

Preliminary Production and Partial Purification of Laccase from a White Rot Fungus and Its Application in Dye Degradation

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CIN and GAE designed the study. Author GAE carried out most of the laboratory work and together with author COA wrote the first draft. Author CIN wrote the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

In this study, a laccase producing white rot fungus from the wood degrading family of dermatophytic *Trichophyton* sp. was isolated. The laccase which was produced via solid state fermentation using rice bran as the lignocellulosic support was partially purified before its effectiveness in degrading an azo dye (methyl orange) was examined. Time course study showed that day nine (day 9) gave the maximum enzyme production. The crude laccase was purified by ammonium sulfate precipitation and dialyzed against sodium phosphate buffer pH 7. Optimum pH and temperature of the were 4.5 and 55°C respectively as assayed using ABTS (2,2 azino bis-3-ethyle benzothiazoline-6-sulfonic acid) as substrate. Dye degradation assay was carried out by introducing 0.5 ml of the enzyme into three dye concentrations: 50 mg/L, 100 mg/L and 200 mg/L and monitoring them for up to 48 hours by taking their absorbances at intervals at 5mins, 5hours, 24hours and 48 hours after enzyme introduction. At the end of the experiment, good dye degradation by laccase from *Trichophyton* sp. was observed to be positive with 50 mg/L being the most effective concentration during the study. The result are positive indicators of the fact that locally isolated white rot fungi has the capacity to be used for many biotechnological dye degradation and removal studies.

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1. INTRODUCTION

In the recent years, the use of enzymes in the diverse fields of industrial applications have increased. Many of such important enzymes are widely distributed in the nature; laccases are among them. Laccases are among the oldest and most studied enzyme systems [1,2]. Of all the large blue copper-containing proteins presently known, laccase is the most widely spread. They contain between 15–30% carbohydrate and usually have a molecule mass of 60–90 kDa with acidic isoelectric point around pH 4.0 [3]. These properties contribute to the high stability the enzyme possesses [2]. Laccases initiate one-electron catalyzation of a wide variety of substances ranging from diphenols, polyphenols, diamines, aromatic amines and many other compounds that are rich in electrons using molecular oxygen [4].

Laccases occur widely in higher plants, bacteria, fungi, and insects among others. In plants, laccases are found mainly in cabbages, turnip, potatoes, pears, apples, and other vegetables. They have also been isolated from different classes of fungi including *Ascomycetes*, *Deuteromyctes* and *Basidiomycetes* where over 60 fungal strains belong [5]. It has been found that the white-rot fungi which belong to the *Basidiomycetes* more efficiently degrade lignin when compared to the *Ascomyceteous* and *Deuteromyceteous* fungi, which tend to oxidize phenolic compounds to give phenoxy radicals and quinines more [6].

Since laccases have wide substrate ranges and use only oxygen as the final electron receptor, they have widespread applications in various industries, such as textile, food, biofuel, organic synthesis, bioremediation, paper and pulp, pharmaceutical, and cosmetic industries [7]. These explains the increasing efforts to isolate and characterize as many of them as are possible from as many sources as can be found. Our objective here is to ascertain the ability of a locally isolated fungus to produce laccase and to carry out preliminary studies on a few characteristics of the enzyme.

2. MATERIALS AND METHODS

2.1 Sample Collection

The exterior surface of fruiting bodies of trees were the sources of samples used in this work.

They were collected from the Department of Plant Sciences and Biology, University of Nigeria Nsukka, Enugu State. The sample was appropriately identified, labeled and immediately used to avoid deterioration.

2.2 Fungi Isolation and Identification

The exterior surface of the fruiting body used for the isolation were first sterilized with 1% mercuric chloride solution before being repeatedly washed with sterile distilled water (for disinfection). The fruiting bodies were opened and the internal tissues transferred with sterilized scalpel to a sterile culture medium containing 2% malt extract agar (MEA) and chloramphenicol (250 mg/ml) and incubated at 30°C for 7-10 days to ensure growth of culturable fungi. When growth was observed, the culture was purified by sub-culturing until a pure mono culture was obtained. The pure culture was maintained on potato dextrose agar slant stored at 4°C. Standard techniques were thereafter used to identify the microorganism of choice that showed the best ability to produce laccase. The observed fungal morphology and other tests showed that the organism is the dermatophyte *Trichophyton* sp. with septate hyphae and micro conidia scattered around its tips.

