CHAPTER IV

RESULTS AND DISCUSSION

Before attempting the immobilization of laccase on various polymer supports it was decided the enzyme should be first purified to eliminate other proteins as much as possible. Attempts will also be made to study some properties of the enzyme.

4.1 Partial purification of laccase

Laccase from *P. sapidus* in Figure 4.1 was purified 40 folds by ammonium sulphate precipitation, ion exchange chromatography and gel filtration as outlined in Figure 3.2. Starting from 1500 grams of *P. sapidus* and 300 ml. of buffer we finally obtained 760 ml. of supernatant. This indicated that the mushroom contained a considerable amount of water. The total activity of laccase in the supernatant was 1702.4 unit. The specific activity of the enzyme was 0.374 unit/mg. protein (Table 4.1). The supernatant was brought to 80 % ammonium sulphate solution. The precipitated protein was dissolved in 245 ml. of buffer and was found to have 1519 unit of laccase with a specificity of 1.77 unit/mg.protein. The protein was dialysed 3 times against buffer and finally concentrated to 93 ml. The total activity of laccase was 1488 unit having the specific activity of 1.88 unit/mg.protein. At this stage the degree of purification was 5 folds. Further purification was achieved through the use of ion exchange chromatography.

FIGURE 4.1 : Haed Nangrom (*Pleurotus sapidus*).

TABLE 4.1 : Partial Purification of Laccase.

STEP	Total protein (mg)	Total activity (ΔOD/min)	Yield activity[1] (%)	Specific[2] activity	Degree of[3] Purification
1. Supernatant	760 ml, 4544.8 mg.	0.056/25 μl (1702.4)	100	0.374	1
2. $(NH_4)_2SO_4$ precipitation	245 ml, 857.5 mg.	0.155/25 μl (1519)	89.23	1.771	4.74
3. Dialysis	93 ml, 790.5 mg.	0.400/25 μl (1488)	87.41	1.882	5.03
4. Ion-exchange	225 ml, 157.5 mg.	0.082/25 μl (738)	43.35	4.686	12.53
5. Ultrafiltration	63 ml, 100.8 mg.	0.242/25 μl (609.84)	35.82	6.050	16.18
6. Gel filtration	43.5 ml, 23.93 mg.	0.207/25 μl (360.18)	21.15	15.05	40.24

(1) % Yield = total activity in step / total activity in first step.

(2) Specific activity = unit / mg. protein.

(3) Degree of Purification = Specific activity / original specific activity.

4.1.1 Anion exchange chromatography using DEAE Sephadex A-50

The protein solution was loaded on Sephadex A-50 and washed with 0.01 M. phosphate buffer, pH 6.0. The enzyme was eluted from the anion exchange resin by a continuous gradient starting with 0.1 M. sodium phosphate buffer, pH 6.0 to 0.5 M. sodium phosphate buffer, pH 6.0. The chromatographic profile is shown in Figure 4.2. There were numerous peaks of laccase indicating that the laccase is isoenzyme in nature. De Vries et al. [28] also reported that laccase from *Schizophyllum Commune* exhibited isoenzyme properties. It should be noted that at pH 6.0 most forms of laccase were negatively charged so that they could bind to the positively charged Sephadex A-50. The assay for laccase in the buffer used in washing the column prior to salt gradient elution did confirm the above statement since no laccase activity was detected. It is also evident from the plot of A_{280} that a fair amount of other proteins was eliminated in this purification step. The total activity in the pooled fraction was 738 unit having a specific activity of 4.69 unit/mg.protein. The enzyme was now purified 12.5 folds.

4.1.2 Gel permeation chromatography on Sephadex G-100

The pooled fractions of laccase after anion exchange chromatography was concentrated by ultrafiltration using the membrane having a molecular weight cut off 10,000 daltons. The solution was forced through the membrane by the pressure of N_2 gas. The concentrated protein of 10 ml. was applied to the column of Sephadex G-100. The elution profile is shown in Figure 4.3. Laccase was eluted between fraction 15-32 showing a

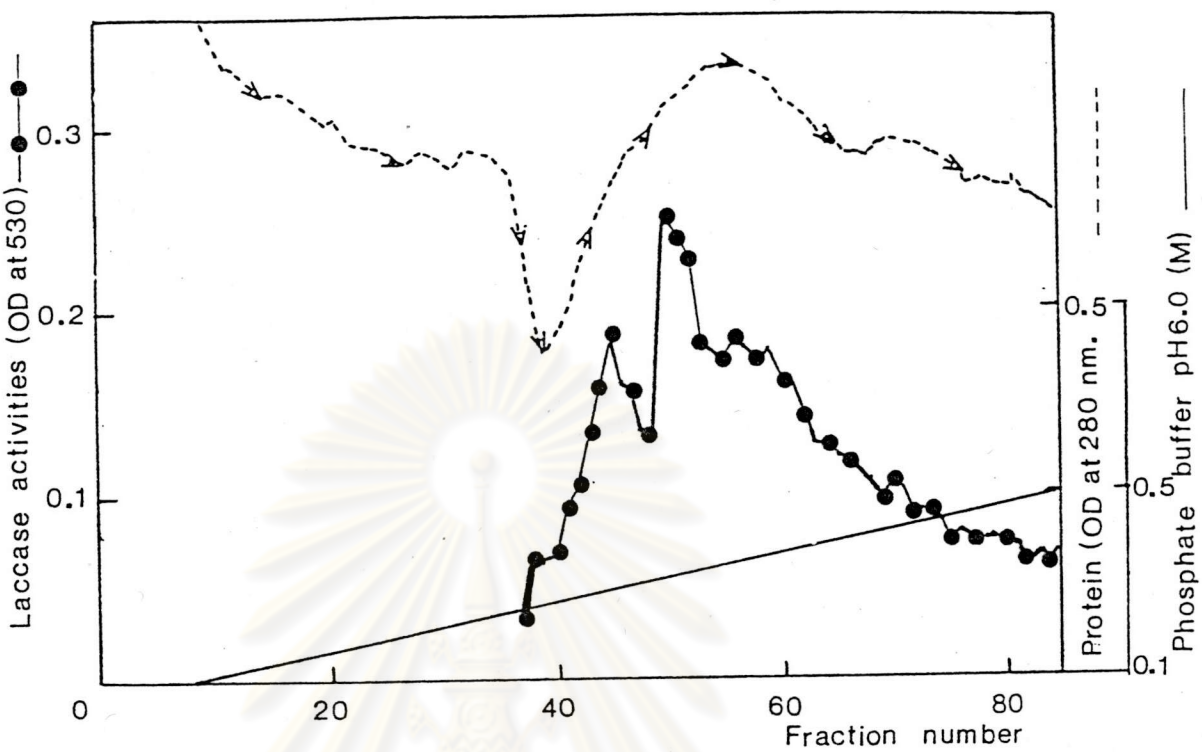


FIGURE 4.2 : Purification of laccase using an anion-exchanger chromatography DEAE Sephadex A-50. 87.5 ml. of precipitated laccase was applied to DEAE Sephadex A-50 column (2.5x40 cm) and equilibrated with 0.01 M. sodium phosphate buffer pH 6.0 at 4°C.

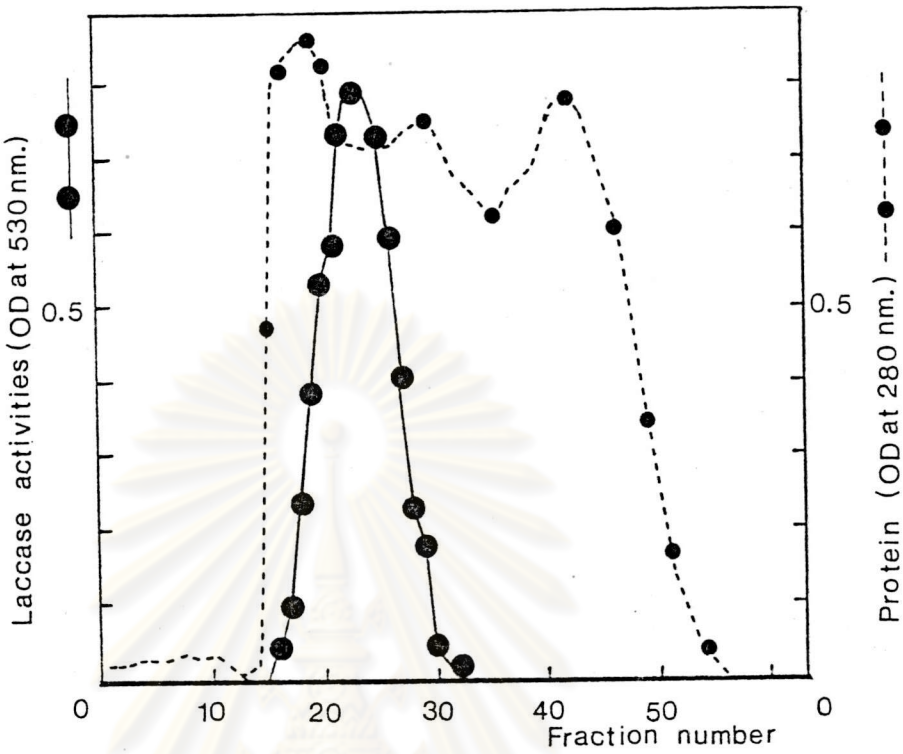


FIGURE 4.3 : Purification of laccase using Sephadex G-100 chromatography.

