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Full Length Research Paper

Purification and characterization of Laccase Enzyme from a Novel Species of Ascomycete

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Abstract
Laccases are one of the most important enzymes which has got potent environmental applications. The present work aims at the purification of one of the isoenzymes of laccase from a potent strain of ascomycete sp. Consistent laccase activity was detected in the culture supernatant of a newly isolated species of ascomycete. Ricebran has shown remarkable inducing effect on the production of laccase when compared with the control medium. One of the laccase isoenzymes produced by this Ascomycete was purified to homogeneity by gel filtration chromatography. The active purified protein showed an apparent molecular weight of 74 KDa. The purified laccase isoenzyme was stable at room temperature and alkaline pH. Chemical characterization was also conducted to find the stability of the purified enzyme. Inhibition of purified laccase at different concentrations of EDTA was also studied.

Key words: *Laccase, Isoenzyme, Ascomycete, Ricebran, Gel permeation Chromatography.*

Introduction

The existence of laccase enzyme was first demonstrated by Yoshida (1883) in a plant, the Japanese lacquer tree *Rhus vernicifera*. As stated in review by Thurston (1994) and Yaropolov et al. (1994). Laccase (p-diphenol : Oxygen oxidoreductase; EC 1.10.3.2) is a copper containing enzyme that catalyzes the oxidation of a phenolic substarte by coupling it to the reduction of oxygen to water. Fungal; laccases display a wide substrate range, are known to catalyze the polymerization, depolymerization, and methylation and/or demethylation of phenolic compounds (1, 2) and may play role in plant pathogenity (3,4) or lignin degradation. Laccase is a dimeric or tetrameric glycoprotein, which usually contain four copper atoms per monomer distributed in these redox sites (Gianfreda et al., 1999; Baldran, 2006).The four Cu atoms differ from each other in their characteristic electronic paramagnetic resonance (EPR) signals; type 1and Type 2 Cu have strong electronic adsorption and well characterized EPR signals. In the presence of an appropriate redox mediator , such as 2,2'-azino –bis (3-ethylbenzothiazoline-6-sulfoanate) (ABTS) or 1-hydroxybenzotrizole (HBT), laccase also catalyze the oxidation of non phenolic lignin mode compounds (6) and degrades polcyclic aromatic hydrocarbons (7) and various dye pollutants. Therefore, these enzymes have recently been attracting broad attention owing to their potential industrial application in biopulping textile dye bleaching, the degradation of aromatic pollutants and detoxification of polluted water (8, 9, 6, 10, and 11). Thus fungal strains with a high level of enzymatic activity and substrate affinity are essential for the further application of biotechnology. Accordingly this study describes the purification and characterization of the laccase from the culture supernatant of novel species of Ascomycete strain.

Materials and Methods

Microorganisms and Culture conditions.

A novel species of Ascomycete strain was grown on modified VMM at 27°C for 5 days and maintained at 4 °C. Microscopic characteristics were studied by slide culture technique. For laccase production, Fungus was inoculated into modified VMM and in inducer supplemented medium. Here rice bran was used as an inducer.

Plate Assays

Laccase activity was visualised from modified VMM plates containing different substrates such as (Guaiacol, p- Coumaric Acid, Catechol and ABTS) at 27°C for 4 days.

Enzyme and Protein Assays

Laccase activity was also studied from modified VMM and the medium supplemented with inducer. Laccase activity was determined using ABTS as the substrate. The assay mixture contains 2 mM ABTS .1 M sodium citrate buffer (pH 3) and enzyme samples. Oxidation of ABTS was followed by absorbance increase at 420 nm (E = 36,000 M-1 CM -1). Enzyme activity was expressed in Iu . The protein concentration was estimated using Lowry's method, with bovine serum albumin as standard.