2.3 Production of Laccase

Solid state fermentation using the method of Tien and Kirk [8] was used to produce laccase. The basal medium comprise (g/L), Ammonium tartarate (0.22), KH_2PO_4 (0.21), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05), CaCl_2 (0.01), Thiamine (0.001), CuSO_4 (0.08), Na_2MoO_4 (0.05), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.07), $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ (0.043), FeSO_4 (0.03), Glucose (10), pH 4.5 and supplemented with 5g of rice bran. Three agar plugs of 0.8 cm cut from the growing edge of the fungal Potato Dextrose Agar (PDA) culture were inoculated into 20 ml of basal medium contained in a 250 ml conical flask. The sample were then statically incubated at room temperature (28°C). Samples were thereafter harvested after 10 days of incubation using sodium acetate buffer, pH 5.0 with shaking for 30 minutes. Equal volume (100 ml) of buffer were added into each conical flask and left to shake for another 30 min using an electric shaker before the contents were filtered through a cheese cloth. The filtrate was centrifuged at 4°C at 4000rpm for 20 minutes to remove mycelia

mat. The supernatant was then assayed for laccase activity.

2.4 Determination of Laccase Activity and Protein Content

Laccase activity was assayed using the modified method of Shin and Lee [9]. This involves monitoring the rate of oxidation of 2,2-azino bis (3-ethylbenzothiozoline)-6 sulphonic acid (ABTS) to the cation radical (ABTS-azine) at 420 nm by the fungal culture supernatant at pH 4.5 and 30°C. The concentration of cation radical responsible for the intense blue green color can be correlated to enzyme activity. Protein content of the enzyme was estimated by the method of Lowry et al. [10] using bovine serum albumin (BSA) as standard.

2.5 Partial Purification of Laccase

Laccase was partially purified by ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) precipitation and dialysis. First, the ammonium sulphate precipitation profile was carried out to determine the appropriate concentration that will precipitate laccase. To do this, different concentrations of $(\text{NH}_4)_2\text{SO}_4$ ranging from 20-100% ammonium sulfate were added to each test tube containing 10 μl of crude enzyme. This was allowed to stand at temperature of 4°C for 30 hours before being centrifuged at 4000 rpm for 20 minutes and the pellets were re-dissolved in 1.0 ml volumes of 0.1M sodium acetate buffer pH 5.0. Laccase activity of the precipitate was assayed to determine the percentage of ammonium sulfate saturation that has the highest activity. From the study of ammonium sulfate precipitation profile, maximum enzyme activity was achieved at 90% ammonium sulfate saturation. That concentration was therefore used to precipitate 165 ml of crude laccase. This was done by adding 99.49 g of ammonium sulfate in 165 ml of crude laccase and stirred vigorously until the salt dissolves completely. The precipitate was centrifuged after 24 hours of incubation and the precipitate collected and re-dissolved in 10 ml of 0.1M sodium acetate buffer pH 5.0 and then dialyzed for 12 hours against the same buffer. The buffer was changed after 6 hours and dialysis completed after the next 6 hours.

2.6 Determination of Optimum pH

Laccase was assayed at different pH ranging from 3.0 to 7.5 using 0.1 M sodium acetate buffer

(3.0-5.5) and phosphate buffer (6.0-7.5) at interval of 0.5 to determine the optimum pH. Test mixture contained 2.8 ml buffer of each pH inside a test tube, 0.1 ml ABTS as substrate and 0.1 ml of enzyme. Enzyme activity was determined at 420 nm. The pH at which the highest enzyme activity was recorded was taken as the optimum pH, (the highest enzyme activity was obtained at pH 4.5).

