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Extraction in Two-Phase Systems and Some Properties of Laccase from *Lentinus polychrous*

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***Abstract*—** Extraction of laccase produced by *L. polychrous* in an aqueous two-phase system, composed of polyethylene glycol and phosphate salt at pH 7.0 and 25^0C was investigated. The effect of PEG molecular weight, PEG concentration and phosphate concentration was determined. Laccase preferentially partitioned to the top phase. Good extraction of laccase to the top phase was observed with PEG 4000. The optimum system was found in the system containing 12% w/w PEG 4000 and 16% w/w phosphate salt with K_E of 88.3, purification factor of 3.0-fold and 99.1% yield. Some properties of the enzyme such as thermal stability, effect of heavy metal ions and kinetic constants were also presented in this work. The thermal stability decreased sharply with high temperature above 60 ^{0}C. The enzyme was inhibited by Cd^{2+}, Pb^{2+}, Zn^{2+} and Cu^{2+}. The Vmax and Km values of the enzyme were 74.70 µmol/min/ml and 9.066 mM respectively.

***Keywords*—**Aqueous Two Phase System, Laccase, *Lentinus polychrous*,

I. Introduction

LACCASES (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multinuclear copper-containing enzyme which catalyze the one-electron oxidation of a wide variety of phenolic and non-phenolic substrates. They have been extensively applied in several fields such as decolourization of dyes [1, 2], degradation of xenobiotics [3], pulp and paper industry [4], denim bleaching [5], food industry [6, 7] and organic synthesis [8]. Laccase can be obtained in some plants, insects, a few bacteria, and especially abundant in whit-rot fungi [9].

Many researches have characterized laccase activity. However, laccase derived from various sources probably secretes different forms and catalytic properties. L. polychrous is an edible mushroom and widely cultivated in many regions of Thailand. Therefore, it is worth to select L. polychrous from the groups of white-rot fungi as the source of laccase in this study.

Enzyme recovery and purification are considered to be the most expensive part of production. The techniques such as chromatography, electrophoresis and precipitation have been widely employed. However, these methods results in high costs of operation, providing low yields and not suitable for large scale production. An aqueous two phase system (ATPS) is an attractive and mild method for extraction of enzyme since it constitutes mild environmental condition containing high water content in each of the liquid phase up to 70-90% and surface tension between the two phases is low [10], resulting in high mass transfer and decreasing the possibility of denaturation of labile biomolecules [11]. Many polymers used in the system have protein-stabilizing properties. In continuous extraction with ATPS is also straightforward and requires relatively simple equipment which are easy to operate [12]. Moreover, the conditions for separation on a large scale do not considerably change from small scale, thus easy in scale-up.The aim of this study is to extract laccase using PEG-phosphate system and to characterize thermal stability, influence of heavy metals and kinetic constants of the enzyme.

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II. Experimental

A. Cell Cultivation and Crude Enzyme Preparation

The active fungal form was cultivated on rice bran and rice husk (2:1 by weight) for 14 days. 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. The obtained supernatant was referred as crude enzyme and used through experiments.

B. Preparation of Aqueous Two Phase System

Aqueous two phase systems were set up at room temperature by mixing required quantities of PEG and phosphate salt (pH 7.0) and 1 ml crude enzyme, adjusting the total volume of the system to 10 ml with DI water. After mixing thoroughly the system was allowed to separate into two phase for 10 min and then centrifuged at 3000 rpm for 10 min to accelerate the phase separation and to reach the eqilibrium state. After clear separation, the volumes of top and bottom phases were measured and analyzed for enzyme activity and protein concentration. The results given are averages of three experiments. The partitioning parameters in ATPS were calculated as follows:

The volume ratio (Vr) 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 in the bottom phase (E_B).

