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Isolation, Purification and Characterization of Laccase from the White-rot Fungus *Trametes Versicolor*

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Running title: **Purification of Laccase produced by *Trametes versicolor***

Abstract
Laccase (benzendiol:oxygen oxidoreductase; E.C.1.10.3.2) is a copper – containing enzyme, part of the group of so-called blue oxidases. The enzyme catalyzes the oxidation of many phenolic compounds and aromatic amines using molecular oxygen as a terminal electron acceptor. Laccases are widely distributed in higher plants, bacteria and fungi. *Trametes versicolor* is a white-rot fungus that produces laccase as extracellular enzyme. In this study different methods for purification of laccase from new isolate of *Trametes versicolor* were tested. Optimal results were achieved when the enzyme was precipitated with 60% acetone followed by size-exclusion chromatography (SEC). The partially purified enzyme showed pH optimum at 5.0 and temperature optimum at 42.5 ˚C. The kinetic constants K_m and V_{max} for syringaldazine were also determined - 0.5 mM and 5000 μM/min respectively. The laccase was stable in broad pH and temperature ranges. Moreover the enzyme remained fully active in 25 % acetone and ethanol for 48 hours. Cd^{2+} inhibited 60 % of enzyme activity.

Practical applications
The wide substrate specificity of laccases is the main reason for their intensive study in last few years. Laccases are successfully used for oxidative detoxification or removal of various aromatic xenobiotics and pollutants found in industrial waste and contaminated soils or water. The enzyme is capable of direct dechlorination, cleavage of aromatic rings of polycyclic aromatic hydrocarbons, decolorization of pulp and dyes. The process includes polymerization among pollutants themselves or copolymerization with other nontoxic substances such as humic materials. Thus polymerized materials often become insoluble or immobilized and easy to remove by using methods as adsorption, sedimentation or filtration.

Key words: laccase, *Trametes versicolor*, isolation, characterization, purification

Introduction

Laccase (benzendiol:oxygen oxidoreductase, EC 1.10.3.2) is a multicopper containing "blue" ligninolytic enzyme, which is able to catalyze the one-electron oxidation of many phenolic compounds with concomitant reduction of oxygen resulting in water formation (Kunamneni et al., 2008a). Laccase is also capable of oxidizing several non-phenolic compounds such as industrial dyes, pesticides, polycyclic aromatic hydrocarbons (PAHs) etc. In the environment laccases are found in many groups of organisms, but fungal laccases are the most studied ones in the last few decades due to their enormous potential for various environmental and industrial applications (Minussi et al., 2007).

The white-rot basidiomycete *Trametes versicolor* has the ability to produce laccase which is part of the ligninolytic enzyme complex along with the manganese-dependent peroxidase.

The aim of the present study is to evaluate the best conditions for purification and characterization of laccase produced by new isolate of *T. versicolor*.

Materials and Methods

All of the chemicals in this section were with analytical grade of purity unless otherwise stated.

Fungal growth and maintenance

Cultural medium

The *Trametes versicolor* isolate was provided from the Department of Biotechnology, University of Food Technologies, Plovdiv. The culture is maintained on Czapek-Dox medium containing sucrose - 30g/dm^3 yeast extract - 5g/dm^3, NaNO□- 2 g/dm^3, K_2HPO_4 - 1g/dm^3, KCl - 0,5 g/dm^3, MgSO□x $7H_2O$ - 0.5 g/dm^3 and $FeSO_4$ x $7H_2O$ - 0.01 g/dm^3 and pH prior sterilization 6.5.

