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Contents

A Study on Enzymatic Activities of Laccse Extracted from Thisi (Natural Lacquer)

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Abstract

Laccases (EC 1.10.3.2) are oxidoreductases enzymes and are present in fungi, some species of higher plants and insects. In recent years, these enzymes have also been found in some bacterial organisms.This research is mainly related to extraction of Laccase enzyme from Myanmar Lacquer tree (Thitsi) and conducted to determine the enzymatic activities of this partially purified laccase by spectroscopic method using Guaiacol (O-methoxy phenol) as substrate. Optimum conditions for catalytic activities of Laccase are pH 6.0 and 40^{o}C for 150minutes incubation time .The enzyme activity is found to have a linear relationship with different concentration ranging between 0.0033 mg/ mL and 0.0296mg/mL of enzyme.

Keywords: Laccase , enzymatic activity, Guaiacol

Introduction

Enzymes

Enzymes speed up the rate of a reaction by a definite amount, proportional toquantity of enzyme present. Simple measurements of enzyme reactions include activity, specific activity (activity per unit mass) and turnover number, activity per mole of enzyme. Turnover number also represents the actual number of times anenzyme molecule reacts per second. To measure the reaction rate, some property difference between reactant and product must be identified. Rate can be measured as disappearance of reactant or accumulation of product. (Nelson , 2004)

Classification of Enzymes

The types of the reaction of enzyme catalysts are

(1) oxidation reduction (oxidoredutases) (2) transfer of chemical groups (transferases) (3) hydrolysis (hydrolases (4) addition to double bonds (lyases)(5) isomerization (isomerases) (6) formation of new bonds (ligases) (Kroschwitz,1989).

Polyphenol Oxidases (Phenoloxidases)

Phenoloxidases are oxidoreductases that catalyze oxidation of phenolic compounds. They are subdivided into two subclasses, laccases and catechol oxidases (tyrosinases). Both are characterized by the involvement of copper ligands and a role in the biosynthesis of melanins and other polyphenols. Cross specificity exists between Laccases and catechol oxidases with respect to the oxidation of ortho-diphenol substrates.

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Laccase Enzyme

Laccases are copper-containing oxidase enzymes that are found in many plants, fungi and microorganisms. Laccase is a phenol oxidase that catalyze the oxidation of several aromatic and inorganic substances (particularly phenols) with the concomitant reduction of oxygen to water. Laccases can be polymeric and the enzymatically active form can be a dimer or trimer. In general, the Laccases exhibit four neighbor copper atoms, which are distributed among different binding-sites and they are classified into three types; Copper type 1,2 and 3 which are differentiated by specific characteristic properties that allow them to play an important role in the catalytic mechanism of the enzyme (D.R.Mcmillin,1997) (M.A. McGuirl, 1999).

The Laccase enzyme is present in very small quantities in thitsi, at the same time it is vitally important as the normal lacquer curing process will not occur in its absence. As a general class of biocatalyst, it is a p-quinol-o_2-oxidorductase enzyme with a redox potential of 514mV at 25ºC and a pH 7.0. The overall structure of the enzyme is a copper glycoprotein with a molecular weight of about 120,000 Da and a composition of 45% sugars and 55% common amino acids with four Cu^{+2} ions/molecule) (Donald,1989).

In this work, laccase enzyme was extracted from Thitsi (sap of *Melanorrhoea usitata* Wall).

Figure1. Photographs of Thitsi (sap of *Melanorrhoea usitata* Wall).

Catalytic Activity of Laccase

Laccase can catalyze the oxidation of various compounds, including o,p-diphenols, aminophenols, polyphenols, polyamines, lignin, some inorganic ions and aryl diamines. It also catalyzes demethylation of lignin and methoxyphenol acids. The second substrate of the enzymatic reaction is molecular oxygen. All known Laccases can also catalyze with equally high efficiencies, both the oxidation of ascorbic acid and phenol substrate. Unlike the reactions catalyzed by other oxidoreductases, oxygen is reduced directly to water by a four-electrons-mediator-less mechanism.