2.7 Determination of Optimum Temperature

Laccase activity at different temperature was determined by incubating the enzyme at temperatures of 30 to 100°C in sodium acetate buffer pH 4.5 for 5 minutes. The activity was read using ABTS as mediator. The ABTS was added after about 5 minutes incubation of the enzyme in a buffer at optimum pH in a water bath at each particular temperature and enzyme activity read immediately as stated above. The temperature at which the enzyme showed the highest enzyme activity becomes the optimum temperature for its activity.

2.8 Substrate Concentration

Effect of substrate concentration on laccase activity was assayed by varying the concentration of substrates with that of the buffer and determining its activity at the optimum pH and temperature. The substrate concentration was varied from 0.01 – 0.2 μM with the volume of buffer decreasing as substrate concentration increases to give a total reacting volume of 2.9 ml without the enzyme and 3.0 ml with enzyme. The enzyme (0.1 ml) was added last to the test mixture and activity read at 420 nm with UV spectrophotometer.

2.9 Decolorization of Methyl Orange Dye by Laccase

Dye decolorization by laccase was carried out preparing three different concentration of methyl orange from a stock concentration of 400 mg/L. The stock solution was prepared by dissolving 0.04 g of methyl orange in 100 ml of sodium acetate buffer pH 4.5. The three test concentrations were 50 mg/L, 100 mg/L and 200 mg/L. the three concentrations were prepared by taking 0.625 ml, 1.25 ml and 2.5 ml respectively from the stock solution, making it up to 4.5 ml with the buffer and then adding 0.5ml of purified laccase enzyme to make a total of 5ml working volume.

The decolorization efficiency (R%) were calculated as follows:

$$\text{Dye decolorization (\%)} = (A_{\text{bs initial}} - A_{\text{bs final}}) / A_{\text{bs initial}} \times 100$$

Where, $A_{\text{bs initial}}$ = initial absorbance of the untreated dye at their characteristic peak

$A_{\text{bs final}}$ = absorbance after treatment with laccase at the same peak

3. RESULTS

3.1 Fungal Isolation

After several screening and rescreening, we obtained a white rot fungus which showed good ability to produce laccase. The fungus was isolated using 2% malt extract agar and sub-cultured on a PDA plate to obtain a pure culture (Fig. 1).

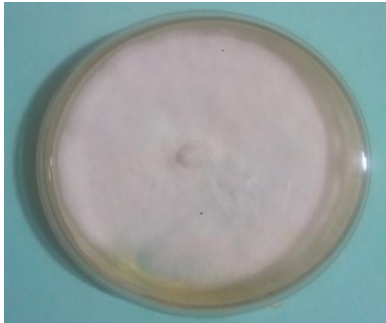


Fig. 1. Pure culture of a white rot fungi, identified as *Trichophyton* sp. obtained after several screening and subculturing

3.2 Identification of the Fungal Isolate

The fungal isolate was identified by examining both macroscopic and microscopic features of a three-day old pure culture. The color, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation, spore shapes etc. were examined. Based on these characteristics, *Trichophyton* sp. was identified and confirmed as the fungal isolates.

3.3 Laccase Production

Laccase was produced from *Trichophyton* sp. under solid state fermentation system using rice bran as lignocellulosic support (Fig. 2). The figure showed that rice bran fully supported the growth of the microorganism.



Fig. 2. Laccase production via solid state fermentation using rice bran as carbon source

3.4 Partial Purification of Laccase

Laccase from *Trichophyton* sp. was partially purified by Ammonium sulphate precipitation and dialysis. Ninety percent (90%) of ammonium sulphate saturation was found to be most suitable for laccase precipitation, at which point it showed an activity of $154 \mu\text{mol}/\text{min}$. (Fig. 3). The figure shows that the least laccase was precipitated when the least concentration of ammonium sulphate used, which was 20%. As the concentration of ammonium sulphate was increased, more of the enzyme was precipitated up until the highest precipitation which happened when the concentration of ammonium sulphate used reaches 90%. Beyond that, as can be seen in the figure, the amount of laccase precipitated decreases. The partially purified laccase obtained after that final point of precipitation was subsequently dialyzed to remove the ammonium sulphate used to precipitate it.

