pH was at 7.5. The reduction at this pH might be due to the enzyme denaturation at high pH. Sarnthima and Khammuang (2007) reported that the optimum activity of laccase from *L. polychrous* was at pH 3.0 and was inactive at pH 8.0. From the results from this study it can be concluded that the optimal values for the partition were obtained at pH 7.0.

### 3.5 Effect of sodium chloride addition

The addition of neutral salt affects the properties of protein partitioning between two phases by decreasing protein hydrophobicity (Yücekan and Önal, 2011). Moreover, the ion of neutral salt distributed unequally between the phases causes an electrostatic potential difference and leads to the change of partition coefficient of specific proteins according to their charge (Albertsson, 1990; Engel *et al.*, 2000). To determine the effect of neutral salt on the partition behavior of laccase, the addition of 0-5% w/w NaCl in the 12% w/w PEG 4,000-16% w/w phosphate system was studied. The pH system was at 7.0. According to obtained results (Figure 5), an increase in NaCl concentration favored the partitioning of laccase in the bottom phase resulting in a lowering of the partition coefficient. The percent yield tended to be decreased in the presence of NaCl as well. The highest $K_E$ (88.30) and percent yield (99.08%) were obtained at no addition of NaCl. It was report that addition of NaCl often changed the partitioning of a specific protein and contaminating proteins in the same direction if they had the similar surface hydrophobicity, resulting in a decrease of the purification factor (Chen *et al.*, 2008). Ketnawa *et al.* (2010) has pointed out that the activity of enzyme decreases and enzyme structure is changed after exposing it to NaCl. The addition of salt at high concentrations may lead to aggregation followed by protein precipitation, because most of the water molecules are strongly bound to the salts. Therefore, the interactions between proteins become more powerful than between protein and water.

Srinivas *et al.* (2002) also observed the same behavior for the partition of Ipomoea peroxidase in PEG-phosphate system. Gunduz and Korkmaz (2000) found BSA favorably partitioned to the bottom phase with increasing NaCl concentration up to 0.2 M. However, the differential response of proteins in the presence of added salt in the two phases has also been investigated, for example, NaCl enhances the partitioning and recovery of amylase in the top phase (Schmidt *et al.*, 1994).

## 4. Conclusions

The laccase produced by *L. polychrous* was extracted and purified by partitioning in ATPSs composed of PEG and potassium phosphate. The parameters such as molecular weight and concentration of PEG, concentration of potassium phosphate, system pH, and NaCl influenced laccase partitioning. The PEG 4,000 and potassium phosphate system showed the highest $K_E$ and laccase recovery in the top phase. The best value of obtained PF was 3.01-fold. This study indicated that ATPSs proved to be a suitable technique for primary laccase purification. However, the SDS-PAGE electrophoresis should be further investigated for confirmation of the extracted laccase purity.

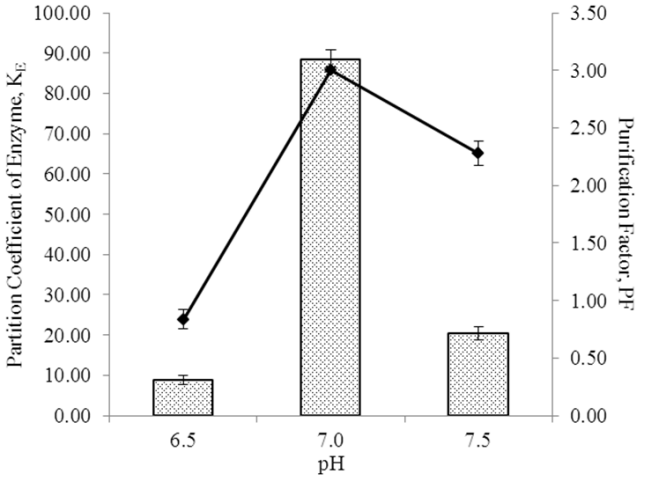


Figure 4. Effect of system pH on the partition coefficient of laccase (▒) and purification factor (♦) in the PEG 4,000-phosphate system.

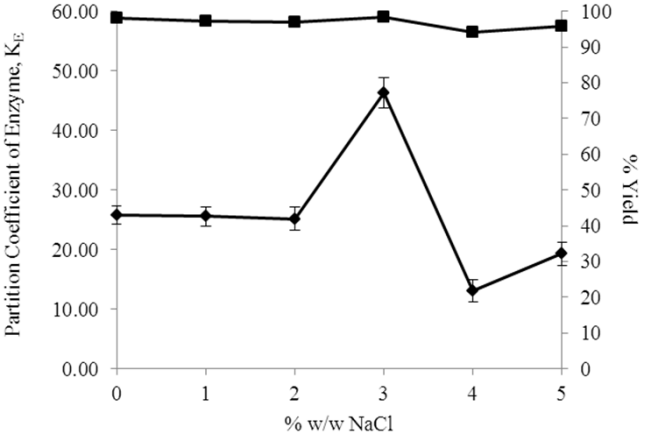


Figure 5. Effect of NaCl on the partition coefficient of laccase (♦) and %-yield (■) in the PEG 4,000-phosphate system and pH 7.0.

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