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

The laccase yield recovery was defined as:

$$Yield(\%) = \frac{100}{1 + \frac{1}{V_r K_E}} \quad (3)$$

C. Determination of Enzyme Activity and Protein Concentration

Laccase activity was determined following the change in optical density at 420 nm using ABTS as a substrate. 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 ^{0}C for 10 min and stopped the reaction with 50% (w/v) TCA. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol ABTS per min. Protein concentration was measured according to the Coomassie Blue G-250 method described by Bradford [13] at 595 nm. Bovine Serum Albumin was used as a standard protein.

D. Thermal Stability and Half Life Time

Thermal stability of enzyme was carried out at pH 4.5 and temperature range of 4-70 ^{0}C. The aliquots of sample were taken and checked residual activity at different time intervals in order to determine half-life of enzyme.

E. Effect of Heavy Metals on Enzyme Activity

The effect of Cd (II), Pb(II), Zn(II) and Cu(II) ions over the activity of laccase was monitored through estimation of the activity reduction in the presence of these metal ions. The enzyme was incubated in buffer (pH 4.5) containing 10 mM ABTS and different concentrations of given metal ions (0.1-10 mM) for 10min. The reaction was stopped and determined the activity.

F. Determination of Kinetic Constants

Km and Vmax constants were determined using various concentrations ABTS in the range of 0-50 mM.

III. Results And Discussion

A. Extraction of Laccase by ATPS

Effect of PEG on Laccase Partitioning

The laccase partitioning in the ATPS containing different molecular weights (MW.1000, 4000 and 6000) and concentrations of PEG (16-20%w/w) with 14%w/w phosphate salt is shown in Table 1. The system pH was controlled at 7.0. The molecular weight of PEG influences protein partitioning by changing the number of hydrophobic interactions between PEG and protein [14,15]. 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 the exclusion effect, which lead to the reduction in the free volume. Thus, polymer acquires a more compact conformation with intramolecular hydrophobic bonds and hinders the partition of protein into the top phase. Laccase partitioning depended on the MW of PEG (Table 1). The ratio volume between the top and bottom phase changed slightly in range of 1.00 to 1.56. The K_E values from all systems were 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. In general rule at high MW polymer, the interactions between PEG and hydrophobic enzyme decrease leading to a decrease in K_E. On the other hand, at low MW polymer is probably unsuitable since the interfacial tension between the phase decreases and as a result the polymer can attract all the desired and contaminated proteins to the same phase. In this work, the system consisted of PEG 4000 provided better results in K_E value (in range of 63.4-66.9) and percent yield than those of PEG 1000 and 6000 comparing at the same concentration. This implied that the selection of intermediate MW of PEG was appropriate for laccase extraction in this study.

The effect of PEG concentration on enzyme partitioning was also investigated. Increasing the PEG concentration enhances the hydrophobic interaction between PEG and the surface of protein. However, high PEG concentration in the system results in the increase of viscosity and the interfacial tension. Then the partition of the enzyme molecules to the top phase is more difficult [15]. In overall, the results in this study did not provide great different in extraction efficiency after increasing PEG concentration.

TABLE I
EFFECT OF PEG MOLECULAR WEIGHT AND CONCENTRATION ON LACCASE PARTITIONING

Phase concentration (%w/w)	V_R	K_E	% Yield
16%PEG1000-14%Phosphate salt	1.50	30.8	97.9
18%PEG1000-14%Phosphate salt	1.00	17.5	94.6
20%PEG1000-14%Phosphate salt	1.50	24.6	97.4
16%PEG4000-14%Phosphate salt	1.50	66.9	99.0
18%PEG4000-14%Phosphate salt	1.50	66.2	99.0
20%PEG4000-14%Phosphate salt	1.56	63.4	99.0
16%PEG6000-14%Phosphate salt	1.50	45.0	98.5
18%PEG6000-14%Phosphate salt	1.50	26.6	97.6
20%PEG6000-14%Phosphate salt	1.50	26.7	97.6

Effect of Phosphate Salt Concentration on Laccase Partitioning

The ATPS composed of 12% w/w PEG-4000 and phosphate salt concentration in range of 14-22 %w/w was selected to study the effect of phosphate salt concentration on laccase partitioning (Fig 1). The increase in potassium phosphate concentration from 14 %w/w to 16%w/w resulted in an increase in K_E. The highest K_E value (88.3) was

observed at 16%w/w phosphate concentration. Above 16%w/w phosphate salt, the K_E tended to decrease. The highest purification factor was 3.0-fold at 16%w/w phosphate salt.