Inoculum preparation and enzyme production

The inoculation was carried out with pre-cultured fungi. For the inoculum preparation and for the cultivation process 500 cm^3 Erlenmeyer flask were used. Each flask contained 100 cm^3 Czapek-Dox medium, sterilized at 121 ˚C for 20 min. The cultured strain must be 7-days old prior the spore suspension preparation. 5% of this suspension of was used for the inoculation of the flasks for vegetative inoculum preparation. After 5 days of cultivation the formed pellets were collected, washed with distillated water and stored in saline solution at 4 ˚C for further needs. The inoculation of the flask for the enzyme production was carried out using 3% vegetative inoculum for each flask. The cultivation was maintained on a rotary shaker with 220 rpm at 28 ˚C for 72 h. At the end of the cultivation the cultural broth was separated from the biomass by filtration on a Buchner funnel. After the separation, cultural broth was concentrated 10 times under vacuum using rotary vacuum evaporator at 40 ˚C and 200 rpm.

Enzyme activities determination and protein determination.

Laccase activity was determined using 0.216 mM syringaldazine in methanol as substrate at 37˚C. The reaction mixture included 100 mM potassium phosphate buffer solution (pH 4.5), 0.216 mM substrate solution and enzyme solution (cultural broth, concentrate or purified). The oxidation of the substrate was monitored at 530 nm using SpectroStar Nano UV-vis spectrophotometer (BMG Labtech). One unit of enzyme activity corresponds to 0.001 change in OD at the reaction conditions and it is expressed in units per cm^3.

The activity of the manganese-dependent peroxidase (MnP) was determined by measuring the oxidation of Mn (II) to Mn (III) at 270 nm, according to Warshii et al.(1989). The reaction mixture contained 1.0 mM $MnSO_4$ in 50mM sodium malonate buffer (pH4.5) and the reaction was initiated by the addition of 0.5 mM H_2O_2 solution. One unit of enzyme activity was defined as the enzyme quantity required to oxidize 1μmol substrate for 1 minute at the reaction conditions.

The total protein concentration was monitored by using the "Bradford" method (Bradford, 1976)

Enzyme purification

Salting-out method

The precipitation process was carried out at room temperature. Ammonium sulfate (saturated solution) was gradually added to the sample with final concentration of 65%; 70%; 75%; 80%; 85%; 90% and the mixture was stirred for 15 minutes and left for 1 hour at room temperature. The precipitate was collected by centrifugation at 6000 rpm for 15 minutes and then resuspended in 100 mM phosphate buffer with pH 4.5 and tested for laccase activity.

Precipitation with organic solvents

This precipitation was carried out using different percentages of acetone, ethanol or methanol (20%, 40%, 60%, 80%) and 2 cm^3 concentrated cultural broth in total volume of 10 cm^3. The samples were then stored at 4 ˚C for 1 hour and centrifuged at 6000 rpm for 30 minutes at 4 ˚C. The supernatant

was discarded and the precipitate was resuspended in 2 cm^3 100 mM phosphate buffer with pH 4.5 and tested for laccase activity.

Precipitation with polyethylene glycol (PEG)
The precipitation was carried out using different percentages of PEG (20%, 40%, 60%, 80%) and 2 cm^3 concentrated cultural broth in total volume of 5 cm^3. The samples were stored at 4 ˚C for 1 hour, and then centrifuged at 6000 rpm at 4 ˚C for 30 minutes. The supernatant was discarded and the precipitate was resuspended in 2 cm^3 100 mM phosphate buffer with pH 4.5 and tested for laccase activity.

Size exclusion chromatography (SEC)
Size exclusion chromatography was performed in Econo-Column (Bio-Rad Laboratories) 55 x 0.8 cm filled with Sephadex G-75 resin. The column was first equilibrated with 20mM sodium phosphate buffer (pH 8.0). The separation was performed at a flow rate of 0.15 cm^3/min using BioLogic LP low-pressure chromatography system equipped with UV detector, conductivity cell and fraction collector (Bio-Rad Laboratories). Fractions of 0.5 cm^3 were collected and assayed for enzyme activities.