10 ml. of laccase from ultrafiltration was applied to Sephadex G-100 column (1.6 x100 cm) and equilibrated with 0.01 M. sodium phosphate buffer pH 6.0 at 4°C.

single peak. A large amount of other proteins were also eliminated from the enzyme as evident in the plot of A_{280} nm. in Figure 4.3. This brought the specific activity of laccase up to 15 unit/mg.protein. The enzyme was purified by 40 folds at this stage. The total activity remained was 360.2 unit.

The fractions containing laccase were pooled and subjected to lyophilization until the dried-cooled flakes appeared. The freeze-dried laccase was stored in this form for later studies.

4.1.3 Polyacrylamide gel electrophoresis of laccase

Polyacrylamide gel electrophoresis (PAGE) was carried out on supernatant of crude laccase preparation as well as partially purified laccase. The results are shown in Figure 4.4. As evident from the Commasie Brillant Blue stain that the number of proteins bands in partially purified preparation (a/2) are fewer than the number of protein bands in the crude preparation (a/1). This indicated that the purification steps did indeed remove some other proteins from laccase.

The zymogram of laccase obtained from the activity stain is shown in Figure 4.4 b. Four distinct bands could be observed in both the crude laccase preparation (b/1) as well as the partially purified laccase (b/2). These bands were designated, starting from the cathode, as 1,2,3 and 4, respectively. It should be noted that band no.3 is rather broad indicating that this band might also include a number of additional isoenzymes of laccase within it. A more refine technique such as isoelectric focusing would be needed to resolve this problem. All of the bands in the crude enzyme preparation were fainter than the partially purified laccase. This was likely due to the fact that the

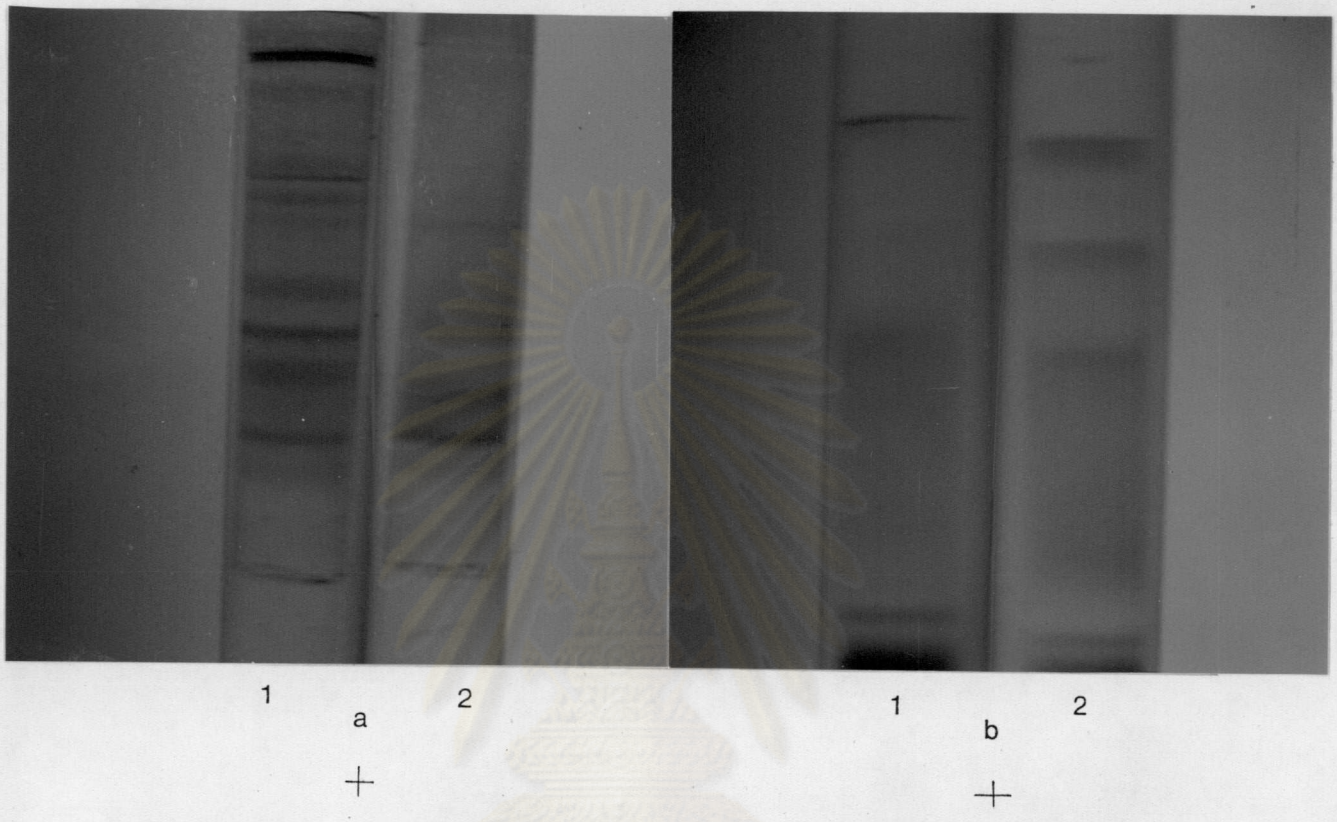


FIGURE 4.4 : Polyacrylamide gel electrophoresis of laccase.

a. Commassie Brilliant Blue stain to see all protein bands in the sample. The protein concentration must be at least 15 μg to be confortably detected.

b. Activity stain detecting only laccase using syringaldazine as the substrate. The method is specific for laccase and can detect the enzyme presented at very low concentration.

1, Crude supernatant of laccase

2, Partially purified laccase

Bromophenol Blue was used as a marker.

concentration of laccase in the partially purified preparation was more concentrate. The intensity of all bands is, therefore, higher. It should be emphasized that band no.2 of the crude preparation did exist eventhough it appeared as a faint band and hardly visible on the photograph (Figure 4.4 b/1). The results obtained from PAGE as shown in Figure 4.4 b confirmed our previous result obtained during the purification using anion exchange chromatography (Figure 4.2) that laccase did consist of several isoenzymes. And the exact number of isoenzymes could be known only if a more refine technique such as the isoelectric focusing be used.

4.2 Spectral properties of partially purified laccase and commercially available Pyricularia oryzae laccase.

To assure ourselves that we were indeed working with laccase we carried out the wavelength scan of the enzyme in the visible region. As mentioned in section 1.2.1 laccase is a copper enzyme that absorbed light at around 600 nm. The result of absorption maximum curve in Figure 4.5 A of partially purified laccase from *P. sapidus* did show a maximum absorption around 600 nm. Furthermore, the scanning of "standard" *P. oryzae* also gave the same pattern of absorption (Figure 4.5 B). Our agreement with that of Bligny & Douce [24], who showed that the native laccase of sycamore cell exhibited a common peak around 600 nm. These data indicated that the partially purified laccase from *P. sapidus* is the blue oxidase consisting of the copper atom complex to the protein molecule.

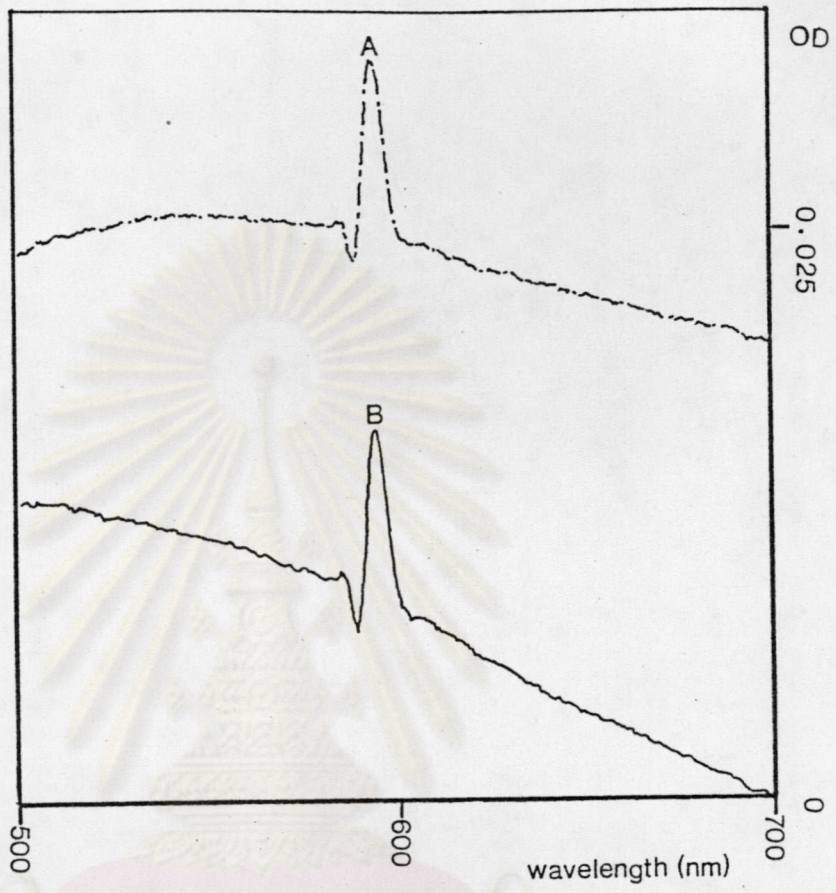


FIGURE 4.5: Scanning spectrum of the partially purified laccase (*P. sapidus*) and commercially prepared enzyme (*Pyricularia oryzae*). The latter enzyme was used as a "Standard laccase" in our study. The wavelength scans were carried out from 500-700 nm using the sequential scanning mode. The followings parameters were set O.D. range 0-0.05, scan speed 20 nm/sec.