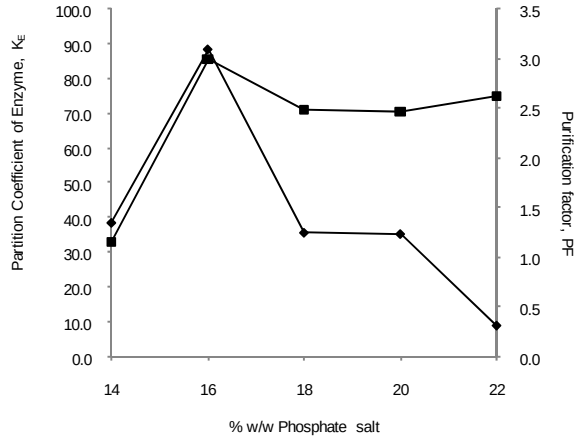

Fig. 1 Effect of salt concentration on laccase partition coefficient, K_E (♦) and %yield recovery (■)

B. Thermal Stability and Half-Life Time

The enzyme reacted with ABTS at the temperature range of 4-70 ^{0}C. The optimum temperature was at 30 ^{0}C (data not shown). The activity decreased gradually with increase in temperature up to 70 ^{0}C. The enzyme was less than 35 % active at above 60 ^{0}C.

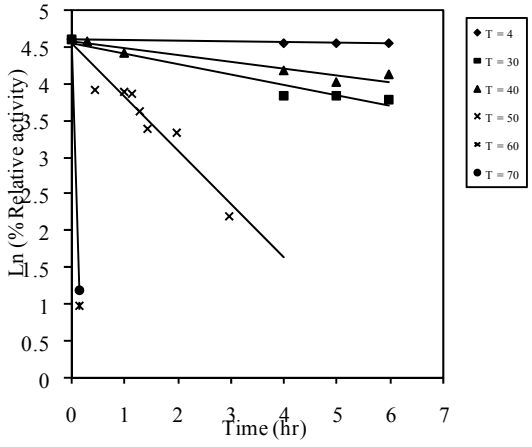

Fig. 2 Effect of different temperatures on the stability of laccase

The thermal stability is one of the most important considering for the use of the enzyme. In the present work, laccase was stable at temperatures up to 40 ^{0}C for several hours incubation. Rapid inactivation occurred above 50 ^{0}C. The natural logarithm of the residual activity of laccase was plotted against the incubation time in Fig 2. The linear behavior of these plots is assumed to follow first-order kinetics. The rate constants (k) were calculated from the slopes. The effect of temperature on half-life (time to lose half of the initial activity) was evaluated from this constant. Half-life of enzyme in the present study was determined at temperature of 4, 30, 40, 50, 60 and 70 ^{0}C and was found to be 84.5, 7.59, 4.84, 0.95, 0.03 and 0.03 hours respectively.

C. Effect of Heavy Metal on Laccase Activity

In general, dyeing effluents contain several heavy metals. The heavy metals may affect the efficiency of enzyme by complexing the substrate, by reacting with active site of laccase, or by forming with the laccase-substrate complex. The effect of Cd (II), Pb (II), Zn (II) and Cu (II) ions is presented in Fig. 3. The highest levels of enzyme activity were found in the control substrate, which had no added heavy metal. Activity levels decreased with the addition of heavy metal ranging from 0.1 to 10 mM. The results showed that it was least sensitive to copper at 0.1 mM. According to their inhibition effect over laccase the heavy metal ions at 10 mM could be ordered in the following sequence: Zn(II) > Pb(II) > Cd(II) > Cu(II). In addition to the type of metal ions, laccase activity highly depends on its source. Though copper is a component of laccases, the similar inhibition effect by copper was also observed in the laccase from *P.desmolyticum* [16] while the laccase from *T. harzianum* [17] and *G.lucidum* [18] showed opposite behaviour. Lorenzo et al [19] found at copper concentration lower than 1 mM stimulated the activity of laccase from *T. versicolor*, however, at high copper level inhibited the effect.

