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Production and Partial Characterization of Laccase from Pleurotus Species

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Abstract
Pleurotus species potential for production of laccase under best levels of physicochemical conditions was studied. The study was conducted at the National Agricultural Biotechnological Research Center, Holetta. Collection of fruiting bodies of fungi were based on their morphology and cultured on potato dextrose agar plate. *Eucalyptus* sawdust and bean straw mixture on equal quantity was used as a main growth substrate for fungi and after extraction a 0.2187 U/ml of laccase enzyme activity was observed. The stability and activity of laccase was estimated under different substrate conditions and concentrations. The optimum pH for laccase activity was 6.0 with an activity of 0.2453 U/ml and it nearly retains 71% of its initial activity at this pH level after 12 hrs incubations. Laccase activity showed an increase in activity towards 50°C with 0.2478 U/ml activity and above 40°C its stability declined sharply and only 32% of its initial activity remain at 60°C overnight. This indicated that crude laccase extract was more stable at lower temperature (laccase only lost 4 and 12% of its initial activity at 20 and 30°C, respectively when incubated overnight) and needed to be stored at low temperatures. There was a linear relation between laccase activity and its substrate concentration. The reciprocal of laccase activity 1/V in U/ml and substrate concentration (1/[S]) in mM was considered to calculate kinetic parameters like Km and Vmax values which was 0.922 U/ml and 4.14 mM, respectively.
Keywords: *Pleurotus* species, Bean straw, *Eucalyptus* sawdust, Laccase, Guaiacol.
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1. Introduction

Oyster mushroom (*Pleurotus* species) belong to phylum Basidiomycota under kingdom Fungi bearing their sexual spores externally on club shaped structures called basidia. They belong to Order Agaricales under Class Agaricomycetes traditionally known as the gilled mushrooms and Family Pleurotaceae, most of which are saprotrophic on wood substrate with some members of the family able to derive nutrition from nematodes (www.bioweb.uwlax.edu/). *Pleurotus* species are common inhabitants of forest litter and fallen trees (Kersten and Cullen, 2007) with their unique capacity of efficiently degrade lignin, which is dependent on the production of oxidative extracellular ligninolytic enzymes. These enzymes primarily comprise lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and hydrogen peroxide-generating oxidases. In addition to the ligninolytic enzymes, they also produce cellulases, xylanases, and other hemicellulases (Hammel and Cullen, 2008).

Enzymes have added great significance in different industries; the one among them which are widely present in the nature is laccases. Laccase is copper-containing protein, which belongs to the diverse and large superfamily of multicopper oxidases (Arora and Sharma, 2010). It is spread widely in plants that are high and fungi (Kiiskinen *et al.* 2004) and have also been found in bacteria as well as insects (Viswanath *et al.* 2014). It can reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines. Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups. These enzymes were known to catalyse the oxidation of a wide range of phenolic compounds and aromatic amines including diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted phenols (Sivakumar et al. 2010).

Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) has been studied since the nineteenth century as one of the few enzymes. The description of Yoshida about laccase in 1883 was when he removed it from the Japanese lacquer tree exudates, *Rhus vernicifera* (Thurston, 1994). Laccase was demonstrated to be a fungal enzyme for the first time in 1896. by both Bertrand and Laborde as reported by Desai and Nityanand, (2011). Laccases of fungi attract considerable attention due to their possible involvement in the transformation of a wide variety of phenolic compounds including the polymeric lignin and humic substances (Baldrian, 2006). Because of its low substrate specificity, laccase can be used to analyse drug, clarification of wine, bioremediation (Mayer and Staples 2002), paper-pulp bleaching, and decolourization of synthetic dyes (Baldrian, 2005), and biosensors (Vianello et. al. 2011). Laccase practical applications in biotechnology have resulted in the need for increasing the microorganisms' spectrum with activities of laccase as well as laccase isolating novel. In general, there is a scientific need to identify different sources of fungal laccases due to its capability to oxidize wide range of

industrially relevant substrates. Keeping this in mind researchers are initiated for lab scale production and partial characterization of laccase produced by *Pleurotus* species. under solid state culture of a mix of *Eucalyptus* sawdust and Bean straw.

2. Materials and Methods

2.1 Culture Collection and Maintenance

Collection of fruiting bodies of fungi was based on their morphology following the approaches of *Pleurotus* species identification guideline given by Consensus Document on the Biology of *Pleurotus* species (OECD, 2005). The main morphological traits considered all through collection of fruiting body were; occurrence, stem/stipe, odor, shape and color of fruiting body. Growing of fungi is in shelf-like clusters, almost absent stem, thick and smooth flesh with shaped cap of whitish kidney having anise odor, were considered. The fungi having the above-mentioned features were collected from diverse trees and were inoculated on PDA (Potato Dextrose Agar) following standard microbiological measures and incubated at 28°C. Observation of the colonies of plates was done for morphological difference and the colony having cylindrical to narrowly kidney shaped features were considered. The colonies with those morphologies were picked with a sterilized inoculating needle and inoculated on a freshly prepared PDA plates in order to obtain pure cultures. Cultures of pure *Pleurotus* species were maintained in PDA slants according to Naraian et al. (2010).