Spectrum A : the partially purified *P. sapidus* laccase.

Spectrum B : the "Standard" *P. sapidus* laccase.

4.3 Optimum pH for partially purified laccase

The relationship between the activity of laccase and pH of incubation mixtures is shown in Figure 4.6. The enzyme exhibited no activity at pH 3.0 while the activities gradually increased from pH 4 onwards and reached a maximum value at pH 6.0. Above this value the enzyme activities decreased to about 20 % at pH 7 and 8. These results indicated that the optimum pH for laccase is around 6.0.

4.4 Effect of temperature on partially purified laccase

If the enzyme is to have any practical application it has to be fairly stable at relatively high temperatures. We, therefore, set out to determine the relationship between laccase activities after the enzyme was incubated at various temperatures for 2 hours. The result is shown in Figure 4.7. As expected the enzyme is most stable at the lowest temperature tested, i.e. 4°C. At higher temperatures ranging from 10-45°C more than 75 % of the activities were retained. The enzyme activities drastically decreased at of 65°C having only 3.88 % of the activity. The activities at 80°C and 100°C were 1.79 and 1.19 %, respectively. It was also reported in the process of the lacquer drying that if the temperature used was more than 60°C there would be a total loss of laccase activity [20].

It can be seen that laccase was relatively stable as evident that at 45°C more than 75 % of the enzyme activity was retained. This observation is not totally all surprising since it is a common feature of enzyme or hormone to be fairly stable if they are glycoprotein in nature. Mayer [8], Nakamura [10], Kumanotani [11], Bligny and Douce [14] all reported that laccase, either from

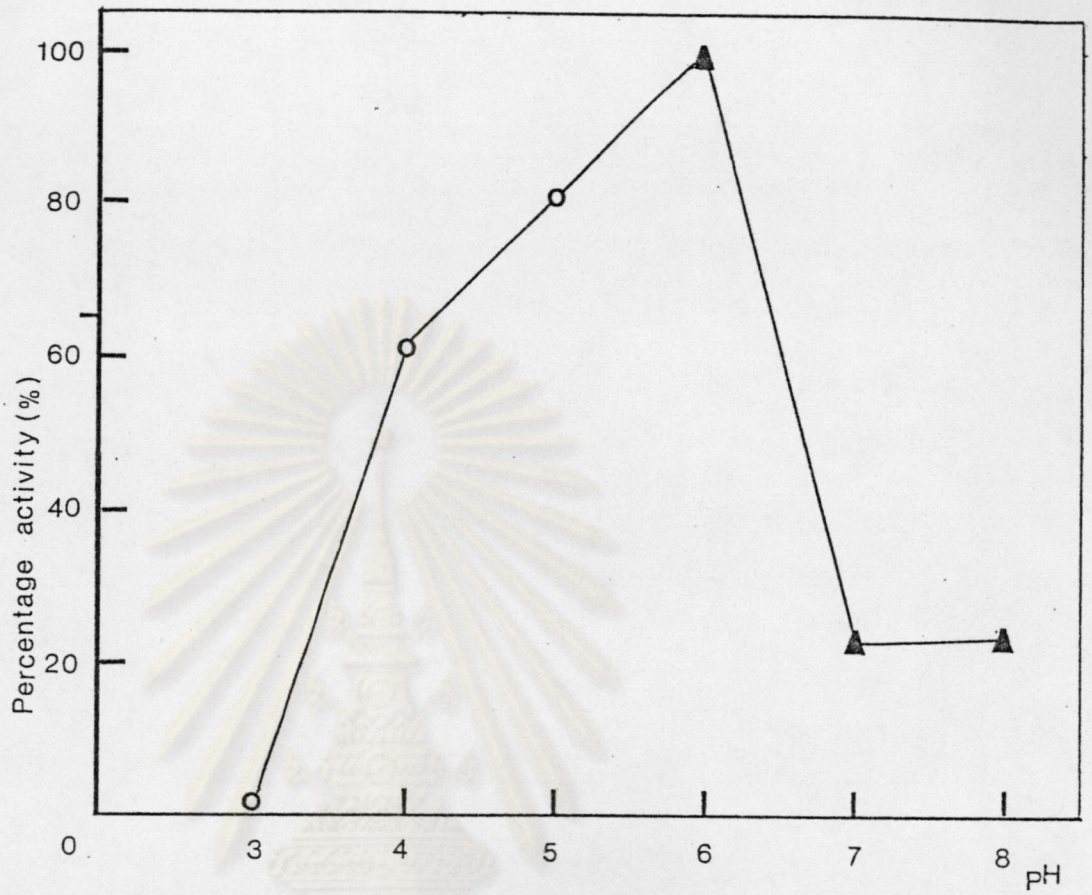


FIGURE 4.6: Effect of pH on reaction of Syringaldazine by partially purified laccase. The percentage of relative enzyme activity was plotted against various pH. The enzyme activities were determined 100 % : 0.462 ΔA_{530}/min.

O—O—O 0.01 M. Acetate buffer.

▲—▲—▲ 0.01 M. Phosphate buffer.

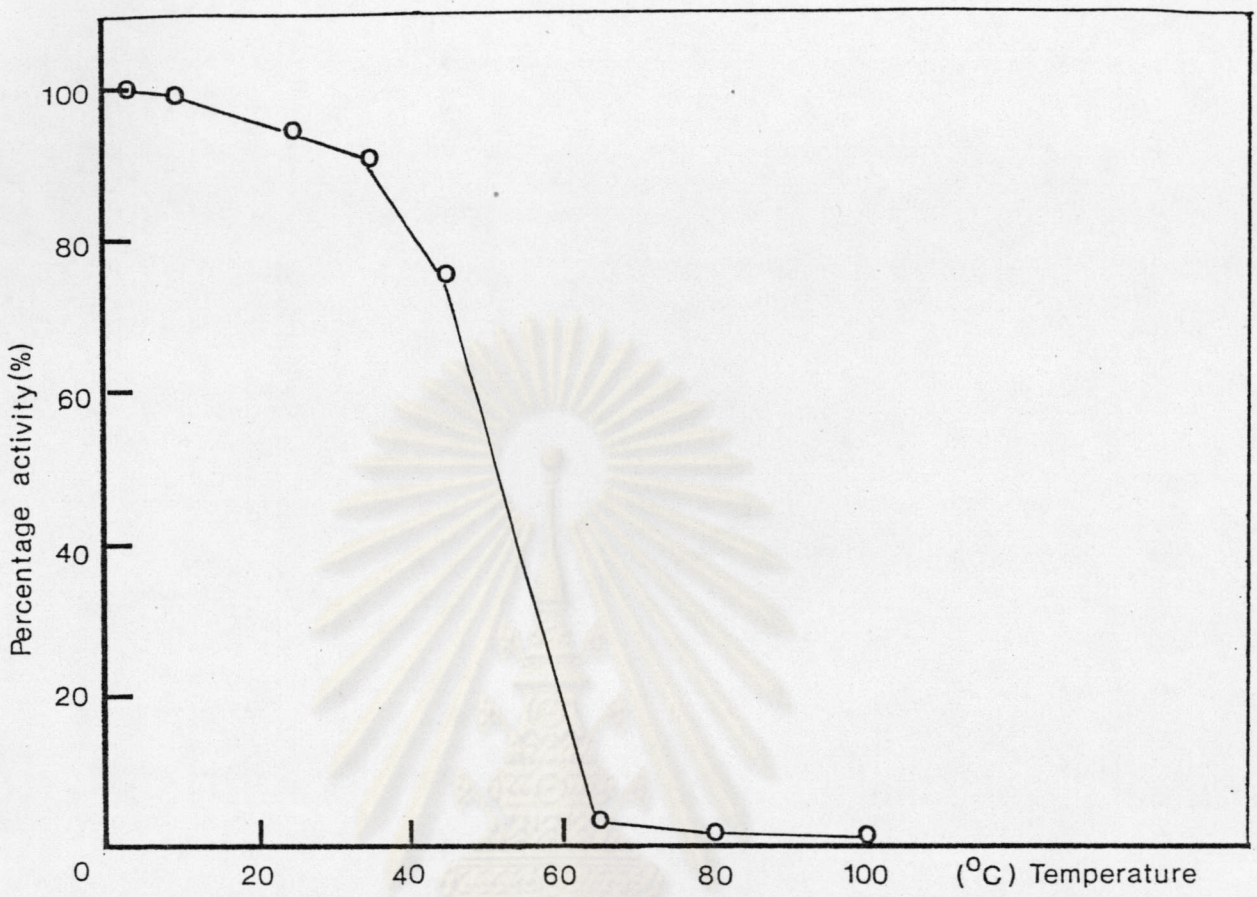


FIGURE 4.7: Effect of temperature on reaction of Syringaldazine by partially purified laccase (Lyophilized laccase).
The percentage of relative enzyme activity was plotted against the various temperatures. The enzyme activities were determined 100 % : 0.352 ΔA_{530}/min.

fungi or Rhus tree did contain a large percentage of carbohydrate attached to the polypeptide. It is a general properties of extracellular enzyme from plants and microorganisms to be stable at a temperature above ambient. This is essential because these enzymes have to function in environments different from inside the cells. One of the obvious function of laccase from *P. sapidus* is to degrade lignin [44,45] so that the mushroom can extract more nutrient from the wood such as using extracellular cellulase to extract glucose from cellulose. The subject of lignin biodegradation was throughly reviewed by Ishihara [26].