Laccase Purification

Unless, otherwise stated, all the procedures were performed at 4°C. On the 25th day, the Culture supernatants from with and without inducer were filtered through a sterile Whatsman No: 1 filter paper. Ammonium Sulfate was added to the both supernatants to give 20%, 40%, 60%, 80%, 100% saturation and Precipitated proteins were collected by Centrifugation at 10,000

rpm for 30 minutes. Enzyme assay and protein estimation of both pellet and supernatant was conducted. The precipitate was then dissolved in an appropriate volume of 0.01M pH5 citrate buffer, dialyzed overnight against the same buffer and concentrated by ultra fitration using a 10 KDa ultra filtration membrane (Amicon, Millpore) and samples were also subjected for lyophilisation. Analysis of isoenzyme as well as enzyme profile was carried out by native and SDS PAGE. After native PAGE, the gels were stained with guaiacol and coomassie brilliant blue R-250. After SDS PAGE, the gels were stained with coomassie brilliant blue R-250 and silver staining. The fraction containing laccase activity was then loaded on to a Sephadex G-50 gel filtration column, pre equilliberated with a 0.01 M pH 5 citrate buffer and eluted with the same buffer at a flow rate of 1.5-2 ml /min. The fractional containing laccase activity was collected, concentrated and used as the purified enzyme preparation.

Molecular Mass Determination

The molecular mass of the enzyme was estimated by SDS PAGE by using a protein marker broad range. After electrophoresis, the gels were stained with silver stain.

Effect of Metal ions and Inhibitors

The activity of laccase was measured using ABTS in the presence of several metal ions, with different molar concentration of NaOH, NaCl and HCl for 1 hour at 4°C. Inhibitory studies were performed by using different molarities of EDTA for 15 minutes at 4°C.

pH and temperature Dependence

The effect of pH on the enzyme stability was measured after 1 hr of incubation at various pHs. The effect of temperature on the enzyme stability was investigated by incubating the enzyme solution for 1hr at various temperatures. After incubation, the remaining activity was determined.

Results and Discussion

White cottony growth was observed on modified VMM agar plates and organism grown on media had dry appearance with entire margin (Fig. 1).

Fig. 1. Growth pattern of the organism on Modified VMM Agar Plate

Creamish white cottony growth was observed on modified VMM media and medium with inducer .(Fig12 &13) In inducer supplemented media, abundant growth was observed. The induced growth of the organism was due to nutritive components present in the Rice bran. It contains significant quantities of starch, proteins, vitamins and minerals. It acts as a complex inducer with 12-13% oil and high level of gum. It also contains ferulic acid and pectin which are known inducers of laccases. Ferulic acid double the production of the enzyme in both *T. versicolor* and *P. ostreatus* and a 3-4 fold increase in enzyme production was observed in *P. mutabilis* (Gianfreda et al., 1999). On 2-3 days of incubation, oxidation zones were observed on modified VMM agar plates with specific substrates. Pedro et al. (1993) demonstrated that the lignin degrading basidiomycetes PM1 (CECT 2971) catalyses the oxidative polymerization of guaiacol to form reddish brown zones in the medium. o- diphenols such as catechol, pyrogallol, guaiacol, protocatechic acid, gallic acid and caffeic acid are known substrates of laccase (Faure et al., 1994). ABTS is a very suitable to screening because its one electron oxidation products are soluble in H2O (Paice et al., 1995; Archibald, 1997). In Botrytis cinera coumaric acid or pectin acted as second inducers when a phenolic substrate such as gallic acid was added to the culture medium (Gianfreda et al., 1999). (Fig. 4, 5, 6, 7, 8, 9).