2.2 Growth Substrate and Optimized Growth Condition

Eucalyptus sawdust (ESD) as a growth substrate was collected from local small wood processing firms in Holeta town and Bean straw (BS) was obtained from Holeta agricultural research center field. First, it was air and oven dried to ease grinding for achieving similar particles sizes and both substrates were mixed on equal ratio (1:1) kept at room temperature in bottles of capped glass.

For fungal cultivation, previously optimized physicochemical growth conditions of *Pleurotus* species for maximal laccase production were used as described in our earlier study (Edae and Alemu, 2017). ESD and BS mixture was weighed in 10g and transferred to flasks of 250 ml, in which 30 ml of distilled water was added to prepare solid state fermentation medium following Demir *et al*. (2011). The substrate was supplemented with 2% soluble starch, 0.2% peptone and 5 mM $CaCl_2$. The pH of media was adjusted to 5.5. When autoclaved, with fungal 5 discs culture of 5-day old under condition of aseptic all flasks were inoculated (Patel et al. 2009), and incubated at 28°C for 10 days for mycelium complete growth. Additionally, 1mM of asparagine was added as a potent inducer on the 3rd day of incubation.

2.3 Extraction of Laccase

Addition of 30 ml of 100 mM acetate buffer (pH 5.0) to the glass bottles containing solid substrates for fungal growth after the tenth incubation day (Kumari and Negi, 2014). Then, the glass bottles were incubated on shaker at 120 rpm for 30 min at room temperature to let the buffer extract the enzyme. The contents were later filtered with Whatman No. 1 filter paper and the culture filtrate was centrifuged at 10,000 rpm for 15 min at 4°C for the removal of insoluble and spores. Collection of clear supernatants (crude enzymes) was done in separate containers by decantation (Elsayed *et al*. 2012). This was used directly to determine enzyme activity.

2.4 Activity of Laccase Assay

For Laccase activity measurement, 0.1 ml of culture filtrate (enzyme source) was added to 4.9 ml of 0.1 M sodium acetate buffer (pH 4.5) and 1 mM guaiacol as substrate (Demir et al. 2011). Prepared reaction mixture was incubated at 50°C for 15 min. Incubation without culture filtrate was used as control. Enzyme activity was measured by reading absorbance in the UV-Visible spectrophotometer adjusted to 465 nm wavelength. Activity of enzyme was expressed as Enzyme units (U), where 1 U is defined as the required enzyme amount to oxidize 1 micromole of guaiacol (substrate) per min and activity of laccase in U/ml was calculated with the following formula (Ping et al. 2008; Kalra et al. 2013).

$U/ml = (A * V) / (\varepsilon * t * l * v)$

Where: U = Enzyme unit; t = time; A = Absorbance; l = sample thickness; V = Total reaction volume; v = Enzyme volume.

2.5 Partial Characterization of Laccase

2.5.1 Effect of pH on the Activity and Stability of Laccase

Optimal conditions of pH for laccase activity were determined over a range of pH 3.5–9.0. The optimum pH for laccase activity was investigated by using three different buffer systems comprising of 100 mM sodium acetate buffer (pH 3.5–5.5); 100 mM sodium phosphate buffer (pH 6.0–8.0) and 100 mM Tris–HCl buffer (pH 8.5–9.0) following Patel *et al.* (2013). The reaction mixture was prepared from 0.1ml of culture filtrate (enzyme source), 4.9 ml of 100mM buffer and 1 mM guaiacol as substrate. Then, the reaction mixtures were incubated at 50°C for

15 minutes and enzyme activity was measured. For determining laccase stability over different pH ranges, similar reaction mixtures were incubated overnight and their activities were measured as an indicator of firmness.

2.5.2 Effect of Temperature on the Activity and Stability of Laccase

Temperature effect of laccase activity was determined by incubating reaction mixture for 15 min at temperatures ranging from 20–80°C with an interval of 10°C at optimal pH. The thermal stability was determined under same assay conditions but incubated overnight in a temperature range of 20–60°C.

2.5.3 Effect of substrate Concentration on Activity of Laccase

Rate of laccase catalysis was determined by incubating substrate guaiacol at various concentrations (2, 4, 6, 8, 10, and 12 mM) with 1 ml of laccase crude extracts. The incubation time was 15 min for determination of the substrate concentration effect on activity of laccase. The enzyme incubated without guaiacol served as control. The kinetic constants Km and Vmax were estimated by curve fitting of the reciprocal plot of reaction rate in enzyme activity (U/ml) versus substrate concentration using the Line Weaver-Burk equation plot transformation of the Michaelis-Menten rate equation (Asgher *et al.,* 2012).