Laccases from *Rhus vernicifera* or *Melanorrhoea usitata* also function outside the plant cells. But in this case to oxidise and polymerize phenol derivatives to form lacquer cover the cut or injuries on the plants. Here again we expect the enzyme to be fairy stable. This finding is an assurance that if one tempt to use laccase for various applications. The enzyme will be in service for relatively long period of time.

4.5 Reaction of laccase on catechol and hydroquinone

In addition to syringaldazine which was a substrate used in this assay. The laccase could also oxidised other substrates such as catechol and hydroquinone as shown as in Figure 4.8-4.9.

In Figure 4.8 catechol was oxidised to its respective quinone with a characteristic maximum absorption at 400 nm. Kumanotanic [11] also observed this product when he used Rhus laccase in his study of catechol oxidation. As the time of reaction proceeded from 1 min to 17 min the spectra showed gradual increases in the heights

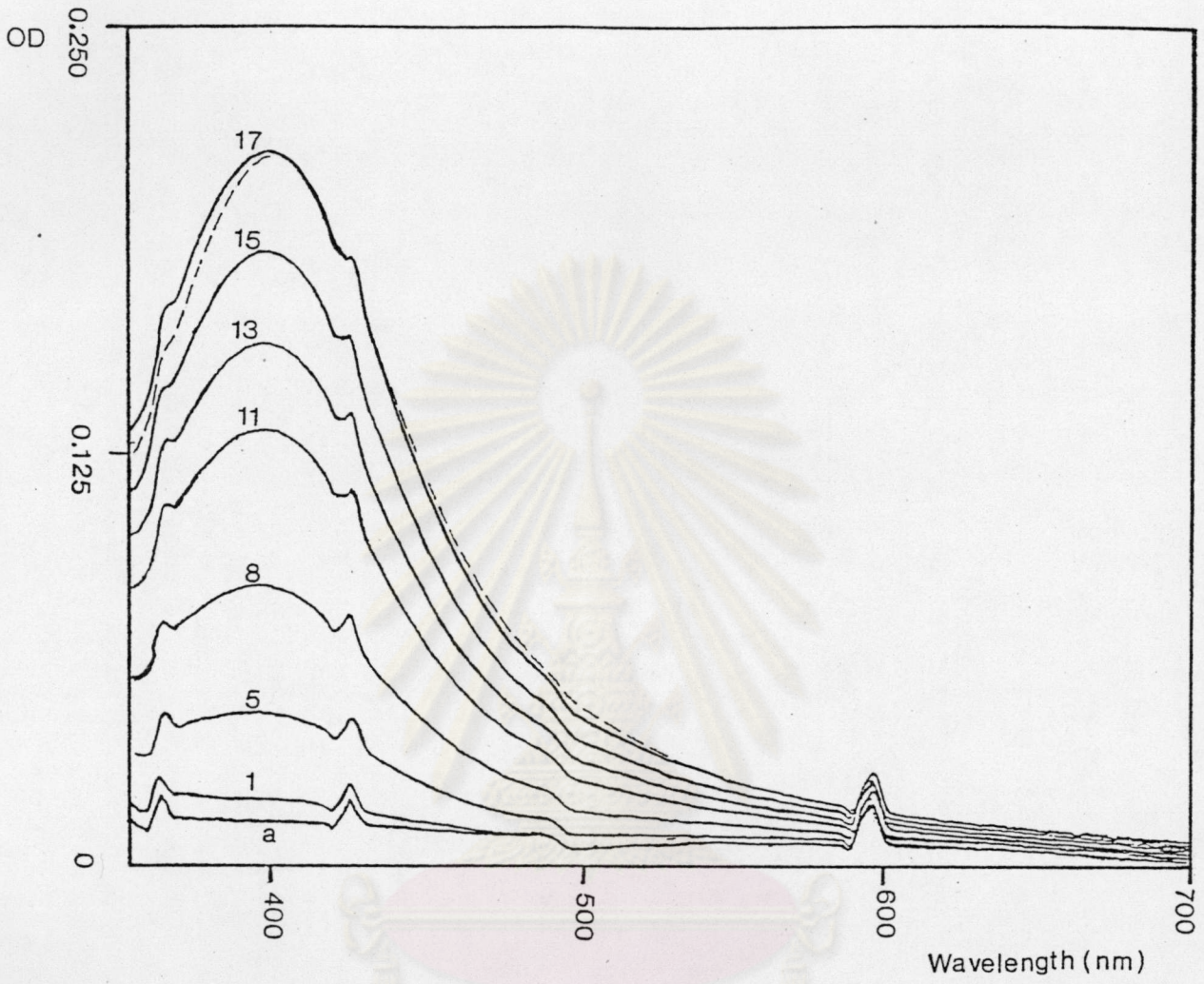


FIGURE 4.8 : Oxidation of catechol by partially purified laccase.

The reaction mixture consisted of 1 mM. catechol in 0.01 M. phosphate buffer, pH 6.0 and 100 μl lyophilized laccase in a total volume of 3.5 ml. and was incubated at 25°C. Spectral scans were carried out from 350-700 nm. after the reaction was allowed to proceed for 1,5,8,11,13,15 and 17 min, respectively. Spectrum a obtained immediately after the laccase was mixed with catechol.

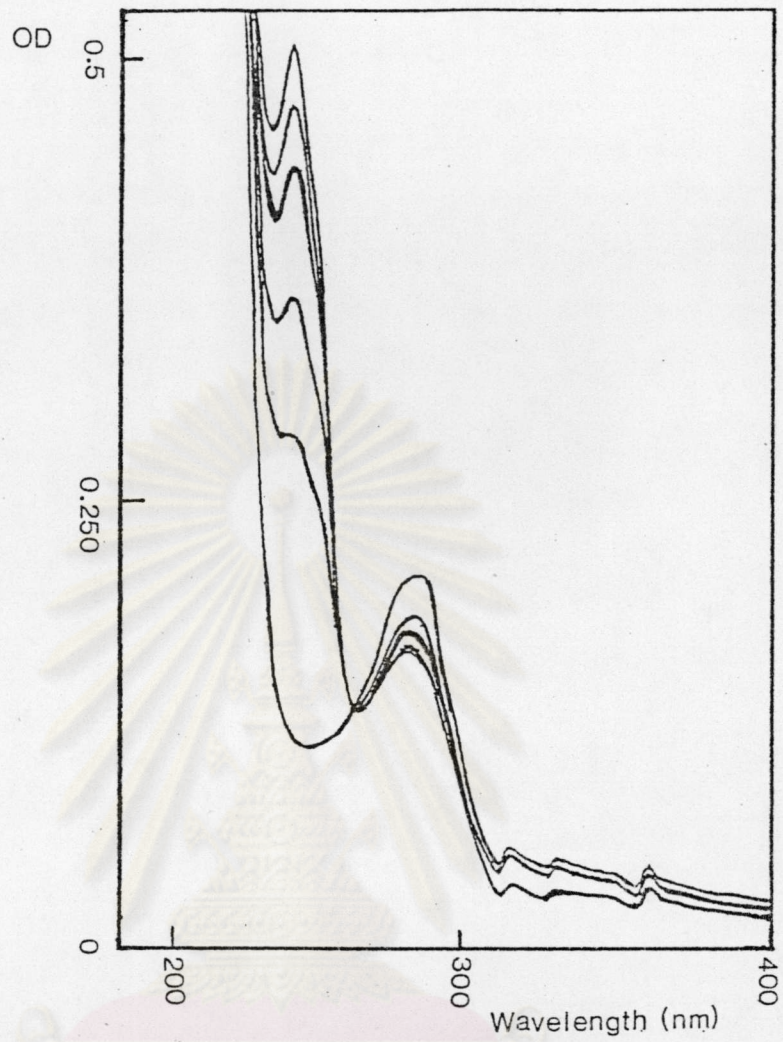


FIGURE 4.9 : Oxidation of hydroquinone by partially purified laccase. The reaction mixture consisted of 0.001 mM. hydroquinone in 0.01 M. phosphate buffer, pH 6.0 and 0.5 ml. lyophilized laccase in a total volume of 3.5 ml. and was incubated at 25°C. Spectral scans were carried out from 190-400 nm after the reaction was allowed to proceed for 20,30,40,50 min, respectively. Spectrum a was obtained immediately after laccase was mixed with hydroquinone.

of 400 nm peaks.