3.5 Characterization of Laccase

The laccase obtained and partially purified from above were subsequently characterized on the basis of pH, temperature and substrate concentration.

3.6 Determination of Optimum pH

The optimum pH for laccase from *Trichophyton* sp. was found to be 4.5 (Fig. 4). The figure shows that the laccase obtained here work best in acid leaning environments. In that sense, it was observed that although the optimum was obtained at pH 4.5, two other more acidic pH which were 4.0 and 3.5 showed the next higher activity after the optimum. Thereafter followed acidic points of 5.0, 5.5 and 6.0 with the laccase

activity falling increasingly at increasing pH points till at pH 7.0 when the activity fell to zero.

3.7 Determination of Optimum Temperature

The optimum temperature of Laccase from *Trichophyton* sp. was found to be 55°C (Fig. 5). Therefore, the optimum temperature at which the enzyme can oxidize ABTS is at 55°C. Only 50°C and 60°C showed the next best laccase activities after the optimum temperature. There were quick falls in activities at temperatures beyond 60°C up until 80°C when it fell completely. Therefore, no activities were found at temperatures between 80°C and 100°C which showed that the enzyme had at those points been denatured.

3.8 Effect of Substrate Concentration

The study on the effect of ABTS concentration on laccase activity obeyed Michaelis-Menten curve (Fig. 6). The result obtained from the effect of substrate concentration on laccase activity was used to plot Lineweaver-Burks plot. Kinetic parameters such as Vmax and Km of the enzyme were calculated from the Lineweaver-Burk plot (Fig. 7). The Vmax and Km values were found to be 592 $\mu\text{mol}/\text{min}$ and 171 μM respectively.

3.9 Dye Decolorization by Laccase

The applicability of laccase enzyme dye decolorization was assayed by reacting it with a

laboratory prepared azo dye (methyl orange) and monitored at varying intervals through 48 hrs. Visible reductions in color intensity were observed as the trend of decolorization was monitored by reading absorbance at 590 nm. Reduction in absorbance was observed with time showing that laccase can to a great extent decolorize methyl orange (Fig. 8).

4. DISCUSSION

Laccases were first described in 1883 from the Japanese lacquer tree *Rhus vernicifera* [11]. After that pioneering effort, several laccases have been studied with respect to such important aspects of laccase properties as their biological functions, substrate specificity, copper binding structure as well as many of their industrial applications [2,7,12,13]. In this work, the fungus *Trichophyton* sp. secreted good levels of laccase in a glucose-ammonium tartarate medium with 2% malt extract and chloramphenicol (250 mg/ml) supplemented with rice bran as lignocellulosic support. Time course study determined that maximum laccase production after nine days (216hr) of incubation. The result is similar to those obtained by Cordi et al. [14] in whose studies the day of maximum production ranged from eight (8) to ten (10) days. That time of nine days for maximum laccase production is also considerably smaller than that obtained by More et al. [15] whose work showed that the highest amount of laccase expressed via activity was obtained after the 19th day. These differences may be as a result of the different

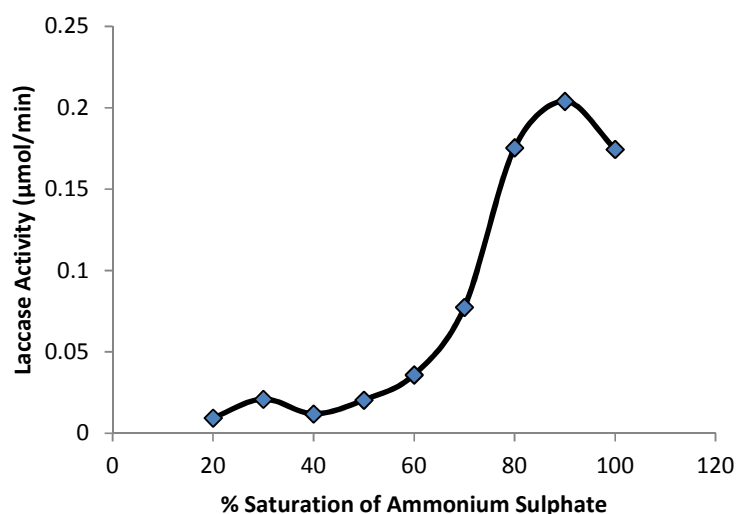


Fig. 3. Percentage saturation of ammonium sulphate for maximum laccase precipitation

