



INDUCTION AND PURIFICATION OF DIPHENOL OXIDASE FROM *Lenzite elegans*

¹Wuyep P.A., ¹Sila Gyang M.D and ²Kutshik, R. J.

¹Department of Plant Science and Technology, University of Jos, Nigeria

²Department of Biochemistry, University of Jos, Nigeria

*Corresponding Email:zhimak.wuyep@gmail.com

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ABSTRACT

Laccase (EC.1.10.3.2. Diphenol oxidase) are multicopper oxidases that catalyze oxidation of many substituted phenolic compounds, aromatic amines including some inorganic substances by using molecular oxygen as electron acceptor. Nevertheless, most laccases that have been studied so far are not well-suited for industrial applications due to their low stability at high temperatures or pH values. This research focused on identifying and characterizing novel fungal laccases that have potential for industrial applications as well as developing efficient production methods for laccases. *Lenzite elegans*, a white rot fungi was screened by plate tests using indicator compound 2,2' Azinobis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS). The ability of *Lenzite elegans* to produce laccase was studied in liquid media. This fungal species produced significant amounts of laccase in titers (958 U), and the enzyme was preliminarily characterized. The novel laccase was found to be rather typical basidiomycete laccase. The novel laccase from *Lenzite elegans* was purified and biochemically characterized and has a relatively high specific activity (30.9 U). This enzyme also showed remarkable stability and active at high temperatures (60°C). Medium I and II supported the elaboration of LiP, MnP and Laccase enzymes than 2 % ME and CCWM. However, the highest Laccase titers were recorded in CCWM. The enzyme was inhibited by NaN₃, EDTA and NaI, and the inhibition was dose dependent. Divalent cations Ca²⁺, Zn²⁺, Mg²⁺, Fe²⁺, Co²⁺ and Cu²⁺ were tested against the enzyme, Fe²⁺, Cu²⁺ exhibited similar characteristics with Fe²⁺ effect higher than all the cations used. Laccase from *Lachnocladium* sp. had a KM of 0.067 μM and Vmax of 0.505 U. with low protein concentrations of 0.118-0.196 mg recorded in this study could be traced to fungal growth and purification procedures.

Keywords: *Lacasse enzymes, diphenol oxidase and Lenzite elegans*

INTRODUCTION

Laccase (EC.1.10.3.2. Diphenol oxidase) is an enzyme belonging to the family of multicopper oxidases, and common in nature. Laccase is widely found in plants, fungi and some bacteria (Thurston 1994; Gianfreda *et al.*, 1999). The physiological functions of this biocatalyst, which is mostly secreted and sometimes intracellular, are different in the organisms but the biocatalyst catalyse the polymerization or depolymerisation processes.

However, laccases have been reported from fungal organisms with most biotechnologically useful laccases originating from fungi. Probably the first report on the presence of laccase in fungi was from Laborde in 1897. Over 60 fungal strains belonging to the phyla Ascomycota, Zygomycota and especially Basidiomycota show laccase activities. The catalytic site of laccase is quite conserved among different species of fungi, but the rest of the enzyme structure shows high diversity. Fungal laccases are mostly inducible, extracellular, monomeric glycoproteins with carbohydrate contents of 10-20% which may contribute to the high stability of laccases.

In the food industry, Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage for the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity in clear fruit juice, beer and wine. Laccase is also employed to ascorbic acid determination, sugar beet pectin gelation, baking and in the treatment of olive mill wastewater. Laccases are able to depolymerize lignin and delignify wood pulps, kraft pulp fibers and chlorine-free in the biopolpation process (Bourbonnais *et al.*, 1997). One of the most studied applications in the industry is the laccases-mediator bleaching of kraft pulp and the efficiency of which has been proven in mill-scale trials. Laccases-mediator system finds potential application in enzymatic modification of dye bleaching in the textile and dyes industries. Most currently existing processes to treat dye wastewater are ineffective and not economical. Therefore, the development of processes based on laccases seems an attractive solution due their potential in degrading dyes of diverse chemical structure, including synthetic dyes currently employed in the industry (Wong and Yu, 1999).

Laccases are involved in green biodegradation due its catalytic properties. The xenobiotic compound is a major source of contamination in soil and laccase degrade it. Moreover, polycyclic aromatic hydrocarbons (PAHs), which arise from natural oil deposits and utilisation of fossil fuels, are also

degraded by laccases. Many PAHs have been found in exhibit cytotoxic, mutagenic and carcinogenic properties that represent serious risk to human health.