The oxidation of hydroquinone as shown in Figure 4.9 resulted is a product with a maximum absorption at 245 nm. In this study the hydroquinone substrate showed a maximum absorption at 285 nm. As the reaction proceeded we could observed reduction of the hydroquinone peak with a concomittant increases in the product peak. Kumanotani [11] reported the suggestion by Yoshida [12] that the machanism of this reaction occured as the followings : (i) There was a formation of semiquinone as an electron was transfered from hydroquinone to the copper atom of laccase, converting Cu^{2+} of laccase to Cu^{1+}. (ii) The Cu^{1+} of laccase was converted back to Cu^{2+} by reacting with endogenous oxygen.

The ability of *P. sapidus* to oxidise both catechol and hydroquinone was in agreement with the definition of laccase as reviewed by Mayer [8] and Mayer and Hartel [9].

4.6 Immobilization of laccase

At this stage laccase was partially purified and kept as freeze-dried powder. The enzyme was then ready to be used in the experiments involved immobilization on synthetic polymer supports. It was decided that two methods of immobilization should be attempted. These were (i) the carrier-binding using a spacer to form covalent bonds between a polymer support and the enzyme molecule, and (ii) the entrapment by mixing the enzyme with monomers and allowed to polymerize. The enzyme would then be entrapped within the network of polymer.

4.6.1 Immobilization of laccase by covalent binding to Amberite IRA-68 using epichrolohydrin as the linking agent.

Amberite IRA-68 is an anion exchanger containing an amine as the functional group to perform the anion exchanging function. These beads of polymer were polymerization product of acrylic monomer and the cross-linking reagent, the divinylbenzene. The amine group was later added to render the anion exchanging ability to the polymer. The polymer consisted of a large amount of carboxyl groups. This availability of COOH's make it possible to link the epichlorohydrin to the polymer according to the mechanism presented in Figure 4.10. The enzyme was then linked to epichlorohydrin via another side of the molecule. The appearance of beads of the Amberite before and after the process of immobilization is shown in Figure 4.10.

(i) Time course of reaction of immobilized laccase

After the immobilization to Amberite IRA-68 the immobilized enzyme was washed thoroughly to get rid of enzyme that might had been associated with the Amberite by other forces. The immobilized laccase was tested for its ability to oxidised syringaldazine at various times of incubation. As shown in Figure 4.12 the oxidation of the substrate was linear upto about 40 min then gradually curved off. It was decided that this 40 minutes-incubation be used in subsequent experiments.

(ii) Stability of immobilized laccase

For practical purpose it is important to know how stable is the enzyme after immobilization. We test this by using the batch system and the

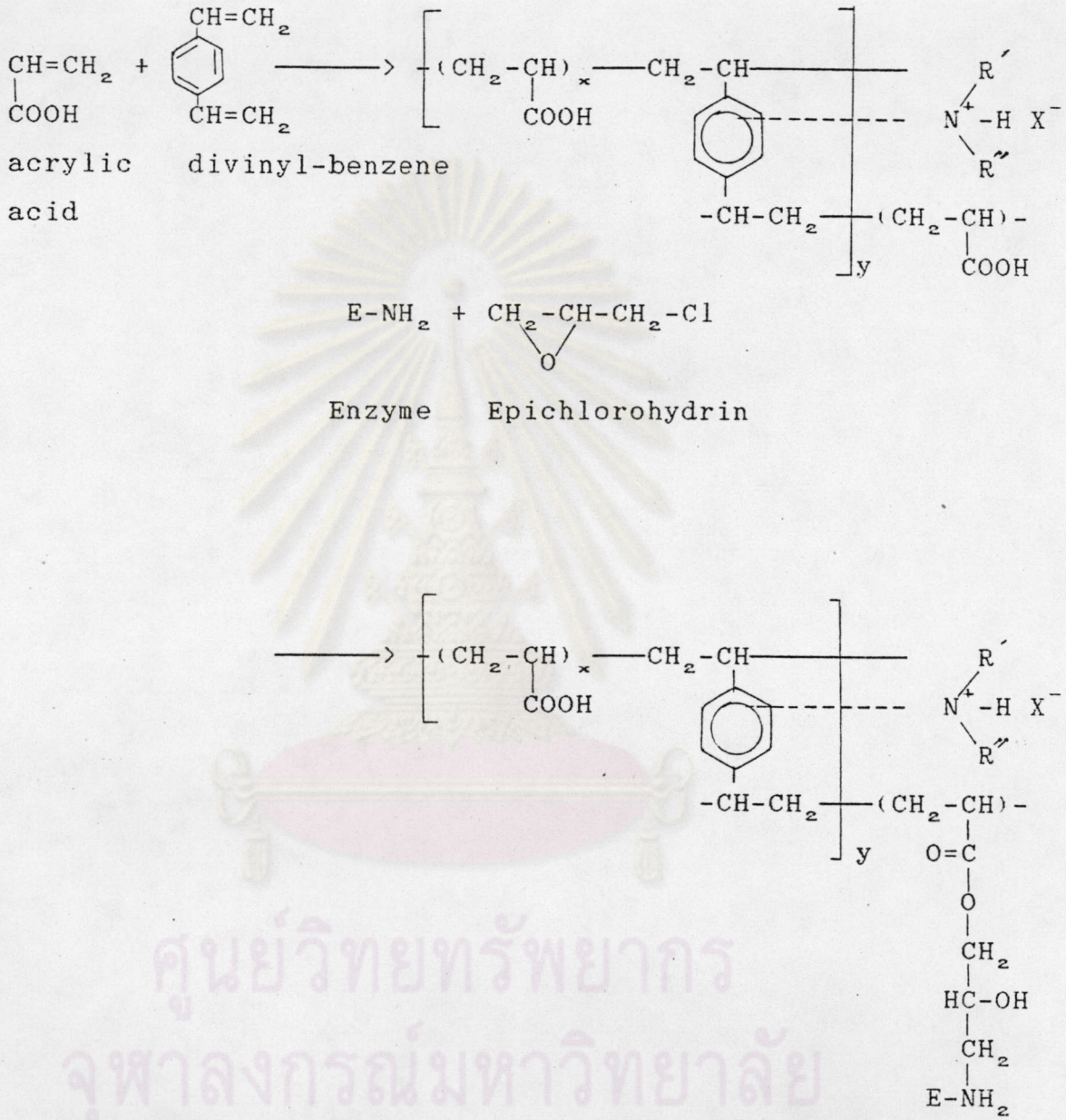
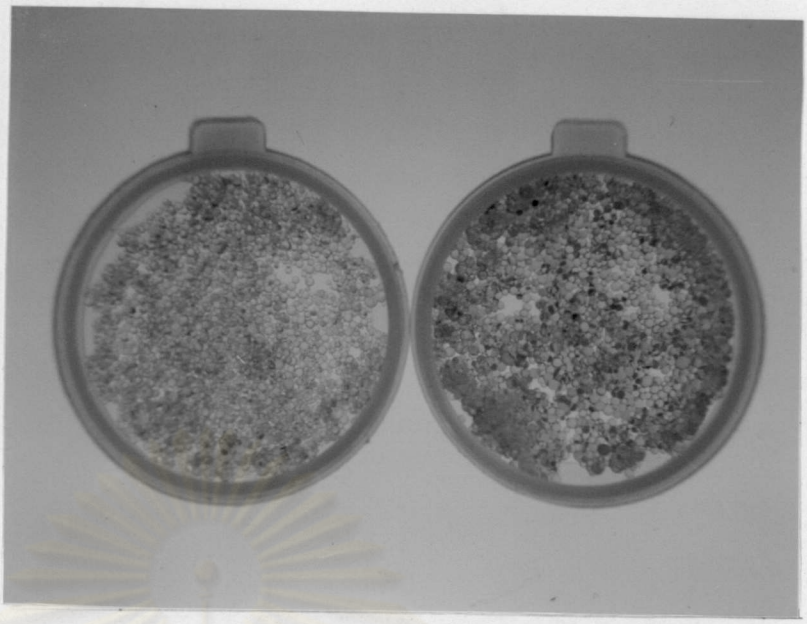


FIGURE 4.10: Immobilize of laccase to Amberite IRA-68 via the cross-linking agent, epichlorohydrin.



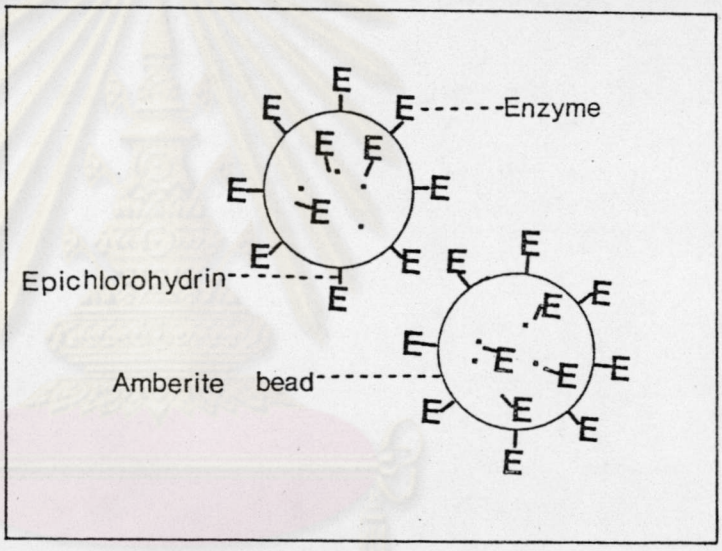


FIGURE 4.11: Immobilization of laccase to Amberite IRA-68 using epichlorohydrin as cross-linking agent.