A number of works have reported a strong pH dependence of both K_m and $V_{max.}$ The Michaelis constant, both by oxygen and electron donor, was pH independent, where as the catalytic constants; both by oxygen and electron donor are pH dependent equally.

Environmental Applications

As mentioned before, Laccase is a phenol oxidase involved (with other enzymes) in the formation of humic material. This assumption is based on the catalytic capacity of the enzyme to affect the oxidation of phenolic substances and phenolic derivatives resulting in the production of polymeric aggregates that usually are less soluble and much more stable than

their compounds. Several researchers have evaluated the potential of Laccase to catalyze the removal of toxic compounds with characteristics similar to those humus-precursors. A wide variety of xenobiotic or xenobiotic-related compounds (including phenol, anilines and their substituted products) were removed effectively and became cross-coupled to naturally occurring humic monomers through the action of a Laccase. Moreover, some pollutants that were relatively insert to enzymatic action were transformed successfully in the bleaching and decolourization industrial processes such as, bleaching of textile dyes, bleaching of kraft pulps.

Materials and Methods

A natural lacquer or thitsi is obtained by tapping the thitsi tree (*Melanorrhoea usitata wall)* which belongs to the family *Anacardiaceace*. It was collected from Phar-an Township, Kayin State. All chemicals used in the experimental work were from British Drug House Chemical Ltd., Poole, England. All chemicals were of reagents grade for preparation of Laccase Enzyme.

Extraction of Latex Acetone Powder from Thitsi

Thitsi (300g) was dissolved in 1L of chilled acetone and was vigorously stirred for 1 hr. The resulting slurry was filtered through a buchner funnel, washed with an excess of chilled acetone and the residue spread out on filter paper. The acetone powder was dried at room temperature and then stored in a desiccator at 4ºC in the refrigerator.

Partial Purification of Laccase Enzyme

Latex acetone powder 15g was dissolved in 300ml of 0.1 M sodium phosphate buffer solution pH 6 and was stirred for 3hrs at 4ºC. The mixture was centrifuged at 5000 rpm for 15 min and the supernatant solution was filtered. The clear green filtrate was 100% saturated with ammonium sulphate at 4ºC, stirred for 30 min, and centrifuged at 5000 rpm for 15 min. The precipitate was dissolved in 0.1 M phosphate buffer and ammonium sulphate was added to 65% saturation. The mixture was then stirred for 30 min and centrifuged at 5000 rpm for 15 min. The supernatant solution was then brought up to 100% saturation with ammonium sulphate, stirred for 30 min and centrifuged at 5000 rpm for 15 min. The precipitate was dissolved in a minimum of distilled water and dialyzed overnight 0.1 M sodium phosphate buffer pH 6, for 2 days in the cold, with two changes of buffer. Then, partially purified enzyme solution was stored at 4ºC in refrigerator.

Laccase Activity Assay

The enzyme activity of Laccase was determined spectrophotometrically using Guaiacol (o-methoxy phenol) as substrate. The guaiacol oxidized product is a colored compound that absorbed at 460nm. Assay mixture contain 10mM of enzyme solution and 1ml of 10mM guaiacol solution (pH 6). Oxidation was followed by the absorbance increase at 460nm. One unit of activity is the amount of enzyme producing a 1.0A increase/min.

Determination of Thermal Stability of Laccase Enzyme

In order to examine the thermal stability of enzyme, solution of Laccase enzyme was exposed at 40ºC, 50ºC, 60ºC and 70ºC in the water bath. A aliquot of each solution was

withdrawn at the appropriate time intervals for the measurement of enzyme activity by assay method. The remaining activity was expressed as a percentage relative to initial enzyme activity with respect to each enzyme.

$$\text{Remaining activity}(\%) = \frac{\text{Activity time at t}}{\text{Initial activity}} \text{ x } 100$$

Determination of Optimum Temperature for Laccase-Catalyzed Reaction

The temperature was fixed variously at 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, 70ºC and 80ºC for each series of experimental runs and the Guaiacol solution of pH 6 was used. The absorbance were measured and the optimum temperature was found from velocity temperature plot.