Fig. 12. Growth of the organism in Production Media without Inducer

Fig. 13. Growth of the organism in Production Media with Ricebran as Inducer

Fig. 4. Growth of the organism on Guaiacol Supplemented Media

Fig. 5. Oxidation of Guaiacol by the Laccase Enzyme

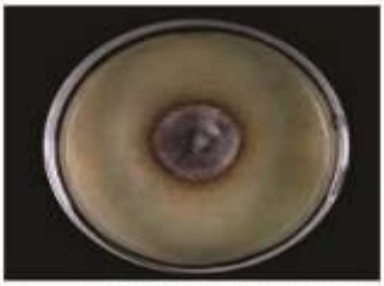


Fig. 6. Growth of the organism on Catechol Supplemented Media

Fig. 7. Oxidation of Catechol by the Laccase Enzyme

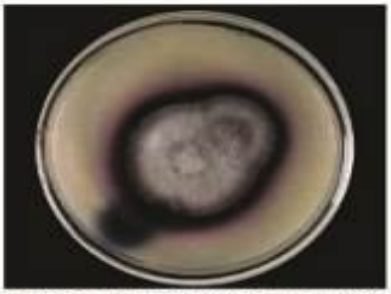


Fig. 8. Growth of the organism on ABTS Supplemented Media

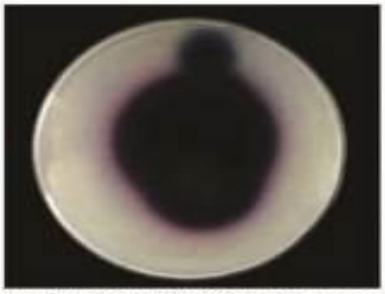
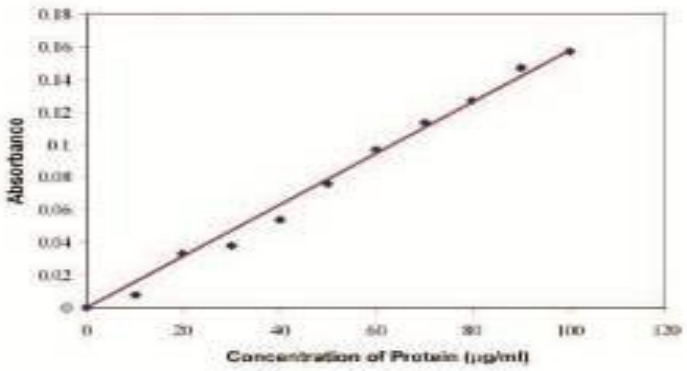


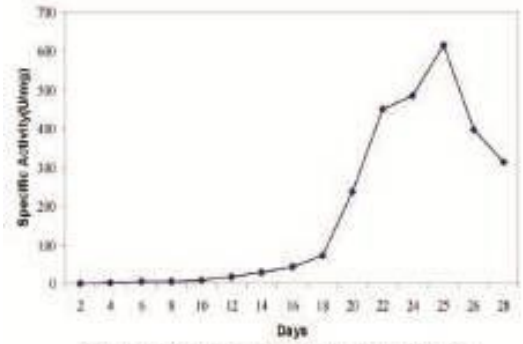
Fig. 9. Oxidation of ABTS by the Laccase Enzyme

Laccase assay was conducted using ABTS as substrate and volume activity was calculated using the formula. The protein estimation was also conducted and the protein content was calculated from the standard graph (Graph 1)

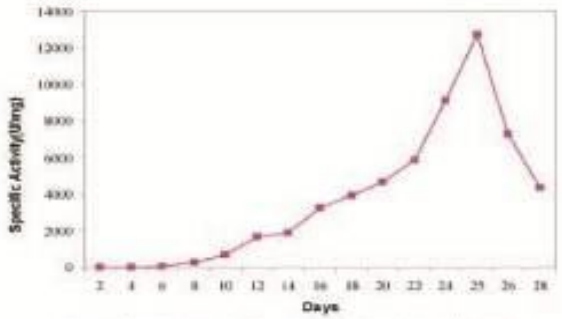


Graph 1. Standard Graph of Protein

Specific activity was calculated for samples taken from modified VMM and inducer supplemented media. It was observed that when inducer was supplemented, there was 20 fold increases in specific activity of the enzyme. The logarithmic increase in specific activity of both media is represented in Graph 2 and 3.