(a) The appearance of Amberite before (left) and after (right) the immobilization.

(b) Schematic drawing depicts the attachment laccase (E) via epichlorohydrin (-) to the individual resin bead (o).

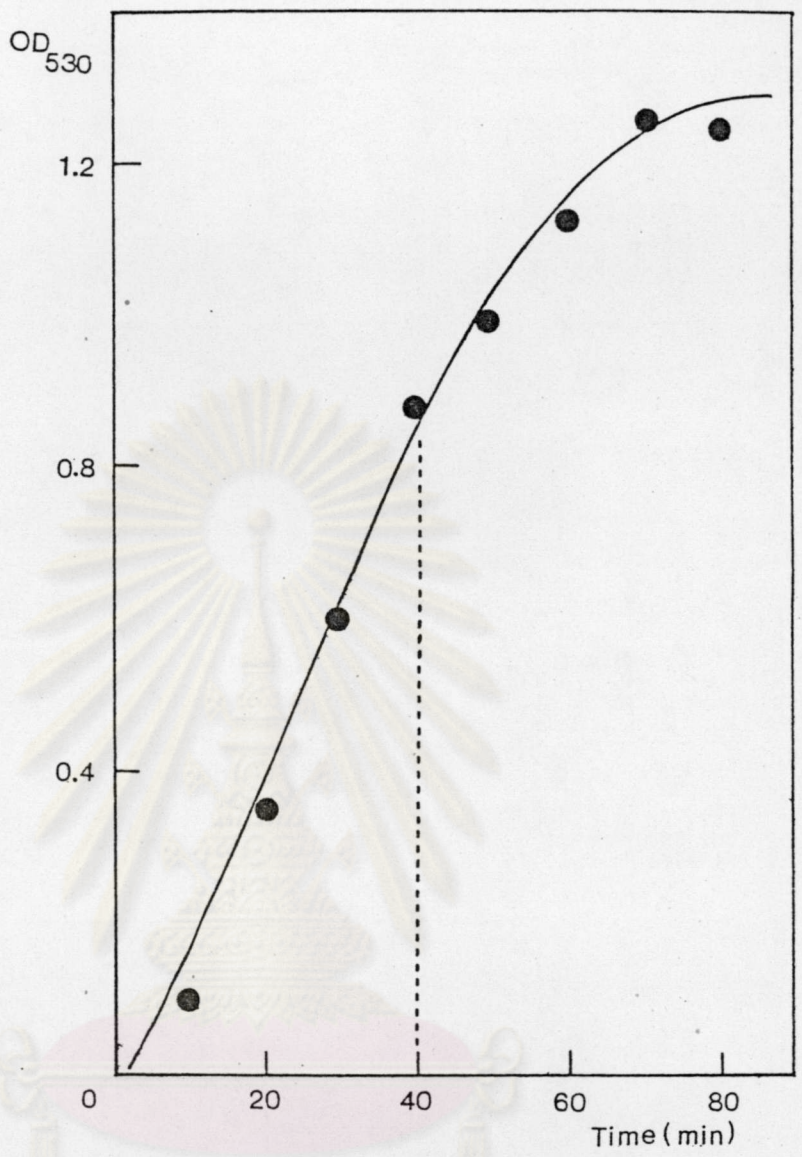


FIGURE 4.12: Time course reaction of immobilized laccase. Each incubation flask consisted of 0.2 gm of laccase-containing resin and 0.1 mM of syringaldazine in 5 ml. of 0.01 M phosphate buffer, pH 6.0. Each flask was incubated at 37°C for different periods of time. The product was measured at 530 nm. Each point was an average from 3 experiments.

continuous system. The results are shown in Figure 4.13 and Figure 4.14, respectively. The result of batch test in Figure 4.13 showed that the activity of immobilized laccase was the highest at the first-time use. The activity then gradually decreased afterwards and was barely detectable at the 8th-time use.

We also tested the stability of immobilized laccase in the continuous system by packing the laccase-containing resin in a glass column. The overall set up is shown in Figure 4.14. The result was shown in Figure 4.15. Starting from the first appearance of the coloured products the system took about 30 min to reach its maximum capacity. The product formation was slowly decreased at first followed by a more rapid decrease afterwards. At the end of the experiment after 11 hours of continuous operation the activity of the immobilized laccase was still declining.

One might argue that the rapid declined of activity indicated that the enzyme did not attach to the Amberite IRA-68 via the covalent linkage but other noncovalent forces. We believe that above explanation was most unlikely since the immobilized laccase was washed exhaustively with salts and buffer solution. This practice is commonly used in other experiments involved covalently-immobilized enzymes [46].

4.6.2 Immobilization of laccase by entrapment within the polyacrylamide gel.

We managed to produce polyacrylamide beads of about 0.5 mm having laccase entrapped within the polymer. The entrapped laccase was tested for its stability first by the batch system. The result was shown in Figure 4.16. The activity of the enzyme was the highest

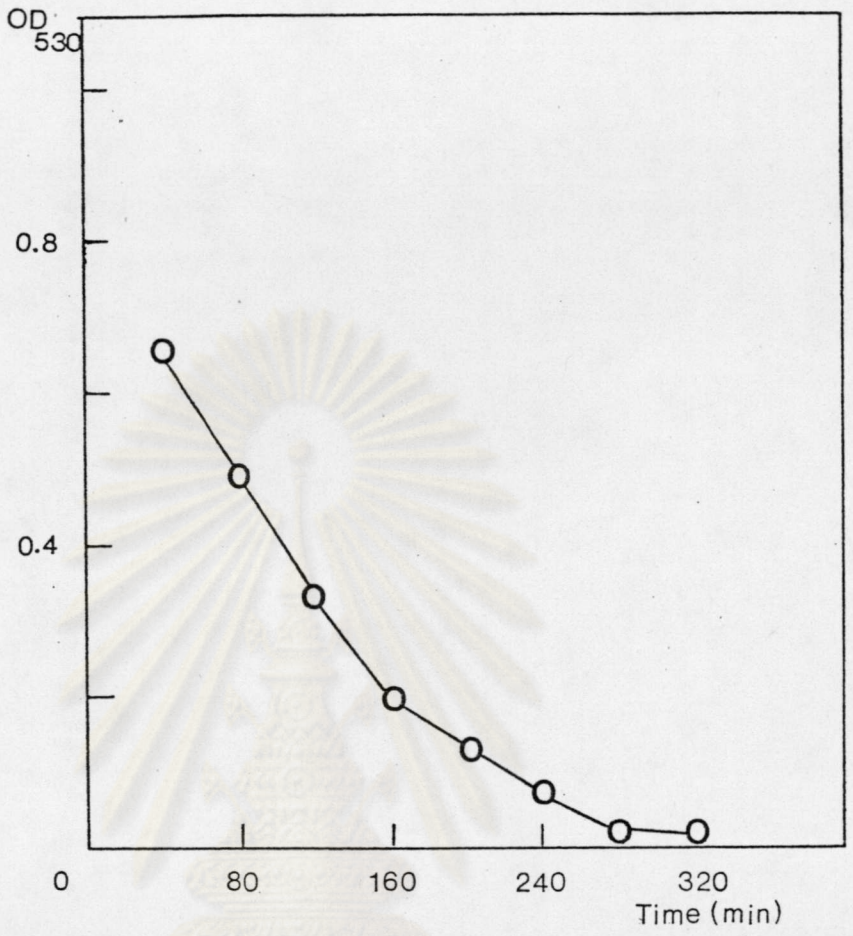


FIGURE 4.13: Stability of covalently immobilized laccase after testing by the batch system. The incubation consisted of 0.02 gm laccase-containing resin and 0.1 mM of syringaldazine in 5 ml 0.01 M phosphate buffer, pH 6.0. The mixture was incubated at 37°C for 40 min. The liquid was taken off the resin and absorption measured at 530 nm. The resin was again incubated with the substrate 9 times and the product formed measured at the end of each incubation.