2.6 Data Analysis

All the experiments were carried out in triplicates and the data presented is the mean value of the triplicates. The standard error was calculated using the mean values.

3. Results and Discussion

3.1 Laccase Production

Culture of *Pleurotus* species was placed on PDA petri-dishes comprising of 4 mM Guaiacol and incubated at 28°C for 5 days. The culture was incompetent to grow fast as anticipated compared to guaiacol free plate; alth ough there was effect of suppression from Guaiacol together with mycelial growth initiation. Culture was able to develop strong brown colour around the colony (Figure 1) because of oxidation of guaiacol by laccase which can be connected to its activity after the 10th day (Kumari and Negi, 2014). Thus, it can be decided that fungus under investigation (*Pleurotus* species) was a producer of laccase.

Figure 1. Detection of laccase production by *Pleurotus* species on PDA plate; PDA plate with guaiacol and PDA plate no guaiacol).

3.2 Laccase Production and Extraction

Production of enzyme is greatly swayed by components of media, particularly nitrogen, carbon sources, as well as physical factors such as inoculum density, temperature, pH, and incubation time. It is vital to produce enzyme in scale of large in inexpensive manner. Therefore, previously optimized physio-chemical conditions were used. The main growth substrate was a mixture of ESD and BS on equal ratio, with 2% soluble starch, 0.2% peptone and 5 mM $CaCl_2$ as supplementary. The pH of media was adjusted to 5.5 and incubated at 28°C for 10 days for complete growth of mycelium. Additionally, 1mM of asparagine was added as a potent inducer on the 3rd day of incubation.

Addition of 30 ml of 100 mM acetate buffer (pH 5.0) to the glass bottles comprising of solid substrates and preserved on shaker at 120 rpm at room temperature for 30 min to allow the buffer extract the enzyme after 10th day of incubation. The contents were filtered with Whatman No. 1 filter paper and the culture filtrate was centrifuged at 10,000 rpm for 15 min at 4°C for removal of spores and other insoluble. Collection of clear supernatants (crude enzymes) was then in separate containers by decantation (Elsayed *et al*. 2012). This was used directly to determine activity of enzyme.

3.3 Laccase Spectrophotometric Analysis

For laccase activity measurement, 0.1 ml of culture filtrate (enzyme source) was added to 4.9 ml of 0.1 M sodium acetate buffer (pH 4.5) and 1 mM guaiacol as substrate (Demir et al. 2011). Preparation of reaction mixture was incubated at 50°C for 15 min. Incubation without culture filtrate was used as control. Measurement of enzyme activity was by reading absorbance in the UV-Visible spectrophotometer adjusted to 465 nm and it was 0.2187 U/ml. Expression of activity of enzyme was as Enzyme units (U), where 1 U is defined as the enzyme amount required to oxidize 1 micromole of guaiacol (substrate) per min. For partial characterization of laccase, a series of experiments was done to check whether its activity is declining or rising following change in substrate conditions as well as concentration. The classical strategy was adopted; therefore, a single parameter was varied at a time and previously optimized parameter was kept at optimal level in the next.

3.4 Partial Characterization of Laccase

3.4.1 Effect of pH on Laccase Activity

In order to determine the optimum working pH for laccase enzyme, the crude extract was first harvested and incubated at 50°C within different pH range from 3.5-9.0 for 15 minutes. The results of this test showed that the optimum pH for laccase activity was 6.0 with an activity of 0.2453 U/ml and there was a sharp decline in activity with further increase in pH (Fig 2). It was also observed that there was less activity of laccase in acidic pH values compared to slight acidic pH ranges. There was statistically significant variation in laccase activity recorded in different pH, which indicate that the level of working pH had direct influence on laccase catalysis.