MATERIALS AND METHODS

Optimization of Laccase production

The white rot fungi that showed moderate to high ABTS oxidizing activity were selected and further cultured on four media types, mainly liquid culture. The composition of the media employed for this procedure is as enumerated below:

1. 2% ME: 20 g/l Malt extract and 1000 ml distilled H₂O (Ainsworth 1995).
2. CCWM: 1 Kg Cabbage, 2 Kg Carrots, 2 Kg Water melon, 1000 ml of 25 mM Acetate buffer pH4.5 and 10 mg ABTS.
3. Medium I: Glucose 5 g/l, malt extract 2 g/l, yeast extract 1 g/l, KH₂PO₄ 0.5 g/l, (NH₄)₂HPO₄ 0.25 g/l MgSO₄·7H₂O 0.15 g/l, CaCl₂·2H₂O (1 % Solution) 5 ml and NaCl (1 % Solution). 2.5 ml. (Ainsworth 1995).

Optimization of laccase production by *Lenzite elegans*

A factorial experiment with a completely randomized design was done to optimize laccase production by *Lenzite elegans* Three factors (media type, inducer and pH for assay) were replicated three times. Data were subjected to analysis of variance and means tested for significant differences with students' T- test. CCWM: 1 Kg Cabbage, 2 Kg Carrots, 2 Kg Water melon, 1000 ml of 25 mM Acetate buffer pH4.5 and 10 mg ABTS was evaluated for laccase production. The effect of pH of the culture at incubation on laccase production was conducted at three different pH values (pH 4, pH 6, and pH 7).

Isolation of Laccase from *Lenzites elegans*.

1000 ml liquid culture was homogenized in Blender. Homogenate was clarified by passing through cheese cloth, this served as crude laccase.

Ammonium sulphate fractionation of crude of Laccase from *Lenzite elegans*.

The method for the purification of laccase from *Lenzite elegans* was adapted from a Protocol described by Fukishima and Kirk (1995). The culture was centrifuged at 10,000 g; for 30 min to remove the mycelial mass from the supernatant. The supernatant was frozen overnight at -4 °C, thawed and centrifuged at 10,000g for 30 minutes (Centrifuge) to remove unwanted long-chain polysaccharides. The supernatant was then fractionated with ammonium sulphate in order to remove unwanted proteins. An

experiment was conducted to determine the degree of ammonium sulphate saturation required to precipitate laccase from the supernatant. Ammonium sulphate was dissolved in 15 ml of supernatant and centrifuged (7300 x g; 10 minutes). The amount of ammonium sulphate was increased by 5 % intervals ranging from 30 % to 95 % saturation. The amount of laccase activity was determined spectrophotometrically using the ABTS. The amounts of solid ammonium sulphate required (grams per 100 ml of supernatant) to obtain different levels of saturation were obtained from 'Methods in protein purification' (Harris and Angal, 1989). After fractionation, the supernatant containing the laccase activity was centrifuged at 10,000g; for 30 minutes and dialyzed, using a dialysis tube with a molecular weight cut-off of 10 000 KDa, against 0.01 M sodium acetate buffer (pH 6) at 4 °C. The buffer was changed at least three times.

Enzyme and protein assays

ABTS assay method for laccase

This assay method is commonly used and is described by Bourbonnais and Paice (1990). The nonphenolic dye 2, 2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to enzyme activity and is most often read between 415 nm and 420 nm. ABTS (0.4 mM) was dissolved in sodium acetate buffer (pH 4.5; 25°C). The absorbance of the cation radical was monitored at 420 nm ($\text{cm}^{-1} = 36 \text{ mM}^{-1} \text{cm}^{-1}$) and 25°C using a JENWAY spectrophotometer. Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1 μmole of product per minute. The reaction mixture contained 580 μl of substrate and 20 radical released. $\text{min}^{-1} \cdot \text{ml}^{-1}$ enzyme) was calculated as follows:

$$U/ml = 2 \left(\frac{V}{v \times \epsilon \times d \times \Delta A \cdot \text{min}^{-1}} \right)$$

$$U/ml = 2 \left(\frac{0.6}{0.02 \times 36 \times 1 \times \Delta A \cdot \text{min}^{-1}} \right)$$

$$= 1.667 \times \Delta A \cdot \text{min}^{-1},$$

Where:

V = Total reaction volume (ml)

v = Enzyme volume (ml)

ϵ = Extinction coefficient of ABTS at 420 nm = 36 $\text{mM}^{-1} \text{cm}^{-1}$ (Bourbonnais and Paice, 1990).