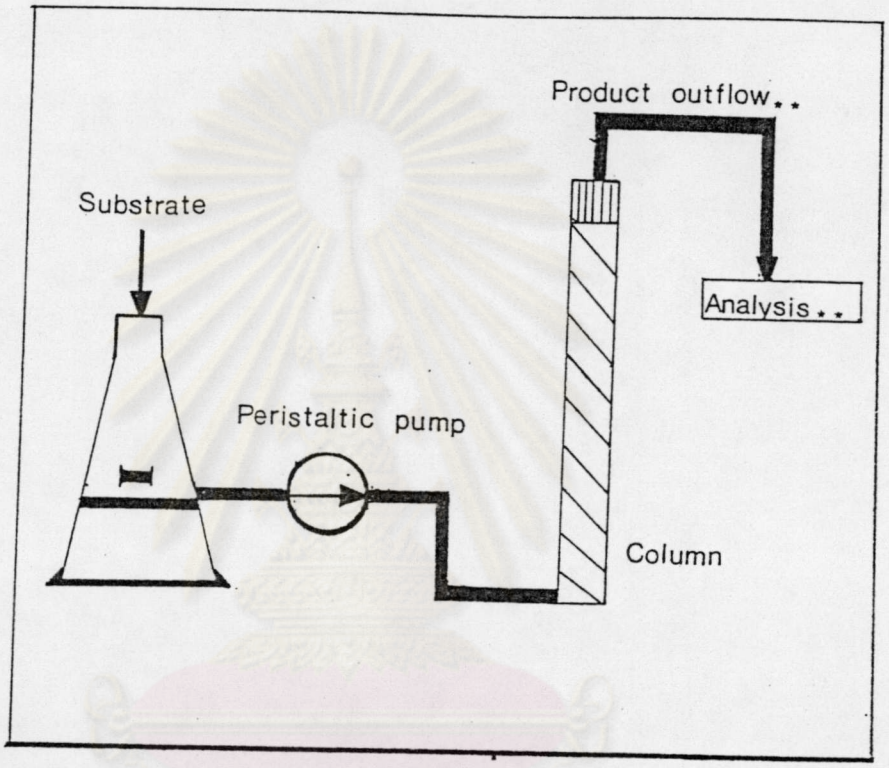


FIGURE 4.14: Diagram of continuous system.

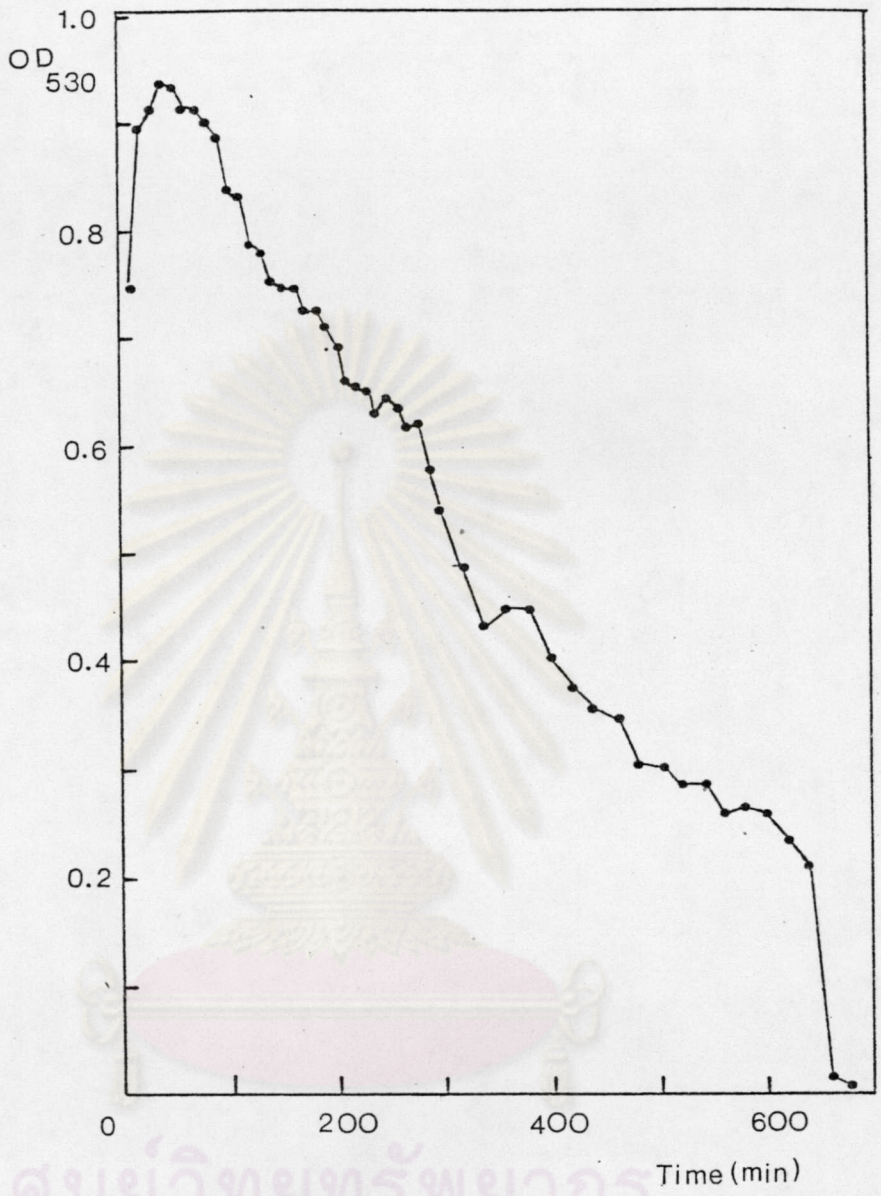


FIGURE 4.15: Stability of covalently-immobilized laccase testing by the continuous system. 7 gm. of laccase-containing resin was packed in a column of 1x10 cm. The substrate was forced through the bottom of the product emerged from the top was collected every 10 min and measured at 530 nm.

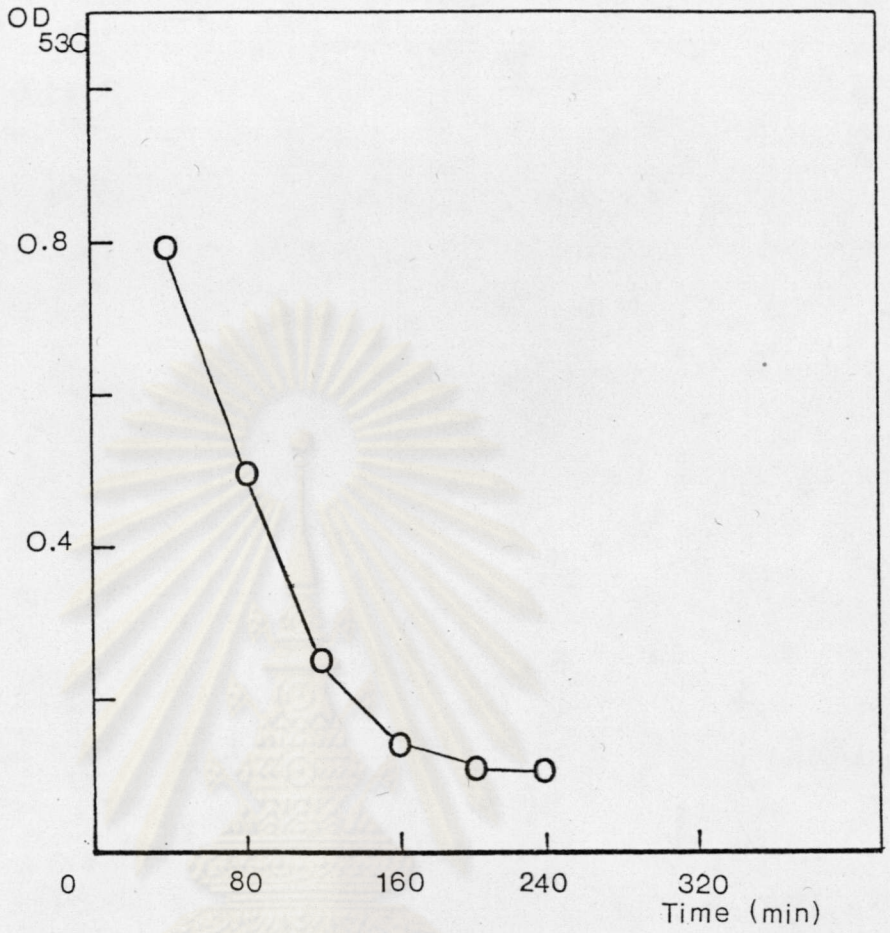


FIGURE 4.16: Stability of entrapped laccase after testing by batch system. The incubation consisted of 0.2 gm laccase containing bead and 0.1 mM of syringaldazine in 5 ml 0.01 M phosphate buffer, pH 6.0. The mixture was incubated at 37°C for 40 min. The liquid was taken off the bead and absorption measured at 530 nm. The bead was again incubated with the substrate 6 times and the product formed measured at the end of each incubation.

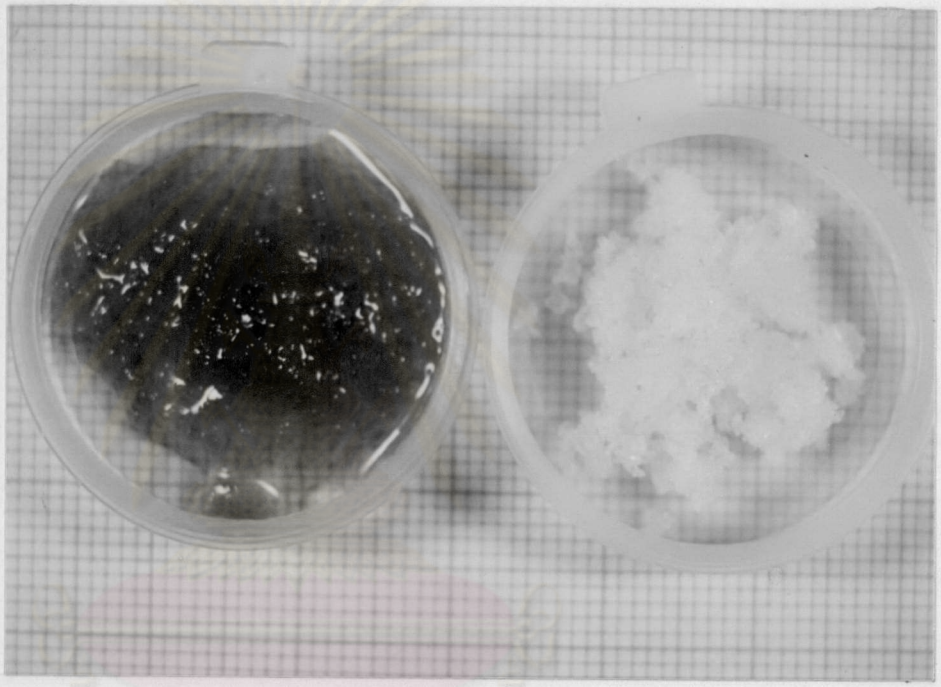


FIGURE 4.17: Immobilization of laccase to polyacrylamide gel using entrapment method.