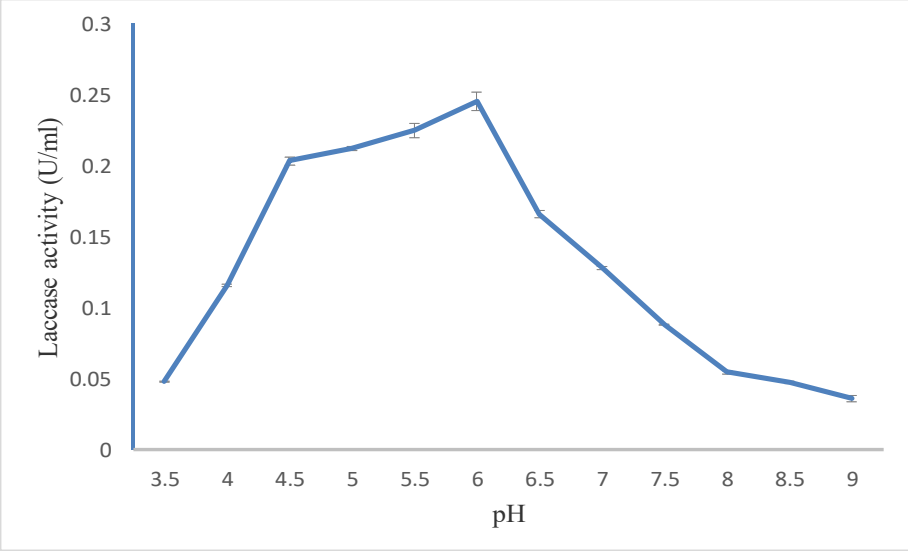

Figure 2 Effect of pH on laccase activity

The result is in agreement with Abdulah (2008) who indicated that enzyme activity has a bell-shaped profile with an optimum pH that varies considerably. The report also showed that the optimum pH for laccase activity was 6.0. The dependence of laccase activity on pH usually renders a bell-shaped profile. This bi-phasic profile is the result of two opposing effects. The first is because of the redox potential difference amid a reducing substrate and the Type 1 copper center of laccase, where the substrate dock. Here the electron transfer rate is favored for phenolic substrates at a high pH. The second is generated by the binding of a hydroxide anion to the Type 2/ Type 3 copper center of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased amount of OH^- ions (Bar, 2001). In addition, Ravikumar *et.al*. (2012) also reported that the optimum pH of the purified laccase was found to be 6.0, and also Ashger *et al*. (2012) indicated that laccase activity increased initially with pH and peaked up at 5.0. The enzyme had a fairly good activity over a wide pH range (5.0 to 8.0), which may be a useful characteristic for various industrial and biotechnological applications.

3.4.2 Effect of pH on Laccase Stability

To determine the stability of laccase over a range of pH, crude laccase extracts was incubated overnight (nearly 12 hrs) at 50°C and pH range from 3.5-9.0. Laccase activity was recorded and compared with the initial activity. Table 1 shows initial & final activity of laccase and percentage of remaining activity after 12 hrs incubation. There was significant difference in laccase activity recorded initially and after overnight incubation. This indicate that overnight incubation had an effect on laccase activity of *Pleurotus* species. Result of this test confirmed that crude laccase nearly retains 71 % of its initial activity at pH 6.0 when incubated overnight. At pH 6.5-7.5 it maintains 66-56% of its initial activity, while it was 33-43% at lower pH values (3.5-4.5).

The reason for declining in enzyme stability at acidic pH is due to the effect of acidic environment in enzymatic structure which cause ionizing groups in active site. The declining in enzyme activity at pH above the optimum pH may be due to irreversible denaturation of enzyme molecule that leads to change in enzyme structure associated with the formation of enzyme dimerization that leads to enzyme autolysis (Abdulah, 2008).
In favor of the current study, Sivakami et al. (2012) indicated that laccase enzyme retains more than 50% activity in the pH range 4.0-6.0. Naturally laccase is very stable over a broad range of pH. The pH stability profile indicated that the enzyme was unstable at pH values above 6.0, but reasonably stable over pH values from 1.5 to 5, maintaining 80% of its original activity after incubation for 1h as reported by Junyao He et al. (2014). They also cited that most laccase from fungi such as *Coltricia perennis*, *Paraconiothyrium variabile* and *Scytalidium thermophilum* have an ideal pH range of 3−6 and have decent stability in near neutral conditions. Othman et al. (2014), in line with the current study also report that, laccase of *Pleurotus ostreatus* was stable at pH of different values and more stable at range of alkaline and it keeps about 85-88% of its initial activity after incubation of 5 h at pH range from 5-7.

Table 1 Comparison of laccase activity (U/ml) recorded in different pH after overnight incubation (values are mean ±standard error, n=3).

	Laccase activity (U/ml)			
pH	Initial	Final	%Remaining activity	P-value
3.5	0.0477±0.0004	0.0159±0.0004	33.42	0.0003
4	0.1155±0.0009	0.0430±4.1E-5	37.27	0.0079
4.5	0.2033±0.0028	0.0880±0.0017	43.29	0.0025
5	0.2120±0.0015	0.1059±0.0051	49.93	0.0197
5.5	0.2248±0.0049	0.1439±0.0009	64.02	0.0338
6	0.2453±0.0065	0.1737±0.0018	70.82	0.0433
6.5	0.1658±0.0026	0.1102±0.0012	66.47	0.0118
7	0.1277±0.0010	0.0763±0.0021	59.72	0.0077
7.5	0.0879±0.0004	0.0497±0.0019	56.55	0.0269
8	0.0545±0.0012	0.0273±0.0006	50.07	0.0105
8.5	0.0472±0.0006	0.0216±0.0002	45.75	0.0077
9	0.0357±0.0024	0.0147±0.0017	41.15	0.0266

Means having p-value less than 0.05 are statistically different between initial and final activity within pH.