Determination of Optimum pH for Laccase Catalyzed Reaction

Guaiacol solutions with pH (s) of 3, 4, 5, 6, 7 and 8 were employed. The absorbances were measured and the optimum pH was obtained from velocity vs pH plot.

Determination of Optimum Enzyme Concentration for Laccase Catalyzed Reaction

Laccase enzyme solution

The procedure was the same as that described in above expect that different concentration of enzyme solutions were tested.

Effect of Substrate Concentration on Laccase Catalyzed Reaction

Guaiacol (0.062g) was dissolved in pH 6 buffer solution and the volume made up to 100ml in a volumetric flask with pH 6.0 buffer solution to give a 5mM Guaiacol solution. Similarly the guaiacol 10mM, 15mM, 25mM, 30mM and 35mM solutions were also prepared.

Procedure

(1) The temperature was fixed at room temperature, (2) the guaiacol solution used were of pH 6 and of various concentration, viz., 5 mM,10 mM, 15 mM, 20 mM, 25 mM, 30 mM and 35 mM. The absorbance measured for the various concentrations of Guaiacol solutions were plotted against the respective concentration (mM) of guaiacol solution and from this plot the values of maximum velocity (V_{max}) and Michaelis menten constant (Km) were calculated.

Results and Discussion

Table 1 Laccase Activity Assay

Purification Step	Volume (mL)	Activity (EU)	Total Protein (mg)	Specific Activity (EU mg^{-1})	Yield (%)	Purification Fold
I (Acetone powder + buffer)	1000	4540	2754.3	1.65	95	1.0
II $(NH_4)_2SO_4$ ppt	40	3800	496.7	7.65	77	5.0

Laccase activity was determined spectrophotometrically using 10mM guaiacol (o-methoxy phenol) as substrate. Laccase activity was 1.65 $EUmg^{-1}$. By ammonium sulphate precipitation method, purification fold was 5 times greater than crude laccase and its activity was 7.65 $EUmg^{-1}$.

Table 2. Thermal Stability of Laccase Enzyme at Incubation Temperature of 40ºC, 50ºC, 60ºC and 70°C at Different Time Intervals

No.	Time (min)	Remaining activity (%)			
		40 ºC	50 ºC	60 ºC	70 ºC
1	0	100	100	100	100
2	30	71.3	63.2	61	34.2
3	60	60.2	49	45.3	30.1
4	90	59.7	43.3	40.2	22.9
5	120	49.4	31.7	30.3	12.7
6	150	45.3	30	25	9.8

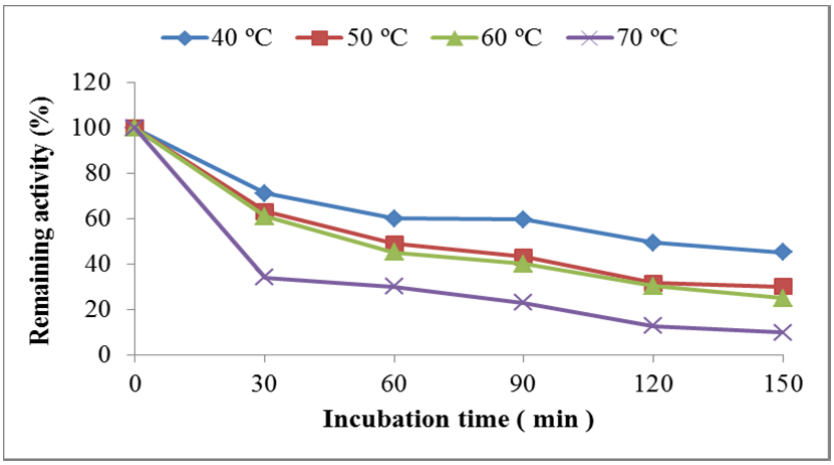

Figure 2. Thermal stability of laccase enzyme at incubation temperature of 40ºC, 50ºC, 60ºC and 70°C