Electrophoresis and molecular weight determination
SDS-PAGE of the concentrated cultural broth, acetone precipitated and SEC fractions was performed as described by Laemmli (1970) using 6.0 % stacking and 10 % resolving gels on a Mini-Protean Tetra Cell system (Bio-Rad Laboratories). Precision Plus Protein Standards pre-stained molecular weight markers (Bio-Rad Laboratories) were used to estimate the molecular weight of separated proteins. Samples were boiled for precipitation of the protein and centrifuged at 10000 rpm for 5 minutes. The samples were then dissolved in sample buffer, containing 5% β-mercaptoethanol added prior use and heated at 100 ˚C for 2 minutes. Electrophoresis was carried out at 20mA until the tracking dye reached the bottom of the gel. SDS-PAGE gels were stained using Coomassie Brilliant Blue Stain (Bollag et al., 1996) and silver staining method (Mortz et al., 2001).

Enzyme characterization
Effect of pH on the enzyme activity and stability
For the determination of the pH-dependence of laccase activity phosphate-citrate buffer (100 mM) was used for pH 3.0 and 4.0 and potassium-phosphate buffer (100 mM) was used for the range 4.5 - 8.0. For pH stability evaluation, the enzyme was pre-incubated at room temperature in different buffers at pH 3.0–8.0 for up to 72h. For the determination of pH optimum the pH range 3.0-6.0 was used and for the stability the pH range was 3.0-8.0.

Effect of temperature on the enzyme activity and stability
The temperature profile of the laccase was studied by measuring the activity in a range of 20 – 60 ˚C. The thermal stability of the enzyme was determined by measuring the activity at optimum pH and temperature after pre-incubation of laccase at temperature range of 20 – 60˚C for up to 48h. Samples for analysis were taken every half hour for the first hour, then every hour until the fifth hour, on the 24^{th} and the 48^{th} hour.

Effect of organic solvents on laccase stability
The enzyme stability in the presence of 25% of acetone, ethanol, butanol and hexane was determined by monitoring of the laccase activity every 24 hours for 72 hours.

Effect of heavy metals on laccase activity
The laccase activity was measured after 30 minutes of incubation in the presence of 1, 5 and 10 mM of Zn^{2+}, Cu^{2+}, Cd^{2+} and Mn^{2+} salts at room temperature.

Kinetic constants determination
The laccase activity was measured at substrate concentration ranging from 0.05 – 0.2 mM. K_m and V_{max} were calculated according to Lineweaver and Burk (1934).

Results

Enzyme purification
Precipitations with ammonium sulfate, organic solvents and PEG were performed. Enzyme activity and protein concentration were determined after each stage of precipitation, and the yield was calculated and taken into account for the evaluation of the methods. The summarized results are shown on Figure1.
The results obtained were similar with all percentages used. Only when 75% ammonium sulfate was used the purification was slightly high in comparison with the other results – 1.54 folds.
For the precipitation with organic solvents acetone, ethanol and methanol were used. The most effective organic solvent for protein precipitation

was acetone with 60% concentration where the purification rate reached 4.9 folds.
When polyethylene glycol was used as precipitation agent the best purification rate reached was when 80% concentration of the polymer was used.

Size-exclusion chromatography (SEC)
The partially purified laccase (after 60% acetone precipitation) was subjected to further purification on Sephadex G75. The corresponding elution profile is presented on Figure 2. Protein peaks were identified by their UV absorbance at 280 nm. Laccase activity was detected in fractions 15 to 20 and manganese-dependent peroxidase activity was also detected in the same fractions.

Determination of the molecular weight
SDS-PAGE was performed for molecular weight determination. Figure 3 shows the electrophoretical profiles of the proteins molecular weight standards and the partially purified laccase after SEC. The three protein bands detected had molecular weights 30 kDa, 49 kDa and 80 kDa.