3.4.3 Effect of Temperature on Laccase Activity

Determination of optimal working temperature was tested by incubating crude enzyme extract at different temperature ranging from 20-80°C with 10°C interval (Fig 3). Phosphate buffer (pH 6.0) which was an optimized pH for laccase activity under current study was used.

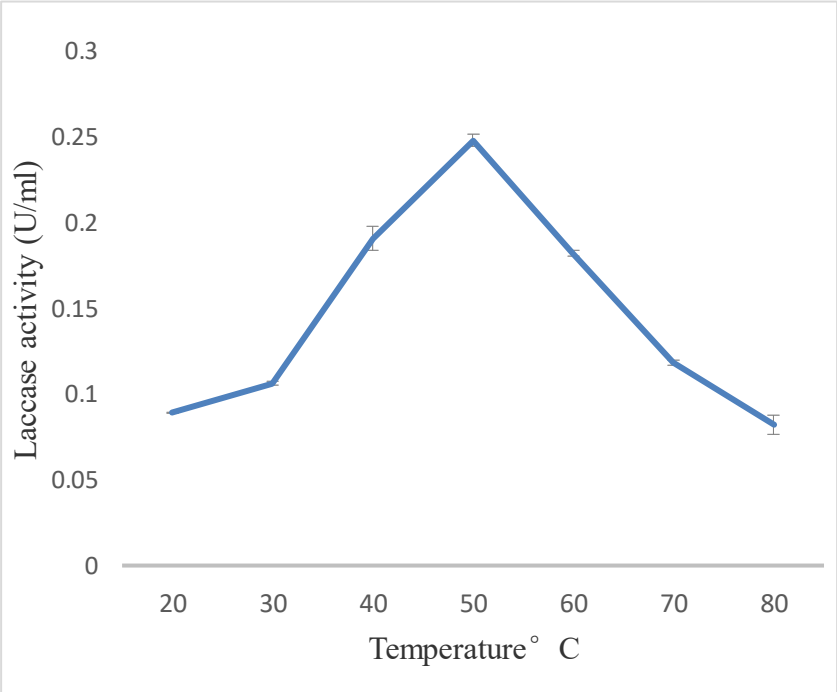

Figure 3 Effect of temperature on laccase activity

The temperature versus laccase activity curve showed an increase in activity towards 50°C with an activity of 0.2478 U/ml. After 50°C the activity decline and the minimum activity was recorded at 80°C which was 0.0824 U/ml that was only 33.3% of its optimum activity at 50°C.

The reason for declining of an activity towards higher temperature is that the speed of enzyme interaction increases with increasing temperature within a certain range due to increased energy kinetics and the collisions between enzyme molecules and substrate, except that the high temperatures within certain limits lead to denaturation of the enzyme and loss of three-dimensional structure and then decline in enzyme activity (Abdulah 2008). Generally, there were significant variation in laccase activity obtained at different incubation temperatures. Othman *et al.* (2014) stated that *P. ostreatus* cleansed laccase showed a maximum activity at 50ºC. The best activity for laccase from *Trametes hirsuta* is ranged from 40 to 60°C as reported by Castillo *et al.* (2012). Laccase optimal temperature was determined to be 60°C and activity of laccase declined when the temperature was increased from 60 to 80°C, with 40% of the optimal observation of activity of enzyme at 80°C (Ding *et al.,* 2012). On the other hand, Ashger *et al.* (2012); Junyao He *et al.* (2014) indicated that the optimum temperature for the crude laccase was 40 and 45 °C, respectively.

3.4.4 Effect of Temperature on Laccase Stability

To determine the thermostability of crude laccase extract, incubation was done overnight (nearly 12 hrs) at temperature range from 20-60°C. The result indicated that laccase only lost 4 and 12% of its initial activity at 20 and 30°C, respectively when incubated overnight (Table 2). At 40°C, moderate stability was observed maintaining 60% of its initial activity. Above 40°C the stability declined sharply and only 32% of its initial activity remain at 60°C. This indicates that crude laccase extracts are more stable at lower temperature and needs to be stored at low temperatures. The reduction of activity above 40°C is due to sensitivity to high temperature, reflecting the effect of temperature on the three-dimensional structure of protein by damaging R-groups of amino acids which lead to denaturation of protein and losing its activity. There was significant variation in laccase activity at different temperature after overnight incubation. Table 2 shows activity recorded initially and after overnight incubation and the remaining activity in percentage.

Table 2 Comparison of laccase activity (U/ml) recorded at different temperature after overnight incubation (values are mean ±standard error, n=3).