Graph 2. Specific Activity of the Enzyme without Induction



Graph 3. Specific Activity of the Enzyme when Rice Bran is supplemented as Inducer

Laccase is an inducible enzyme. The production of the enzyme is dependent not only on the type of fungal strain but also on the growth conditions employed. The presence or absence of inducers, induction time, nature and composition of culture medium, type of culture conditions and genetic manipulation can affect the enzyme production (Gianfreda et al., 1999). On ultrafiltration the protein content of without inducer was concentrated 5 times and that of inducer supplemented media by 3 times. The purity of the concentrated laccase enzyme was estimated by spectrum analysis. One of the standards of the purity was satisfied by ultra filtrated samples. . The ratio of the absorbance at 280 nm to that at 600 nm is generally 14-30 and the ratio of the absorbance at 330 nm to that at 600 nm is 0.5-2.0 (Wahleithner et al., 1996)

Purification

Ammonium Sulfate precipitation was carried out from with and without inducer supplemented medium. It was observed that majority of enzyme was precipitated in 60 and 80 % fraction in both medium. But in without inducer, maximum specific activity was obtained in 80% fraction (7472 U/mg). In inducer supplemented medium, maximum specific activity was found in 60% fraction (9206.28 U/mg). After dialysis samples were observed to dilute about 1-5 times and on lyophilization, the protein was more concentrated then dialyzed samples. Native PAGE was conducted from without inducer supplemented medium. On guaiacol staining it was observed that two bands developed for 60% fraction and three clear bands for 80% fraction. On Coommassie staining, it was observed that fraction of ammonium sulfate precipitation containing protein other than laccase and has subjected to further purification techniques (Figure 16).

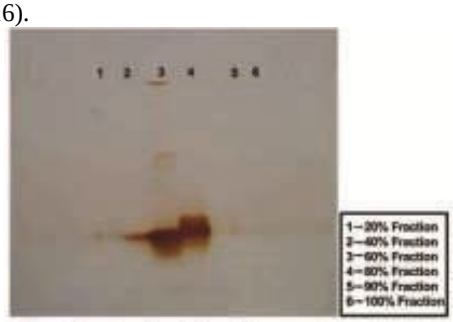


Fig. 16. Guaiacol staining of Isoenzymes of Laccase from the Media with Inducer

Guaiacol staining was also conducted with inducer supplemented medium. It was observed that 60% fraction was comparatively pure it contains only one isoenzyme, where as five bands were observed for 80% fraction. Thus a change was observed in the isoenzyme pattern of the enzyme when inducer was supplemented to the media.

Different aromatic compounds can induce laccase production differentially with respect to expression level and isoenzyme composition (Scheel et al., 2000). On coommassie staining it was observed that there was no other major contaminating protein in the fractions, the number of bands with guaiacol staining and Coommassie staining was similar. (Figure 17).

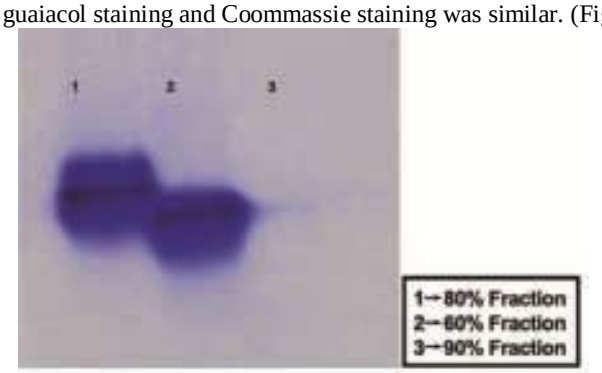


Fig. 17. Coomassie staining of Enzymes from the Media with Inducer

SDS PAGE was conducted with both medium. When the same concentration of the protein was subjected to coommassie and silver staining, it was observed that silver staining was found to be more sensitive to protein at low levels (Fig. 18 , 19).