Enzyme characterization
Effect of pH and temperature on the enzyme activity and stability
For the characterization of the partially purified enzyme pH optimum and pH stability were determined as well as the temperature optimum and stability. The optimal pH of the laccase was found to be 5.0 (Fig.4, a) and the optimal temperature (Fig.4, b) was determined to be 42.5˚C.
Figure 5 shows the stability of the enzyme at different pH and temperature values. The laccase remains stable in the pH range 4.0 - 7.0 for the first 5 hours of the experiment displaying over 80% of its activity (Fig. 5, a). The partially purified enzyme was stable in the studied temperature range for the first 5 hours (Fig.5, b). The enzyme kept more than 90 % of its activity after 48 hour at 20 to 40˚C and more than 70 % at 50˚C.

Effect of organic solvents on laccase stability
The effect of acetone, ethanol, butanol and hexane on the stability of the partially purified enzyme is shown on Figure 6. For 48h the enzyme remains stable in the presence of butanol and hexane and slight decrease of the activity was detected in the presence of acetone and ethanol.

Effect of heavy metals on laccase activity
Table 1 presents the effect of metal ions on laccase activity. Cd^{2+} ions inhibited the laccase activity in concentration-dependent manner. At 10mM - 60 % inhibition was recorded. Cu^{2+} ions had no effect on laccase activity. Zn^{2+} and Mn^{2+} inhibited 13% of laccase activity t the highest concentration tested.

Kinetic constants determination
The K_m and V_{max} were determined by measuring the laccase activity at different substrate concentrations and their double reciprocal plot (Fig. 7). The resulted value for K_m was 0.5 mM and for V_{max} - 5000 μM/min.

Discussion
Laccase produced by *Trametes versicolor* in Czapek-Dox medium was purified and characterized. The purification and characterization are necessary steps in order to assess the enzyme suitability for industrial applications. Industrial processes require more resistant enzymes for extreme temperatures and pH.
Even the ammonium sulfate precipitation is a widely used method for enzyme purification (Saito et al., 2003; More et al., 2011) the results show that it's not efficient for the purposes of the present study. Acetone seems to be the best precipitation solvent which is in agreement with other studies on laccase purification (Sun et al., 2013; Bryjak et al., 2010; Marques De Souza and Peralta, 2003).
The laccase molecule, as an active holoenzyme form, is a dimeric or tetrameric glycoprotein, usually containing -per monomer- four copper (Cu) atoms bound to three redox sites (Kunamneni et al., 2008b). The molecular mass of the monomer ranges from about 50 to 100 kDa. In the present study 3 protein bands were detected corresponding to 30, 49 and 80 kDa. Several laccase isoenzymes have been detected in many fungal species. More than one isoenzyme is produced in most white-rot fungi with molecular weights between 60 and 100 kDa (Bertrand et al., 2015). *T. versicolor* produces manganese-dependent peroxidase (MnP) as a part of its lignin-degrading enzyme complex. This enzyme has molecular weight ranging from 30 – 40 kDa (Paice et al., 1993). The protein band at 30 kDa belongs to the MnP and the other two bands belong to laccase isoforms.
The pH and temperature optimum for the isolated enzyme were determined. The laccase showed optimal activity when pH equals 5.0 and at 42.5˚C. Laccases isolated from *T. versicolor* strains have shown pH optima between 4.0 and 5.0 and

temperature optimums around 40 ˚C (Minussi et al., 2007; Han et al., 2005), which correlates with the results obtained.

The application of an enzyme depends on its stability in harsh environmental conditions. The proteinous nature of the enzyme determines its susceptibilities to long exposure to extreme temperatures and pH. The enzyme has shown stability at harsh conditions such as high temperature and different pH values. Laccase was stable at 60 ˚C for 5 hours. The laccase was inactivated immediately when placed in buffer with pH 3.0. At the other used pH values the enzyme kept around 100% of its relative activity up to the 5^{th} hour after which the activity decreases slowly.