	Average laccase activity (U/ml)			
Temp (°C)	Initial	Final	%Remaining activity	P-value
20	0.0893±8.2E-5	0.0858±0.0021	96.11	0.1707
30	0.1064±0.0014	0.0936±0.0005	88.06	0.0233
40	0.1908±0.0071	0.1146±0.0029	60.04	0.0169
50	0.2478±0.0034	0.0912±0.0032	36.81	0.0005
60	0.1821±0.0017	0.0585±0.0032	32.14	0.0017

Means having p-value less than 0.05 are statistically different between initial and final within temperatures.

For selection of industrial applications, relatively high thermostability is a desirable and attractive enzyme characteristic. Though, most of white rot fungi laccases are stable and practically fully active in a range of 30-50°C temperature (Ashger *et al.* 2012). Othman *et al.* (2014) discovered that the *P. ostreatus* purified laccase could sustain heat up to 30°C without specious loss of activity for 120 min, and the remaining activity decreased regularly as a function of both time of exposure as well as temperature. Enzyme incubated at 40, 50 and 60ºC for 120 min resulted in a loss of about 4, 20 and 40% of its activity, correspondingly. Junyao He *et al.* (2014) also discovered that at 45°C, laccase retained approximately 50% of its initial activity. Nonetheless, it was almost completely inactive at temperatures above 60°C. Ding *et al*. (2012) also found that there was no laccase activity loss discovered over 80 min at 30, 40 or 50°C. At 60°C, 46% of the residual activity of laccase remained after 80 min, indicating that *G. lucidum* laccase is a thermostable enzyme. Less than laccase activity of 10% was retained after 20 min when incubated at 70°C, and complete inactivation happened after 10 min at 80°C. The greatest stability of the enzyme of laccase was observed at 30°C after 48 hrs of incubation (Stoilova *et al.* 2010).

3.4.5 Effect of Substrate Concentration on Laccase Activity and Study of Kinetic Parameters

Concentrations of different guaiacol substrate (from 2-12 mM) were prepared to check the effect of substrate concentration on activity of laccase and to find the kinetic laccase parameters. Indication of the result was that, there was a lined relation between activity of laccase and its substrate concentration. There was activity increase with substrate increasing concentration. After certain concentration, the rate of increase in the velocity decreased because of occupation of enzyme active sites by the substrate and in conclusion there was no rate increase of reaction. More addition of the substrate had no laccase activity effect.

The reciprocal of laccase activity 1/V in U/ml was plotted against reciprocal of substrate concentration (1/[S]) in mM. Kinetic parameters like Km and Vmax values for laccase of *Pleurotus* species were calculated using Lineweaver Burk plot. The regression equation for the Lineweaver Burk slope was y = 4.4898x+1.0842

(R^2 = 0.9862). From this, the apparent calculated Vmax and Km were 0.922 U/ml and 4.14 mM, respectively (Fig 4). The relationship between rate of reaction and substrate concentration depends on the affinity of the enzyme for its substrate expressed as Km (Michaelis constant) of the enzyme.