Partial purification of Laccase from *Lenzite elegans*

with Sephadex G-75 Gel chromatography. 5.0 mls of Ammonium sulphate fractionated sample was loaded on to the pre-equilibrated Sephadex G-75 column (described above) were eluted with 50 mM Acetate buffer pH 4.5, 5.0mls of the eluates were collected in test tubes, and Laccase activity was assayed with ABTS according to Bourbonnais and Paice, 1990. Lignin peroxidase and Manganese peroxidase activity assay was done using UV-spectrophotometer. The assay mixture consisted of 200 microlitres of crude enzyme extract, 0.4 M veratryl alcohol in 25 mM sodium acetate buffer pH 4.5, 0.2 M H_2O_2 and a few drops of phenol red in a test tube at 28° C. Veratryl alcohol oxidation was determined at 469 nm after 30 minutes and 2 hr.

Protein assays

The protein contents of column effluents were estimated by spectrophotometric measurement at 570 nm. Protein concentration (mg/ml) was determined using the Biuret method with Bovine serum albumin as standards in this range 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 mg/ml.

Effect of Temperature on partially purified Laccase from *Lenzite elegans*

The effect of temperature on Laccase activity was measured by incubating the enzyme and substrate at 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 30 min, and activity assayed as described above.

Effect of pH on partially purified Laccase from *Lenzite elegans*

The activity of laccase was determined as function of pH using 0.1 M ABTS as substrate. The assay procedure was modified in terms of pH of the buffers. Buffers used in the assay include 0.005M acetate buffer (pH 3 – 5), phosphate buffer (pH 6 – 7), Tris-HCl buffer (pH 8– 9).

RESULTS AND DISCUSSION

Lenzite elegans was grown in 2% ME, CCWMM, Medium I and II for 20 days. Laccase activity in all the media reached a maximum after twelve days of cultivation (figure 1). Again Medium CCWMM supported higher Laccase production followed by Medium I and II and then 2 % ME. Relatively high titers of laccase were observed even after peak production as shown in figure 4.1.

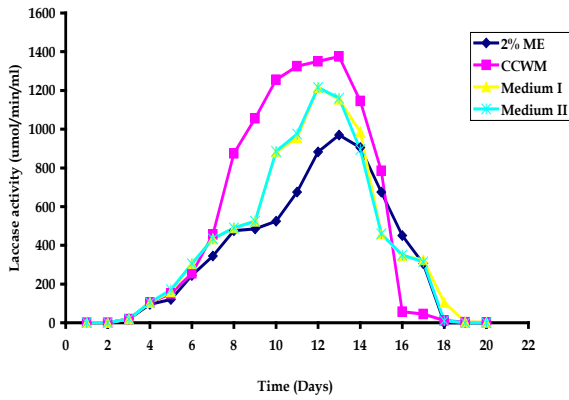


Fig.1: Production of Laccase activity by *Lenzite elegans* in 2 % ME, CCWM, Medium I and Medium II

It was observed that cultivation pH had an impact on initial Laccase induction and subsequent production. Figure 4. 2 showed that Laccase induction began on day two at the selected pH, and the Laccase activity reached a peak on Day 4 for pH6 and day 5 for pH 4 and 7. It was also observed that the culture with pH 6 had higher Laccase titers than those at pH 4 and 7. Generally speaking, cultivation pH has been shown to induce early production of Laccase.

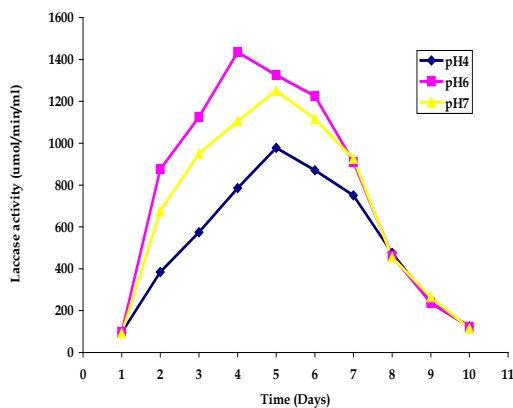


Fig.4.2. The effect of pH on production of laccase in CCWM by *Lachnocladium* sp.