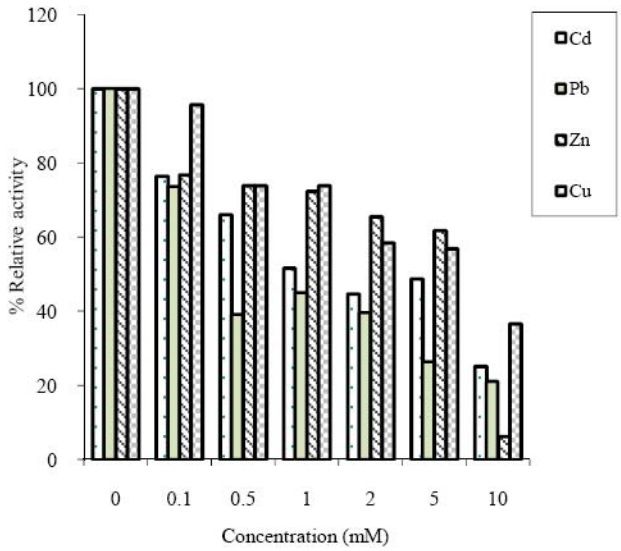

Fig. 3 Effect of different heavy metal concentrations on the activity of laccase

D.Kinetic Studies

The reaction rate of laccase on substrate was measured over a wide concentration range of ABTS at 25 ^{0}C, pH 4.5. It was found that at very low ABTS concentrations, the rate was directly proportional to the ABTS concentration. However, at high concentration the rate was independent. The relationship between reaction rate and ABTS concentration seemed to follow Michaelis-Menten model (data not shown). To evaluate the kinetic constants, the Lineweaver-Burk reciprocal plot was

considered (Fig.4). The kinetic parameters were estimated by linear regression from double-reciprocal plots. The Vmax and Km values were 74.70 µmol/min/ml and 9.066 mM respectively.

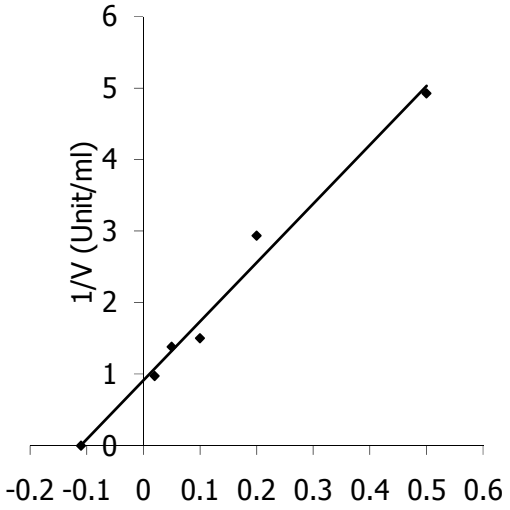

Fig. 4 Double reciprocal Lineweaver-Burk plot of laccase with ABTS

IV. Conclusion

The laccase from L. polychrous was extracted by PEG-phosphate system at pH 7.0 and 25^{0}C. The system parameters such as PEG molecular weight, PEG concentration and phosphate concentration influenced laccase partitioning. The optimum system was found at system pH 7.0 containing 12% w/w PEG4000 and 16% w/w phosphate salt with KE of 88.3, purification factor of 3.0-fold and 99.1% yield in the top phase. The laccase lost thermal stability quickly at temperature above 60 ^{0}C. Heavy metals (Cd (II), Pb (II), Zn (II) and Cu (II) ions) showed inhibitory effect on enzyme activity. The calculated Vmax and Km values according to Lineweaver-Burk plot were 74.70 µmol/min/ml and 9.066 mM respectively.

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