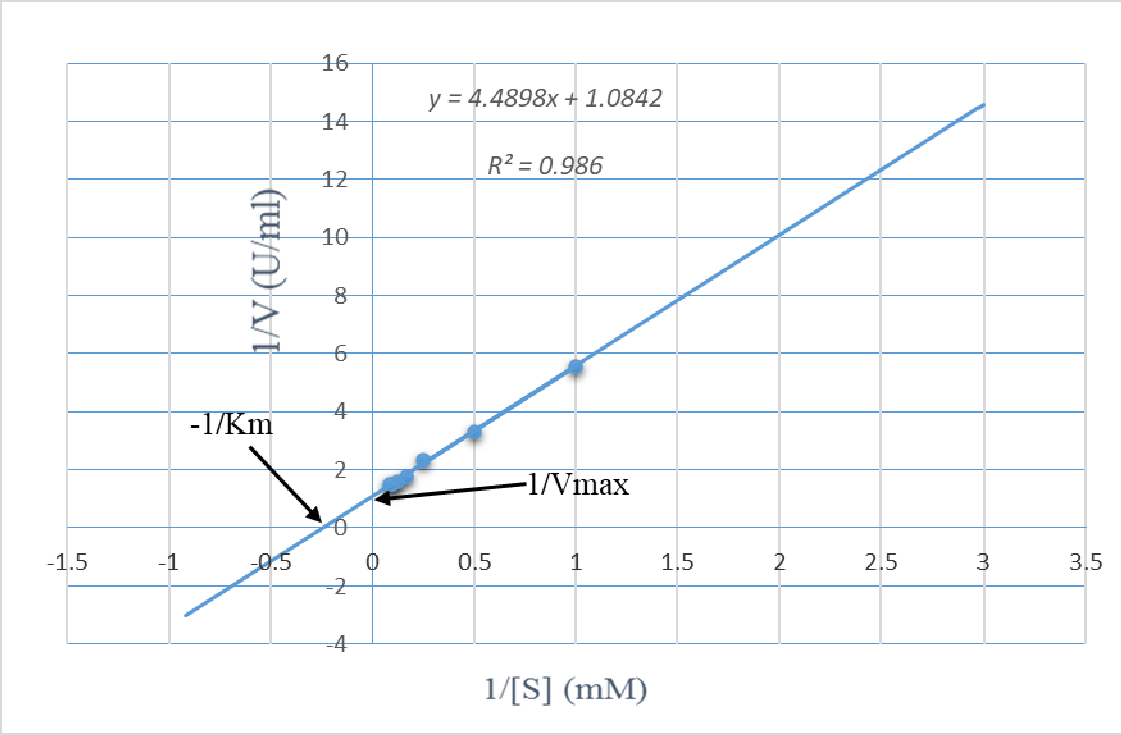

Figure 4 Linweaver Burk plot of laccase activity vs substrate concentration

Laccase has different values of Km and Vmax for different use of substrates kind. Saqib *et al.* (2015) stated that the value calculated of Km and Vmax for laccase were found to be 0.666 mM and 20.8 µM/min, respectively. Shraddha *et al.* (2011) also stated that Km using guaiacol reagent as substrate for laccase found to be 0.405 mM. The Km and Vmax values of *T. versicolor* laccase by using ABTS as a substrate were 73 uM and 780 U/ml, respectively as indicated by Asgher *et al.* (2012). There was significant variation in laccase activity at different guaiacol concentration.

4. Conclusion

Enzyme production is greatly influenced by media components, and hence in current investigation the main growth substrate which was ESD and BS mixture on equal ratio was supplemented with 2% soluble starch, 0.2% peptone and 5 mM $CaCl_2$. Guaiacol was used as a reaction substrate and activity of enzyme was measured by absorbance reading in the UV-Visible spectrophotometer adjusted to 465 nm and it was 0.2187 U/ml. For partial characterization of laccase, a series of experiments was done to check whether its activity is declining or rising following change in substrate conditions as well as concentration. Accordingly, the optimum working pH and temperature of laccase were found to be 6.0 and 50°C, respectively. After 12 hrs incubation at pH 6.0 laccase was found to maintain 71% of its initial activity. Laccase only lost 4 and 12% of its initial activity at 20 and 30°C, respectively, when incubated for 12 hrs at its optimum pH value. Generally, laccases are stable and almost fully and/or partly active in a temperature range of 20-50°C. These characteristics hopefully would make this enzyme potentially attractive in variety of industrial applications like animal feed treatments. There was a lined relation among laccase and its substrate concentration; there is activity increase with substrate concentration increase. The connection amid reaction rate as well as substrate concentration depends on the enzyme affinity for its expressed substrate as Km (Michaelis constant) of the enzyme. The lower the Km, the higher affinity of the enzyme towards its substrate.

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References

Abdulah, A.R. (2008). The use of partially purified laccase produced by some fungal isolates in Aflatoxin B1 degradation. M.Sc. Thesis, University of Baghdad College of Science Department of Biotechnology.

Arora, D.S. and Sharma, R.K. (2010). Ligninolytic fungal laccase and their biotechnological applications. Applied Biochem Biotechnology 160: 1760–1788.

Ashger, M., Nasir Iqbal, H.M. and Asad, M.J. (2012). Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs. Bio Resources 7(1): 1171-1188.

Baldrian, P. (2005). Fungal laccases-occurrence and properties, FEMS Microbiol Rev. 30 (2): 215–42.

Bar, M. (2001). Kinetics and physico-chemical properties of white-rot fungal laccase. Master of Science Thesis, Faculty of Natural and Agricultural Science, Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein.

Castillo, Z.P., Villalonga-Santana, M.L., Tamayo-Cortés, J., Rivera-Muñoz, G. and SolísPereira, S. (2012). Purification and characterization of laccase from *Trametes hirsuta* BM-2 and its contribution to dye and effluent decolorization. African Journal Biotechnology 11(15): 3603-11.

Demir, A., Aytar, P., Gedikli, S., Çabuk, A. and Arisoy M. (2011). Laccase Production with Submerged and Solid-State Fermentation: Benefit and Cost Analysis. Journal of Biology and Chemistry 39(3): 305-313.

Desai, S.S. and Nityanand, C. (2011). Microbial laccases and their applications: Review. Asian Journal of Biotechnology 3(2): 98–124.

Ding, Z., Peng, L., Chen, Y., Zhang, L., Gu, Z., Shi, G. and Zhang, K. (2012). Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. African Journal of Microbiology Research 6(6): 1147-1157.

Elsayed, M.A., Hassan, M.M., Elshafei, A.M., Haroun, B.M. and Othman, A.M. (2012). Optimization of Cultural and Nutritional Parameters for the Production of Laccase by *Pleurotus ostreatus* ARC280. British Biotechnology Journal 2: 115-132.