Fig.2: Time course (Days) on the production of Laccase in CCWM by *Lenzite elegans*

Laccase activity was purified from the culture by Anion exchange and gel chromatography. During the anion chromatography most of the Laccase activity was separated from the proteins (figures 3 and 4). Three activity peaks were resolved. Active fractions from the three identified peaks were pooled. The purification steps are shown in table 1. In the anion exchange chromatography, the specific activity increased from 6.5 U/mg proteins to 30.9 U/mg proteins, but decreased to 27.5 U/mg proteins in the final step and the purification fold from 4.69 to 4.18.

Table 1. Purification of Laccase from *Lenzite elegans* by Gel filtration and anion exchange chromatography.

Purification Steps	Total activity µmol/min	Protein mg	Specific activity µmol/min/ml	Purification fold	Yield %
Culture filtrate	1.29	0.196	6.5	1.00	100
Sephadex G-75	5.75	0.186	30.9	4.69	445.4
Q-Sepharose	3.25	0.118	27.5	4.18	251.7

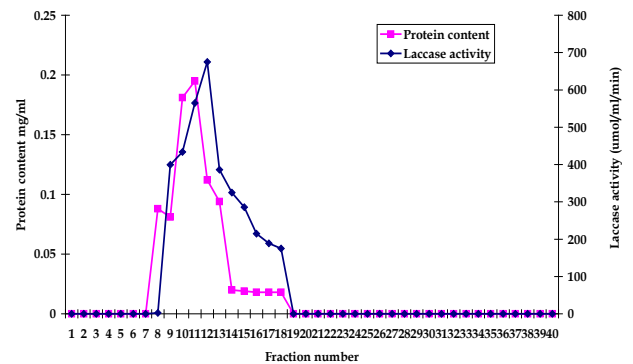


Fig.3. Elution profile of Laccase on Sephadex G-75 gel filtration

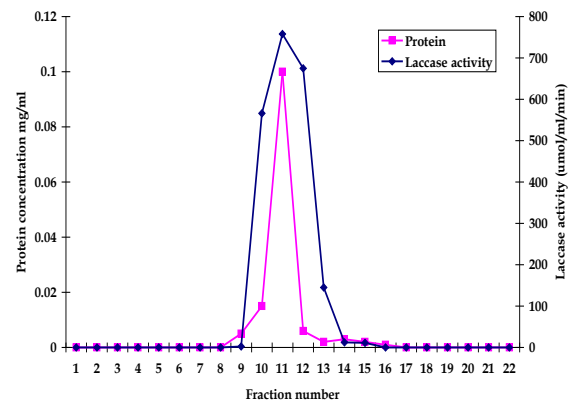


Fig.4: Elution profile of Laccase on Q-Sepharose anion exchange chromatography

It was also observed that the enzyme is active over a remarkably wide temperature range 20 °C to 70 °C where. Significant activity is detected as low as 20 °C and approximately 50 % of as activity is maintained at temperatures as high 70°C. The optimum temperature for Laccase from *Lenzite elegans* was determined to be 60 °C

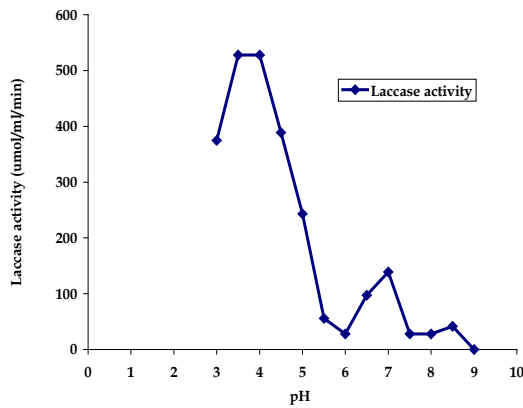


Fig. 5: The effect of pH on Laccase from *Lenzite elegans*

Kinetic Data

The initial velocity data, Laccase from *Lenzite elegans* exhibited a regular hyperbola consistent with the Michealis -Menten progress curve as shown in figure 5. This was further proven when the kinetic data (averages of duplicate data) was fitted to a single-substrate (Michealis-Menten) Kinetics by non-linear regression analysis as shown in figure 6. The KM value (0.067 μ M) indicated a binding affinity toward ABTS. The Vmax (0.505 U) gives an indication of the efficiency of the Laccase enzyme with this substrate.