Generally, high organic solvent concentrations cause laccase denaturation (Stoilova et al., 2010). In the present study, the partially purified laccase displayed extremely high organic solvent stability. The enzyme lost around 20 to 40 % of its relative activity when placed in solution with acetone and ethanol and remained stable for 48^{th} hours in presence of butanol and hexane.

Four metal ion compounds were used in different concentration for the determination of their effect on the enzyme activity. In this experiment $CdCl_2$ showed the biggest inhibition activity over the enzyme where in concentration of 8mM it's able to show 8% inhibition, and when the concentration was 10mM the inhibition increases to 60%.

K_m is equal to the substrate concentration at which the reaction rate is half its maximum value. Therefore, if an enzyme has a small value of K_m, it achieves its maximum catalytic efficiency at low substrate concentrations. Different syringaldazyne concentrations were used for determination of K_m and V_{max} of the partially purified enzyme. Figure 6 illustrates the Lineweaver-Burk plot for representation of enzyme kinetics. With this method K_m was found to be 0.5 mM and V_{max} value was 5000 μM/min

Conclusion

Laccase produced by *Trametes versicolor* was purified and characterized. Different methods for purification were tested. 60% acetone precipitation showed the highest purification fold. SEC was used for further purification. However, more purification steps are needed. The electrophoretical profile revealed 3 protein fractions with molecular weights respectively - 30, 49 and 80 kDa. Partially purified enzyme had pH optimum at pH 5.0 and temperature optimum at 42.5˚C. The laccase was stable in broad pH and temperature ranges. Moreover the enzyme remained fully active in 25 % acetone and ethanol for 48 hours. Cd^{2+} inhibited 60 % of enzyme activity. The kinetic constants of laccase activity using syringaldazyne as substrate were also determined. The stability of the enzyme in organic solvents is of great value when it comes to degradation of persistent environmental pollutants (e.g. PAHs, phenol, organophosphorus pesticides) which are usually hydrophobic.

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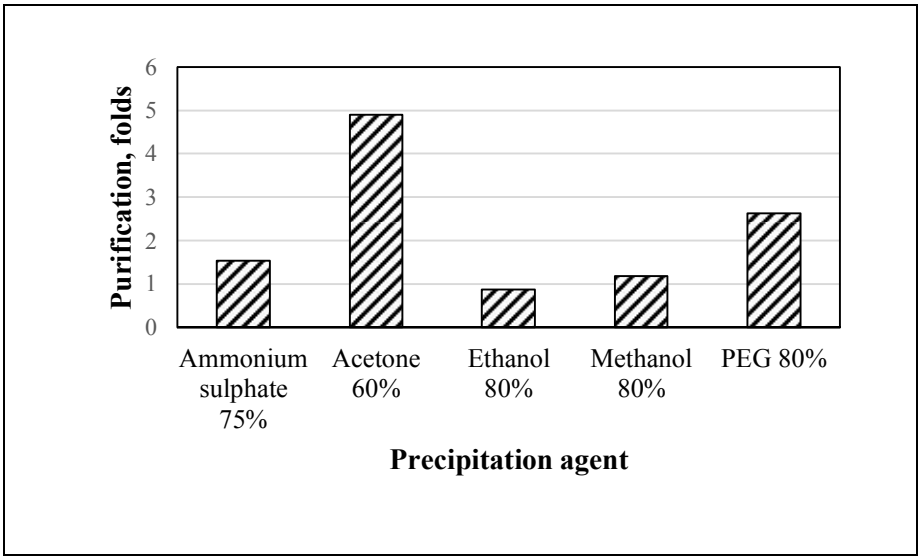


Figure 1. Comparison between used precipitation techniques based on the purification folds