Hammel, K.E. and Cullen, D. (2008). Role of fungal peroxidases in biological ligninolysis. Current Opinion in Plant Biology 11: 349-355.

Junyao He, X., Ling Ye, Q. and Dong, L. (2014). Enhanced production of an acid-tolerant laccase by cultivation of *Armillariella tabescens*. Journal of Chemical and Pharmaceutical Research 6(1): 240-245.

Kalra, K., Chauhan, R., Shavez, M. and Sachdeva, S. (2013). Isolation of Laccase Producing *Trichoderma* Spp. and Effect of pH and Temperature on Its Activity. International Journal of Chem. Tech. Research 5(5): 2229-2235.

Kersten, P. and Cullen, D. (2007). Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Fungal Genetics and Biology 44: 77-87.

Kumari, J. and Negi, S. (2014). Development of bioprocess for the production of laccase by *Pleurotus ostreatus* MTCC 1802 using evolutionary optimization technique. Indian Journal of Experimental Biology 52: 1106-1111.

Kiiskinen, L.L., Palonen, H., Linder, M., Viikari, L. and Kruus, K. (2004). Laccase from *Melanocarpus albomyces* binds effectively to Cellulose. FEBS Letters 576: 251-255.

Mayer, A.M. and Staples, R.C. (2002). Laccase: New functions for an old enzyme. Phytochemistry 60: 00167-00171.

Naraian, R., Singh, D., Verma, A. and Garg, S.K. (2010). Studies on in vitro degradability of mixed crude enzyme extracts produced from *Pleurotus* spp. Journal of Environmental Biology 31(6): 945-951.

Organization for Economic Co-operation and Development (OECD) (2005). Consensus Document on the Biology of *Pleurotus* spp. (Oyster Mushroom). Environment, Health and Safety Publications Series on Harmonization of Regulatory Oversight in Biotechnology. André-Pascal Press, Paris France 34:10-15.

Othman, A.M., Elshafei, A.M., Hassan, M.M., Haroun, B.M., Elsayed, M.A. and Farrag, A.A. (2014). Purification, Biochemical Characterization and Applications of *Pleurotus ostreatus* ARC280 Laccase. British Microbiology Research Journal 4(12): 1418-1439.

Patel, H., Gupte, S., Gahlout, M. and Gupte, A. (2013). Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. Biotechnology 4: 77–84.

Ping, W., Xuerong, F., Li, C., Qiang, W. and Aihui, Z. (2008). Decolorization of reactive dyes by laccase immobilized in alginate/gelatin blent with PEG. Journal of Environmental Sciences 20: 1519–1522.

Ravikumar, G., Gomathi, D., Kalaiselvi, M. and Uma, C. (2012). Production, purification and partial characterization of Laccase from the mushroom *Hypsizygus ulmarius*. International Journal of Pharma and Bio Sciences 3(3): 355-365.

Saqib, H., Asad, M.J., Gulfraz, M., Asghar, M., Minhas, N.M., Mahmood, R.T., Zakia, S. and Mahmood, N. (2015). Solid State Fermentation for the production of Laccase by *Neurospora sitophila* using agro-wastes and its partial purification. International Journal of Biochemistry and Biotechnology 4 (5): 564-573.

Shraddha, R.S., Sehgal, S., Kamthania, M. and Kumar, A. (2011). Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications. Enzyme Research 2011: 2-11.

Sivakami, V., Ramachandran, B., Srivathsan, J., Kesavaperumal, G., Smily, B. and Kumar, J.M. (2012). Production and optimization of laccase and lignin peroxidase by newly isolated *Pleurotus ostreatus* LIG 19.

Journal of Microbiol Biotechnology Research 2(6): 875-881.

Sivakumar, R., Rajendran, R., Balakumar, C. and Tamilvendan, M. (2010). Isolation, Screening and Optimization of Production Medium for Thermostable Laccase Production from *Ganoderma* sp. International Journal of Engineering Science and Technology 2(12): 7133-7141.

Stoilova, I., Krastanov, A. and Stanchev, V. (2010). Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Advances in Bioscience and Biotechnology 1: 208-215.

Teshome, E. and Melaku, A. (2017). Selection and optimization of lignocellulosic substrate for laccase production from *Pleurotus* species. Int. J. of Biotech. and Molec. Bio. Research 8(4): 38-48.

Thurston, C.F. (1994). The Structure and function of fungal laccase. Microbiology-Sgm 140: 19-26.

Vianello, F., Ragusa, S., Cambria, M.T. and Rigo, A. (2011). A high sensitivity Amperometric biosensor using laccase as biorecognition element. Biosens Bioelectron 21: 2155-60.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P. and Narasimha, G. (2014). Fungal Laccase and their Applications in Bioremediation. Enzyme Research 2014: 1-21.