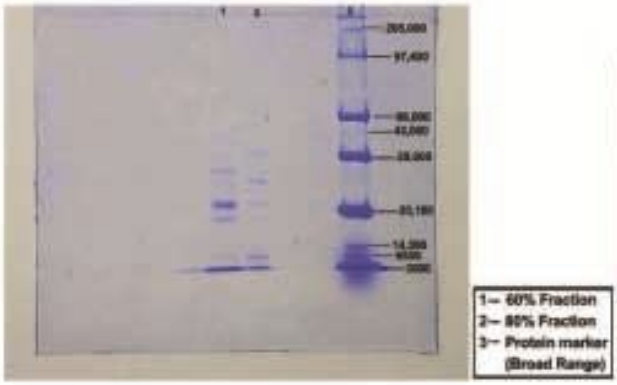


Fig. 18. Coomassie staining of Enzymes from the Media without Inducer

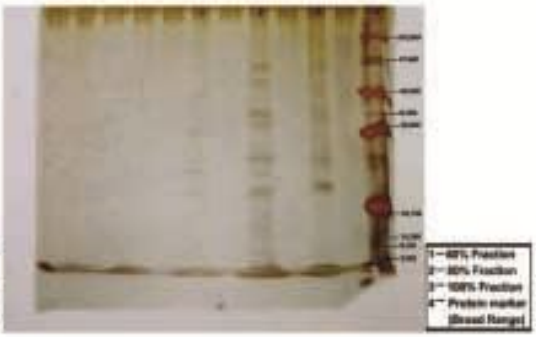


Fig. 19. Silver staining of Enzymes from the Media without Inducer

But in inducer supplemented medium, the number of bands for the 60% fraction was same in both staining techniques. (Fig 20,21).

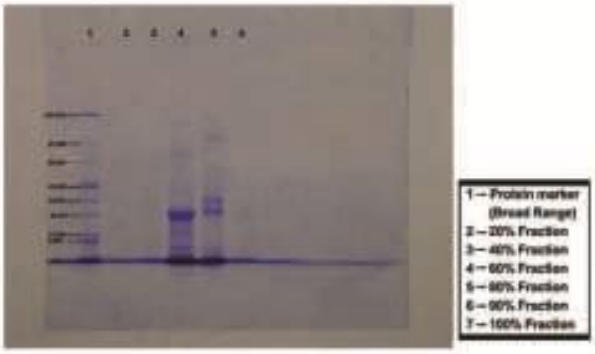


Fig. 20. Coomassie staining of Enzymes from the Media with Inducer

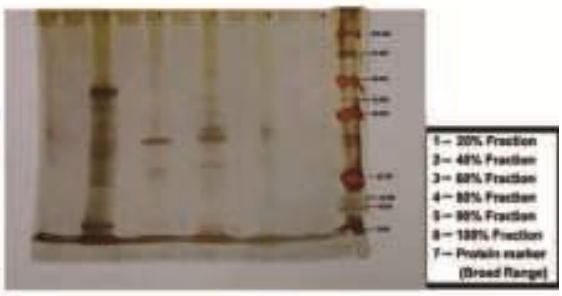


Fig. 21. Silver staining of Enzymes from the Media with Inducer

Study of isoenzyme profile was conducted, it was observed that all the isoenzymes obtained by fractional precipitation at different satutration level of ammonium sulphate were same as that obtained by ultrafiltration. In Ultrafiltration, the protein in the

supernatant was concentrated and thus a complete enzyme profile was obtained. T. gallica produce two laccase isoenzymes when tryptone and glucose was supplemented in the medium (Jia Li Dong and Yi Zheng Zhang, 2004) (Fig 22)