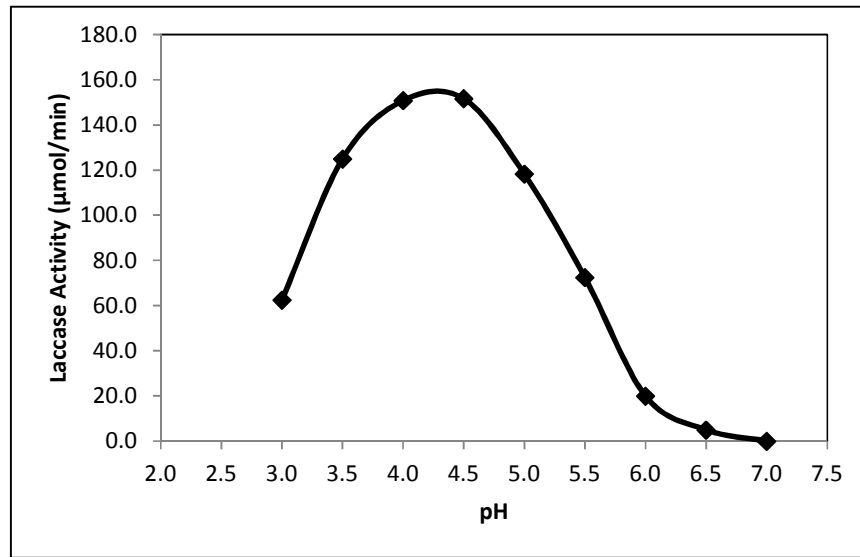


Fig. 4. Optimum pH for laccase from *Trichophyton sp.*

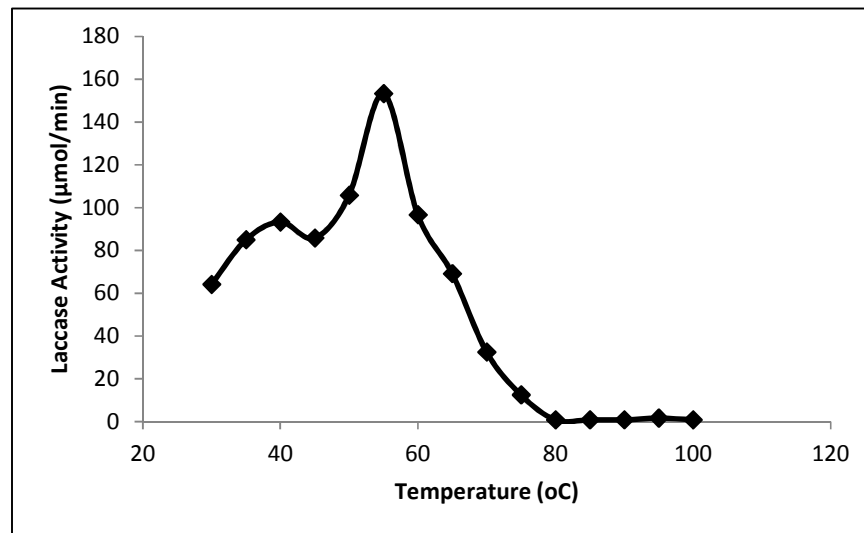


Fig. 5. Optimum temperature for laccase from *Trichophyton sp.*

fungi specie used, temperature, pH, the inducer or the lignocellulosic support used among other factors. It has been observed that solid state fermentation is particularly for the production of many enzymes from filamentous fungi due to the fact that they mimic conditions under which the fungi grow naturally [16]. Not surprisingly therefore, the use of natural various natural substrates, especially lignocellulosic-based agricultural residues as growth substrates have been studied for various enzymes including laccases [17,18]. It has been found that irrespective of whether laccase production is by carried out by either of solid-state or submerged

fermentation, rice bran always gives better activity and higher productivity results than other substrates [2].