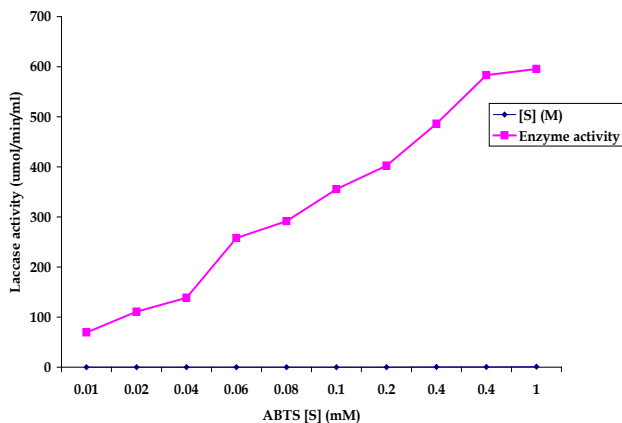


Fig.6.: Michaelis-Menten plot for laccase from *Lenzite elegans*

Inhibition studies

Three potential inhibitors (sodium azide NaN_3 , EDTA and NaI) were evaluated to test the inhibition properties of laccases at 37°C. Sodium azide and EDTA were the most efficient inhibitors (figure 7) with inhibition concentration being significant at 25 mM and 50 mM. However, as the concentration increased to 75 mM there was a decline in inhibition. NaI inhibited the laccases to a lesser extent following a similar pattern with respect to inhibitor concentration. Overall, the inhibition patterns of the inhibitors could be assumed to be concentration dependent. That is,

laccase activity from *Lenzite elegans* is inhibited with small concentration (25 mM -50 mM) of the chosen inhibitors.

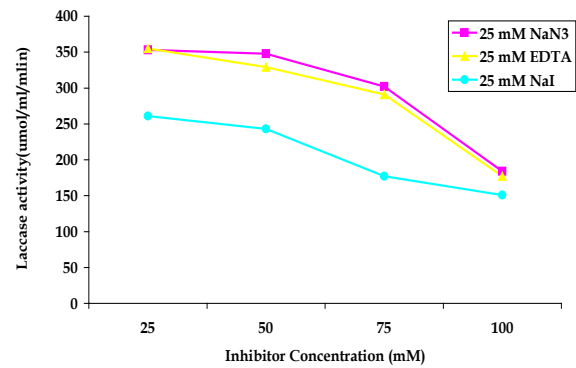


Fig.7. Effect of varying Inhibitor Concentration on Laccase Activity

Effect of divalent cations on Laccase activity

The cofactor requirement revealed that 25 mM Fe^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , Ca^{2+} and Mg^{2+} were activators of laccase enzyme from *Lenzite elegans*. 25 mM Fe^{2+} activated Laccase by about 80 % while Cu^{2+} by about 40 %. The extent of activation of Laccase enzyme by the remaining divalents cations was $\text{Zn}^{2+} > \text{Co}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ in that order, as shown in figure 4.10

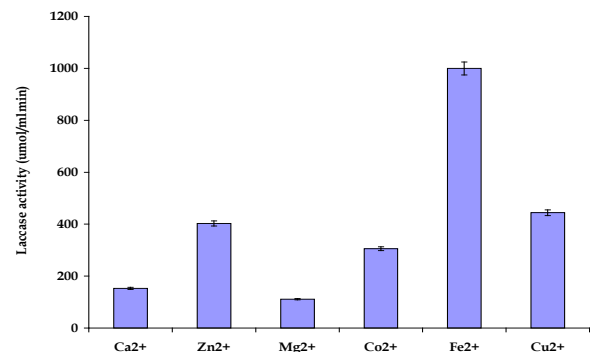


Fig.8: The effect of Divalent ions on Laccase activity. The bars represent standard errors of the mean of two replications

The nitrogen concentrations had no effect on laccase production by *Lenzite elegans*. Among several complex nitrogen sources, Medium I and II did not increase the laccase production compared to CCWMM. In the optimized conditions the maximum laccase activity of *Lenzite elegans* was 985 $\text{U min}^{-1}\text{ml}^{-1}$, this is as high as reported by others. Results from this experiment indicated that laccase produced by *Lenzite elegans* under any method of cultivation performed better at slightly acidic pH.