The appearance of entrapped laccase (right) before reaction with syringaldazine and after reaction with syringaldazine.

at the initial use. The enzyme activity again showed a downwards turn having the lowest activity at the 8th-time use. The result was similar to the one observed in the case of covalently immobilized enzyme (Figure 4.13).

It is interesting to note that in this case of laccase entrapped within the polyacrylamide gel. The gel after incubation with the substrate appeared red pink (Figure 4.17). It was quite difficult to wash off the coloured product indicating that the syringaldazine entered deep inside. The gel and when the substrate met with the entrapped laccase the reaction occured mostly inside. Incontrast to this observation the covalently immiobilized laccase-Amberite system (Figure 4.11) would have laccase covalently attached to the outer surface of the besd of Amberite. The reaction occured mostly on the surface where the substrate encounter the enzyme. Also the pore size of the Amberite would be too small to allow the coloured product to go inside the bead of Amberite. We, therefore, never encountered the red-pink bead. The product appeared outside the bead and was easily washed off the resin.

The stability test in the continuous system again showed a time-lapsed of 20 min for the system to reach a full production. The activity then decreased rather rapidly compare to the covalently immobilized enzyme (Figure 4.18). The immobilized enzyme in polyacrylamide gel bead had very little activity only after 9-10 hours of use.

We had tried two methods for the immobilization of laccase, namely, (i) the covalent binding to Amberite resin and (ii) the entrapment within the polyacrylamide gel beads. We succeeded in the

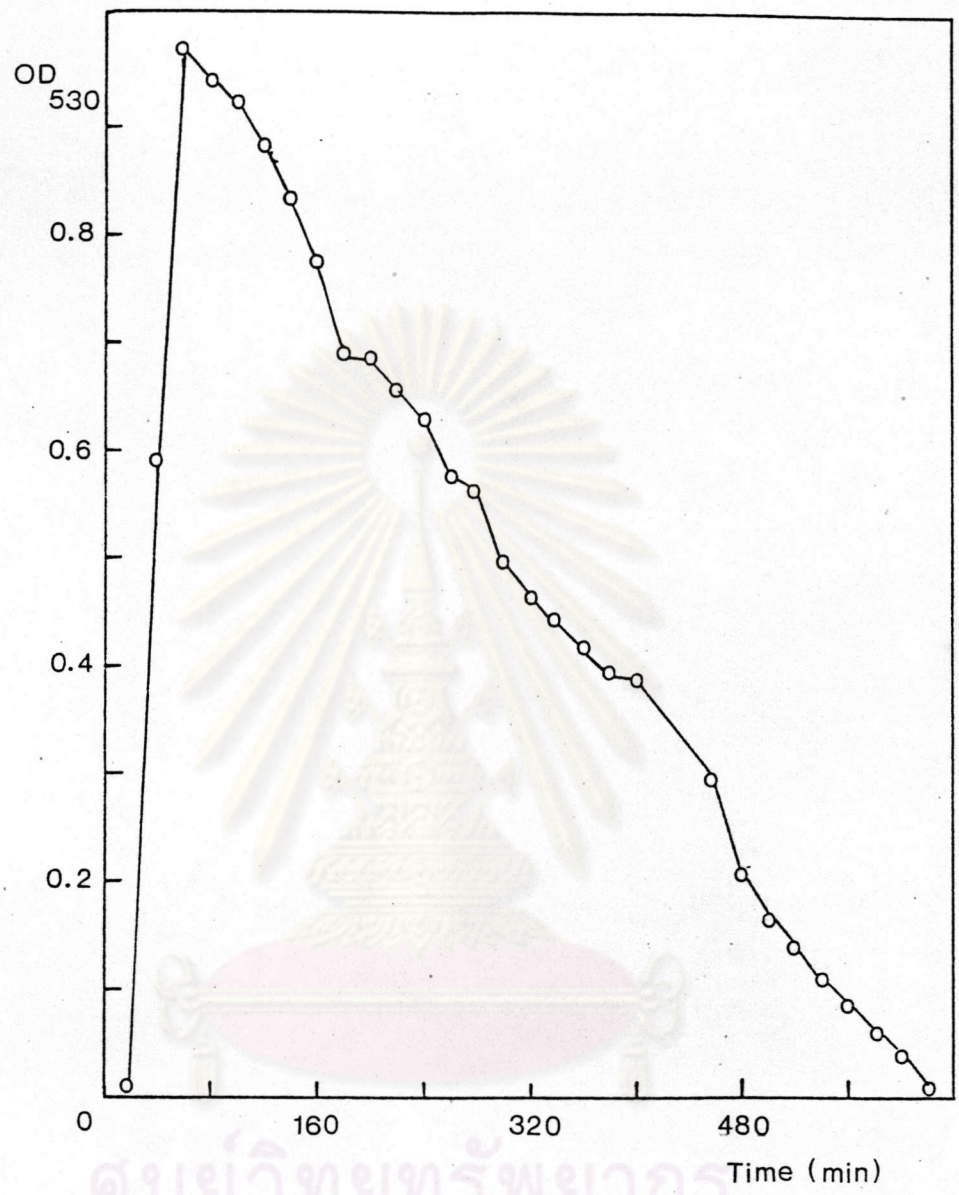


FIGURE 4.18: Stability of entrapped laccase testing by the continuous system. 0.725 gm of laccase-containing resin was packed in a column of 1x100 cm. The substrate was forced through the bottom of the column and 5 ml of the product emerged from the top was collected every 20 min and measured at 530 nm.

immobilization using both methods. However, the stability test showed that the immobilized enzyme was stable for only limited period of time (Figure 4.12,4.13,4.15 and 4.17). The loss in activities after repeated use could well be explained in the terms of the loss in catalytic power of the enzyme. It should be emphasized that the immobilization, especially, the covalent binding does affect conformation of enzymes to a certain degree [47]. The immobilization of laccase in this case might have put strain on the native three dimensional structure of the enzyme. The enzyme could function well at the initial stage of use but the strain imposed on it was getting worse that curtain molecules of the enzyme came to a "breaking point" resulted in the inability to catalyse the reaction. More molecules of the enzyme gradually reached this "breaking point" after a prolonged use. The overall effect was, therefore, a gradual decrease in the catalytic capability of the immobilized laccase.

4.7 Potential applications of laccase

At the completion of experiments involving the partial purification of laccase and its immobilization we could think of two potential application of this study. One is the used of laccase in a soluble form in the process of lacquer drying. Sripapan [20] in fact suggested that a catalyst should be found and added to the Yang Rak to enhance its drying. It was mentioned earlier that Yang Rak from Thailand and Burma usually obtained from *M. usitata* contained low amount of laccase [20]. The obvious solution to this problem is, therefore, to use laccase from other, presumably cheaper, source to help reducing the time needed for drying lacquer. We would like to suggest that

Pleurotus sapidus should be a source of choice for laccase since we can cultivate the species in large quantity in a short period of time. The laccase can, therefore, be produced as much as needed. Also, on the application point of view we do not need a highly purified enzyme. In fact one may use laccase after its precipitation from the crude extract. We have done a preliminary work on this but did not get sufficient results to report at this stage. The reasons are mainly due to the time limitation and the availability of "genuine" raw latex from *M. usitata*. However, we believe the potential is promising in this regard.

Another potential application of laccase lies on its application in waste treatment. The laccase could be used economically in its immobilized form for its allow the use of enzyme more than once. A number of reports already appeared suggesting the use of laccase in treatment of water containing phenols, amines and lignin [48]. The use of laccase would be more economical than the use of peroxidase since the first does not require the use of hydrogen peroxide [49,50]. However, one should do more experiments on the immobilization of laccase to find the method that could preserve laccase activities longer than the present study. Also, to be able to use immobilized laccase in a "rough" conditions such as waste treatment one should look for supports that are mechanically strong. Other supports do exist, for example, porous glass, ceramic and other more durable polymers [51]. We have reached the objects set out for this investigation namely, we succeeded in the partial purification of laccase as well as able to immobilized the enzyme using two different methods. We strongly believe that the above mentioned

potential applications should be urgently explored to its full limit. We are optimistic that laccase will have role to plays both in the lacquerware industry as well as the water treatment industry in the future.