Table 3. Effect of Reaction Temperature on the Specific Activity of Laccase Enzyme for 30 min of Incubation Time

No.	Time (min)	Specific activity ($EUmg^{-1}$)
1	20	7.9
2	30	11.3
3	40	12.6
4	50	13.7
5	60	12.3
6	70	9.2
7	80	4.5

Thermal Stability of Laccase Enzyme

The thermo tolerance of Laccase enzyme was evaluated from the activity retained after heating the enzymes at different temperatures, such as 40ºC, 50ºC, 60ºC and 70ºC for various time intervals. Under these conditions, the activity of Laccase was decreased about nearly 50% at 40ºC for 150min incubation time. Laccase lost 50% of its activity at 50ºC for 60min incubation time. The Laccase enzyme retained over 45.3% of its initial activity at 60ºC after 60min incubation time. The activity of Laccase was decreased at 70ºC within 30min incubation time. Thus, the value of T_{50} for Laccase enzyme, defined as the temperature at which 50% of the initial activity was retained. The T_{50} for laccase is 40ºC.

Optimum Temperature for Laccase-Catalyzed Reaction

The temperature activity profile of Laccase enzyme, determined for the enzymatic activity retained after heating the reacting mixture at different temperatures for a reaction time of 30 minutes as shown in Table 2 and Figure 2. The activities of Laccases gradually increased as temperature was increased from 20ºC to 50ºC. The optimum temperature for laccase-catalyzed reaction was about 50ºC. At 60ºC, the activity of laccase enzyme slowly decreased.

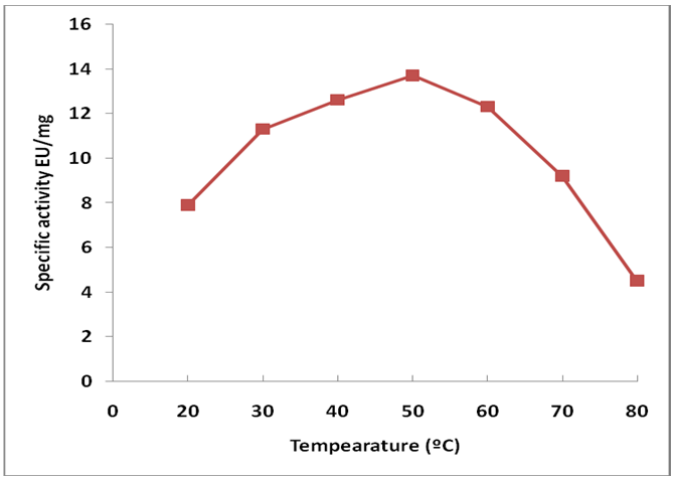

Figure 3. Effect of reaction temperature on the specific activity of laccase enzyme for 30 min of incubation time

Optimum pH For Laccase Catalyzed Oxidation of Guaiacol

In this study the pH value of the phosphate buffer varying from 3 to 8 was used to determine the specific activity of Laccase enzyme. Figure 4 shows the effect of pH on laccase activity. It was found that the optimum pH for enzymatic activity of laccase is pH 6.

Optimum Enzyme Concentration for Laccase Catalyzed Reaction

The enzyme activity was found to have a linear relationship with different enzyme concentration ranging between 3.1mgL^{-1} and 27.3 mgL^{-1} of enzyme. Figure 5 showed the linear relationship of absorbance as a function of laccase enzyme concentration correlation factor is about 0.9776.