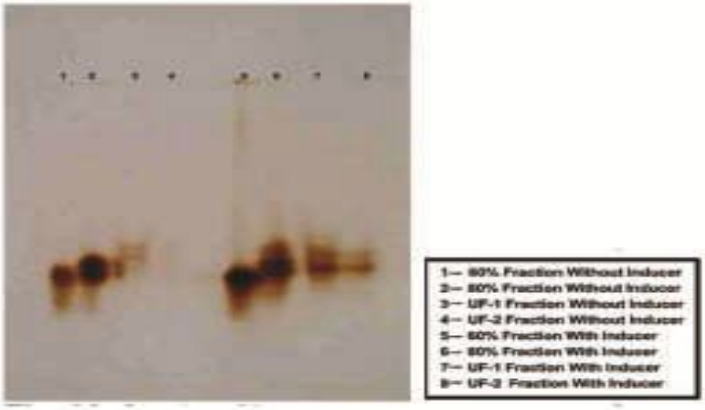


Fig. 22. Study of Isoenzyme Profile

GPC was conducted, It was found that the first and fifth fraction was having maximum specific activity. The GPC results showed the presence of contaminating proteins in the fractions from 6-10. (Table -1).

Table 1 GPC of the Purified Protein from the fractions(1-10)

Fractions	Enzyme Activity(U/mg)	Protein(mg/ml)	Specific Activity(u/mg)
1	6,833	0.4	17,082.5
2	7,430	1	7,430
3	9,625	1.8	5,347.22
4	2,000	1	2,000
5	3,388	0.2	16,940
6	694.4	2.2	315.63
7	236.11	4.2	56.21
8	3,233	3.6	898.06
9	450	2.2	264.54
10	466.7	2.4	194.45

Native page of purified protein was conducted it was confirmed that same isoenzyme was present in all fractions. (Fig 23).

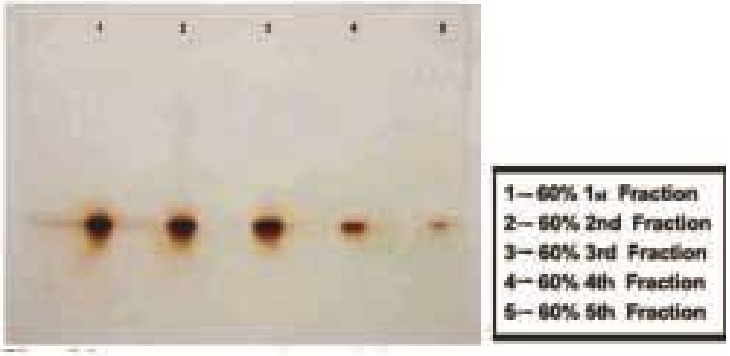


Fig. 23. Native PAGE of Purified Fractions

SDS PAGE of purified protein was also conducted and on silver stanining it was concluded that protein was tetrameric. The laccase molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomers distributed in three redox sites (Gianfreda et al., 1999) (Fig – 24).

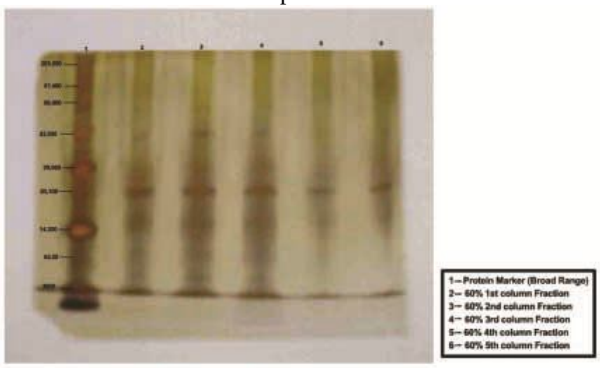
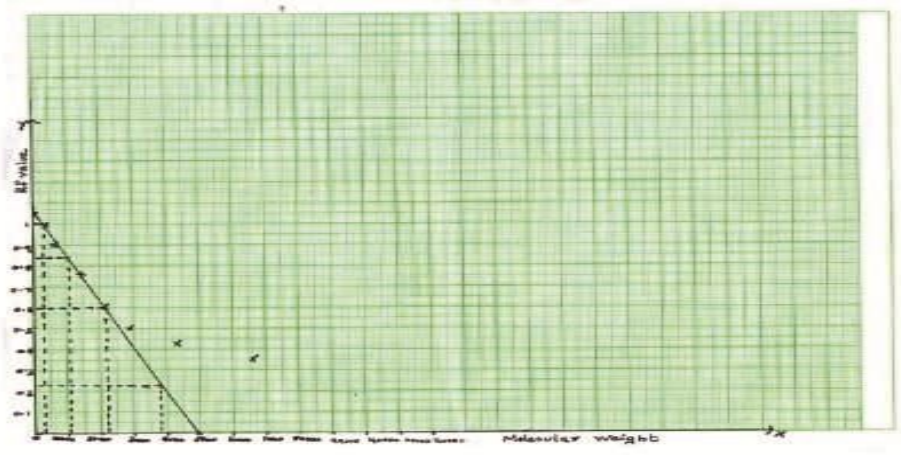