There was no significant difference between 2 % ME, Medium I and II with regards to laccase induction. The biomass was separated

from the supernatant by passing through cheese cloth. The supernatant was frozen and thawed, this was necessary in order to remove long chain polysaccharides from the supernatant, which were produced during growth of the fungi. Ammonium sulphate fractionation to remove *Lenzite elegans* laccase from the supernatant was done at a saturation of 75 %. No laccase activity remained in the supernatant after centrifugation. Dialysis against the running buffer (0.01 M sodium acetate) was done to remove excess ammonium sulphate from the supernatant prior to column chromatography. Three buffer changes were necessary to achieve complete removal of unwanted salt. The Q-sepharose anion exchange chromatography successfully separated the dark brown contaminating colour of the supernatant from the laccase enzyme. The Q-Sepharose anion exchange chromatography successfully separated the laccase enzyme from the contaminating pale brown colour present in the supernatant. The column step resulted in the separation of various proteins peaks that could be separated from the laccase enzyme. The purification fold increased by a factor 1.00 to 4.18 during this step. A typical elution profile for this purification step indicates a single laccase activity peak corresponding to the position of a shoulder in the peak representing protein content.

The optimum temperature of laccase from *Lenzite elegans* was determined to be approximately 60°C. The temperature range where the enzyme is active is remarkably wide. Significant activity is detected as low as 20°C and approximately 95% of activity is maintained at temperatures as high as 70°C. Optimum temperatures are significantly affected by the assay used so this data should be interpreted with care. Temperature stability studies are usually more informative. According to literature the typical optimum temperature range for laccases are 50 °C to 60°C (Luisa *et al.*, 1996). *Lenzite elegans* laccase therefore exhibits a typical optimum temperature. Examples of other laccases with higher temperature optima that what is commonly expected were produced by the fungus *Marasmius quercophilus* (Farnet *et al.*, 2000) and *Coriolus hirsutus* (Shin and Kim, 1998). The profile of the effect of temperature on *M. quercophilus* laccases corresponds to what was found for laccase from *Lenzite elegans*.

Previous studies have shown that fungal laccases are generally active at low pH. However, in this study the laccase activity showed activity over a wide pH range. This could be due to the involvement of multiple laccase isozymes. Xu 1996 carried out a study to elucidate the reason for the different pH

dependencies of laccases using different substrates. He reported that the dependence of laccase on pH usually renders a bell-shaped profile as demonstrated for 2, 6- Dimethoxyphenol, (DMP), syringaldazine and guaiacol. This biphasic profile is the result of two opposing effects. The first is due to redox potential difference between a reducing substrate and Type 1 copper centre of laccase, where the substrates dock. Here the electron transfer rate is favoured for phenolic substrates at high pH. The second is generated by the binding of a hydroxide anion to the Type 2/Type 3 copper centre of laccase, which inhibits the binding O₂, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of increased amount OH ions. ABTS is the only substrate that does not adhere to this principle. With ABTS there is rather a monotonic decline than a bell-shaped profile. ABTS is regarded as a non-phenolic substrate in contrast to the three phenolic substrates mentioned above.

The values reported for the parameter, Kcat, is a representation of the rate of the catalytic process. The ratio Kcat/KM gives an indication of the efficiency of each enzyme and therefore allows us to compare the efficiencies of the different enzymes. Unfortunately, only ABTS was used as a substrate to test the efficiency of laccase from *Lenzite elegans* instead of several substrates. ABTS has a higher redox potential than laccase itself and the mechanism by which laccase-catalyzed oxidation of ABTS is not yet fully understood (Bourbonnais *et al.* 1997).

Laccase from *Lenzite elegans* was inhibited with high degree of affinity by sodium azide (NaN₃). Sodium azide complexes to the copper atoms in the active site, EDTA also had a high inhibitory effect towards this enzyme. EDTA exhibits metal chelating properties and NaI, halides are known to inhibit laccase at the Type 2/3 trinuclear copper site. It is at this site that molecular oxygen is reduced to two molecules of water. The inhibition therefore entails that the oxygen is prohibited from being reduced, causing a break in the terminal electron acceptance, and leading to a decrease in redox potential difference between the two copper sites. However, it has been reported that laccases catalyzes the oxidation of iodide (I⁻) to iodine (I₂). Xu compared the redox potential of the halides (0.535 V- 2.87 V) and typical laccases (0.47 V- 0.74 V), he reasoned that it is possible for iodide to be oxidized by a typical laccase enzyme. This would not be possible for chloride and fluoride as their redox potentials are significantly higher than that of iodide. It has been suggested that iodide acts as a substrate for laccase.

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