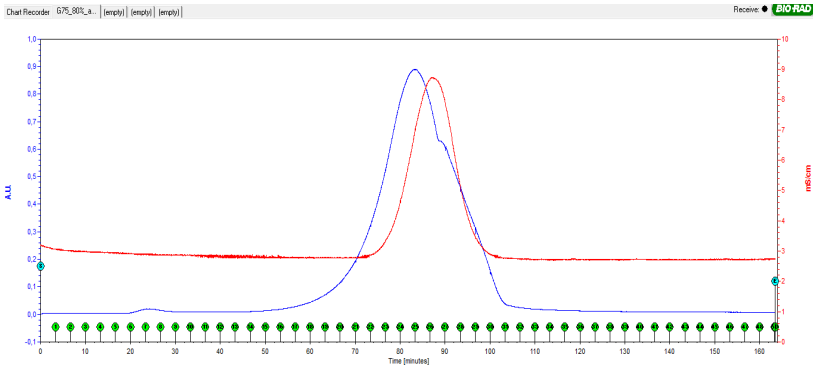


Figure.2 Laccase SEC elution profile

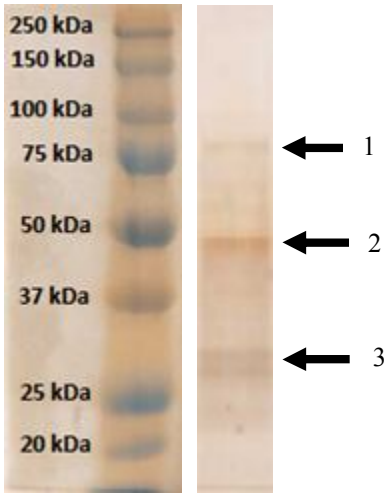
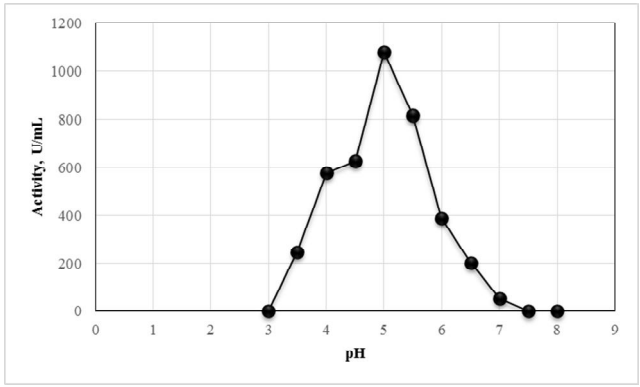
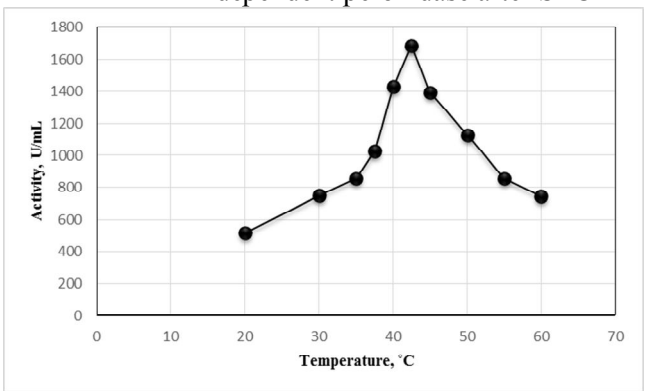


Figure.3 SDS-PAGE after silver staining: A- Molecular weight standards, B-1,2 Laccase isoforms, 3- Manganese-dependent peroxidase after SEC