The optimum pH of the laccase from *Trichophyton sp.* used in this study is 4.5 and has a bell-shaped profile which is similar to those reported by More et al. [15]. Generally, optimum pH for optimum laccase production from fungi is considered ideal at low acidic pH range, typically around 4.0 [19], which is in agreement with our findings in the present work. The optimal temperature obtained here which is 55°C. That result is typical, as it has been found that

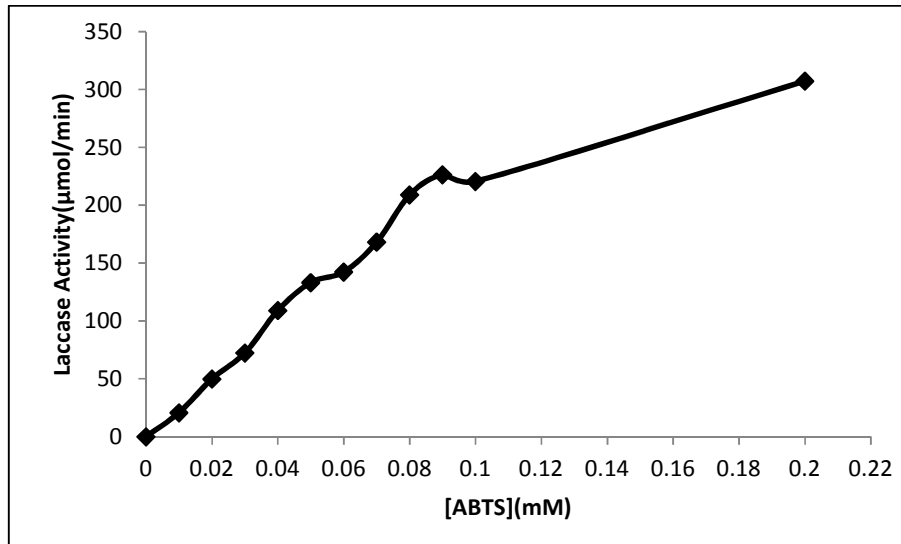


Fig. 6. Effect of ABTS concentration on laccase from *Trichophyton* sp.

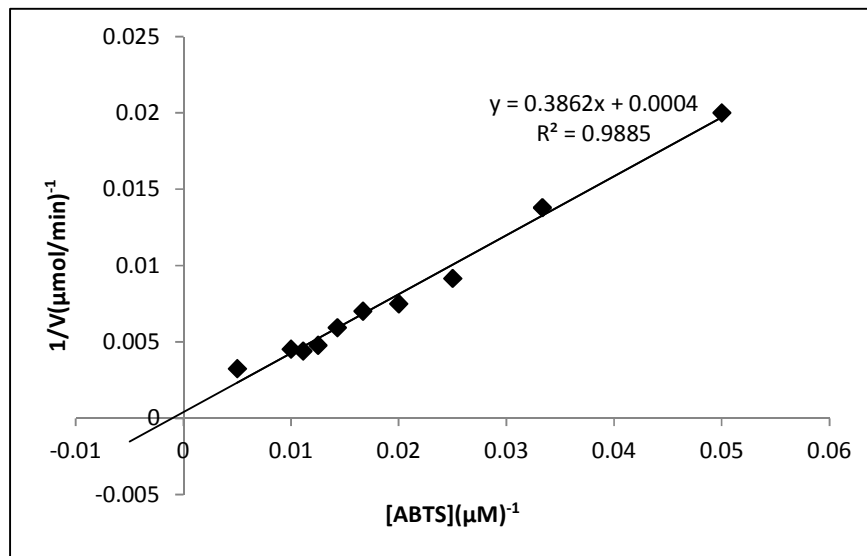


Fig. 7. Line-weaver bulk plot of the effect of ABTS concentration on laccase from *Trichophyton* sp.

temperature profiles of laccase activity usually do not differ from other extracellular ligninolytic enzymes with optima between 50°C and 70°C [19], although a few have been found to be below it, sometimes closer to 30°C [20] especially so for bacterial laccases which typically have their optimal temperatures between 32°C to 45°C. These statements essentially buttress the fact that optimal temperature for laccase activity differ greatly from one organism to another [2].