Effect of pH on the Specific Activity of Laccase Enzyme

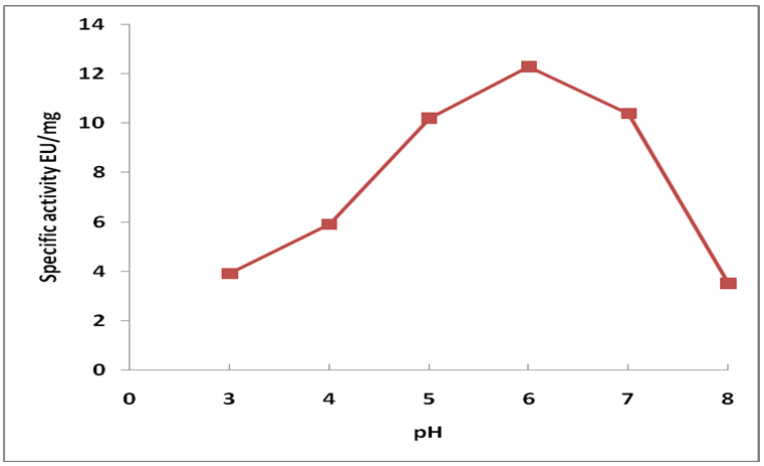

Figure 4. Effect of pH on the specific activity of laccase enzyme

4. Relationship between Absorbance and Enzyme Concentration of Laccase Catalyzed Reaction

No.	Enzyme concentration (mg L^{-1})	Absorbance*
1	3.1	0.125
2	6.5	0.246
3	11.3	0.450
4	15.6	0.631
5	18.4	0.826
6	27.3	1.019

* at λ_{max} =460nm

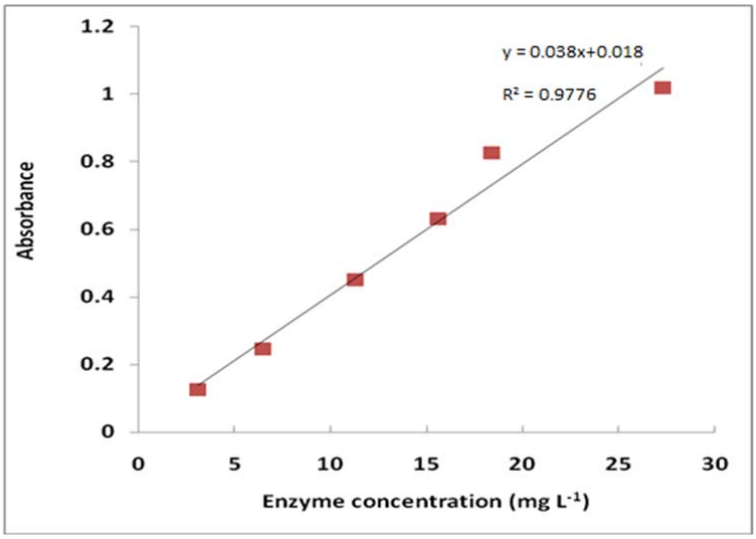

Figure 5. Absorbance as function of enzyme concentration for laccase catalyzed reaction

Conclusion

The results showed the activity and stability of extracted Laccase enzyme from thitsi (Myanmar Lacquer). Firstly Laccase extracted from Myanmar Thitsi was found to be 8.12% in the form of latex acetone powder whereas the crude .Laccase activity was 1.65 $EUmg^{-1}$. By ammonium sulphate precipitation method, purification fold was 5 times greater than with respect to crude Laccase and its activity was 7.65 $EUmg^{-1}$. The thermal stability of Laccase enzyme at 40ºC is about 45.3% after 150 minute incubation time. The optimum pH is 6.0 for Laccase enzyme activity. The optimum temperature for Laccase-catalyzed reaction is about 50ºC. At 60ºC, the activity of Laccase enzyme slowly decreases.

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