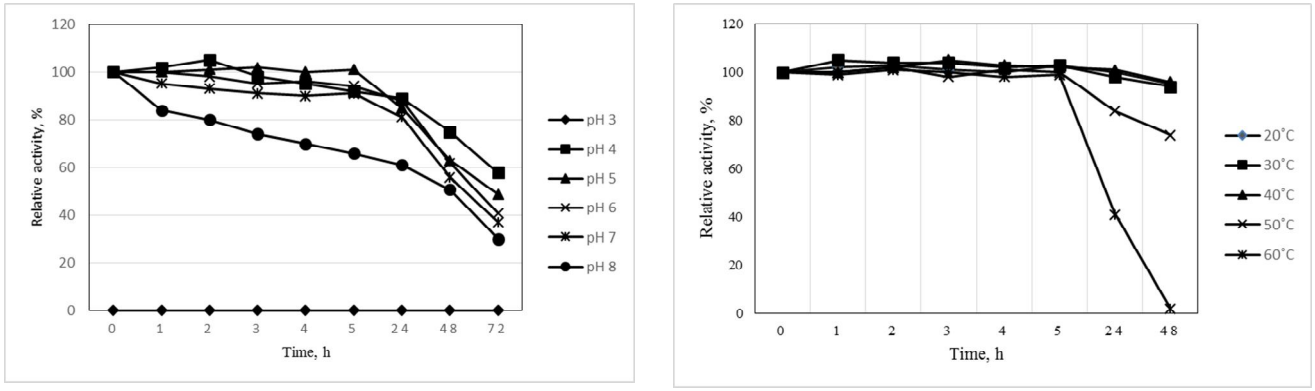


a)



b)

Figure 4. Characterization of Laccase: a) pH optimum determination; b) Temperature optimum determination



a) **b)**

Figure 5. Characterization of Laccase: a) pH and b) temperature effect on laccase stability

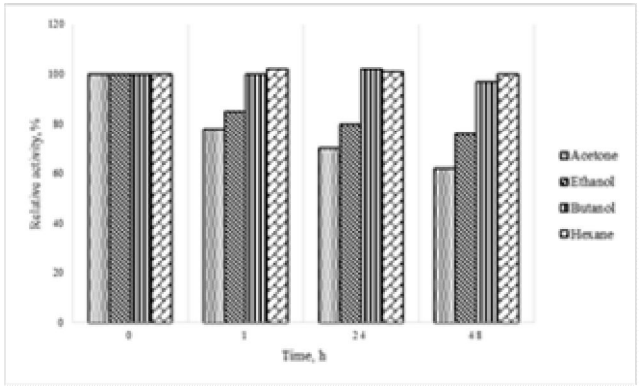


Figure 6. Effect of organic solvents on laccase stability

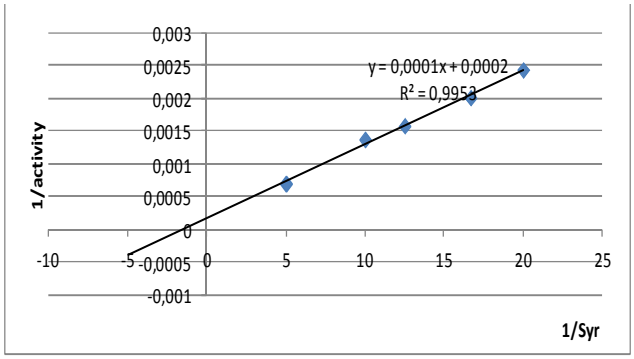


Figure 7. Lineweaver–Burk plot of laccase kinetic

Table 1. Effect of metal ions on Laccase activity

Compound, concentration, mM	Laccase activity, U/cm3	Inhibition, %
Control	1312,52	
$ZnSO_4$ 1 mM	1283,08	2
$ZnSO_4$ 5 mM	1140,48	13
$ZnSO_4$ 10 mM	1143,72	13
$CuSO_4$ 1 mM	1305,92	1
$CuSO_4$ 5 mM	1308,84	0
$CuSO_4$ 10 mM	1302,36	1
$CdCl_2$ 1 mM	1207,08	8
$CdCl_2$ 5 mM	864,64	34
$CdCl_2$ 10 mM	520,44	60
$MnSO_4$ 1 mM	1283,08	2
$MnSO_4$ 5 mM	1240,48	5
$MnSO_4$ 10 mM	1141,62	13