The kinetic study of the dialyzed laccase obtained from a *Trichophyton* sp. gave a K_m

value of 171 μM^{-1} and V_{max} value of 592 $\mu\text{mole}/\text{min}$. That supposedly low K_m value obtained with ABTS correlates strongly with that obtained by Jung et al. [21], who had reported that low K_m value for ABTS is actually a unique kinetic property of laccase from *Trichophyton* sp. Having the low K_m value is a positive attribute as it means that only very small amounts of substrates will be necessary to saturate the enzyme and that it will always function at constant rate irrespective of variations in the concentrations of substrates [22]. With a high K_m value, the opposite is observed as the enzyme activity will depend largely on the concentration

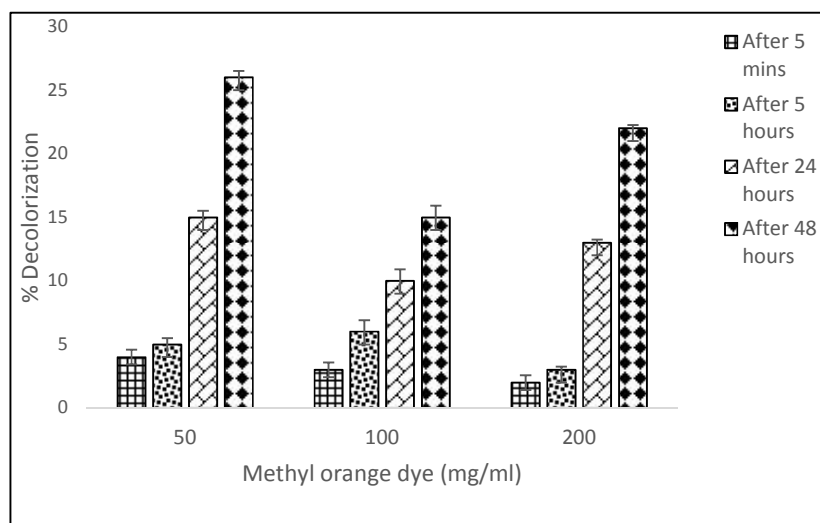


Fig. 8. Decolorization of methyl orange by laccase from *Trichophyton* sp.

of the substrate. As a matter of fact, the activity of the enzyme increased as the concentration of the substrate (ABTS) increases, until it reaches its maximum velocity and remained constant at that point of saturation. This is consistent with typical enzyme observations in line with Michaelis-Menten principle.

Because of their nature, laccases now offer several advantages of great interest for biotechnological applications. They exhibit broad substrate specificity and are thus able to oxidize a broad range of xenobiotic compounds including synthetic dyes, chlorinated phenolics and many other recalcitrant compounds [19]. This explains the attempt made here to ascertain the ability of the partially purified laccase we obtained to decolorize methyl orange. As shown above, the dye decolorization assay showed appreciably good level of methyl orange dye removal. This was observed most with the 50 mg/L concentration. Possible reasons for that concentration being removed the most could be the fact that it is the least concentrated of the three. On the whole however, the result demonstrated is that despite the very partial and minimal purification, the laccase obtained here showed considerably good ability in the removal of methyl orange of varying concentrations. This suggests that it is a good candidate and option in textile and other industries where recalcitrant dyes are predominantly used [23].

5. CONCLUSION

In the present study, the influence of pH, temperature and substrate concentration on a

fungal laccase isolated from *Trichophyton* sp. was studied. Result obtained showed that the laccase has an optimal pH of 4.5, optimal temperature of 55°C, Km value of 171 μM^{-1} and Vmax of 592 $\mu\text{mol}/\text{min}$. The enzyme showed appreciably good dye degradation ability with methyl orange despite the nearly crude nature of the enzyme used, indicating that it has good prospects of being used as a dye removal option.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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