Fig. 24. SDS PAGE of Purified Fractions

On spectrum analysis the purified protein found to satisfy one of the standards of the purity. The molecular weight of the protein was extrapolated from its Rf value. The molecular weight of the purified protein was approximately 74 KDa (Graph 6) The reports about molecular weight of laccase from different fungal source suggest that it can be within a range of 60-100 KDa (Benny Cheeftz, 1998). A 70 KDa extracellular laccase was purified from the rice blast fungus Magnaportha grisea using gel filtration and ion exchange chromatography (Gopal Iyer and Chatoo, 2003). Molecular mass of purified lacccase B from Trametes sp. AH28-2 is determined to be 74 KDa by SDS–PAGE which is larger than that of laccase A (Xiao et al., 2003). Enzyme yield and purification fold of the enzyme was calculated (Table – 2). After GPC the protein content was too less (0.4 mg/ml) as compared to culture supernatant and ultrafiltration. Thus it was concluded that after GPC all the
contaminating proteins are separated from the specific enzyme.

Table 2: Enzyme yield and Purification fold of the purified enzyme.

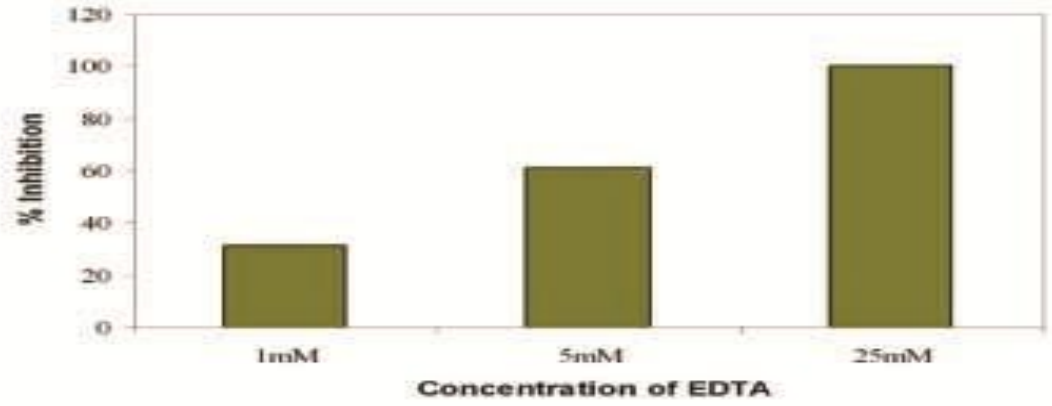
S. No.	Samples	Enzyme Activity	Protein	Specific Activity	Yield	Purification Fold
1	Culture Supernatant	17,341.1	1.36	12751.25	100%	
2	GPC	6833	0.4	17082.5	39.4%	1.33
3	UF	8812.5	4	2203.12	50%	0.17



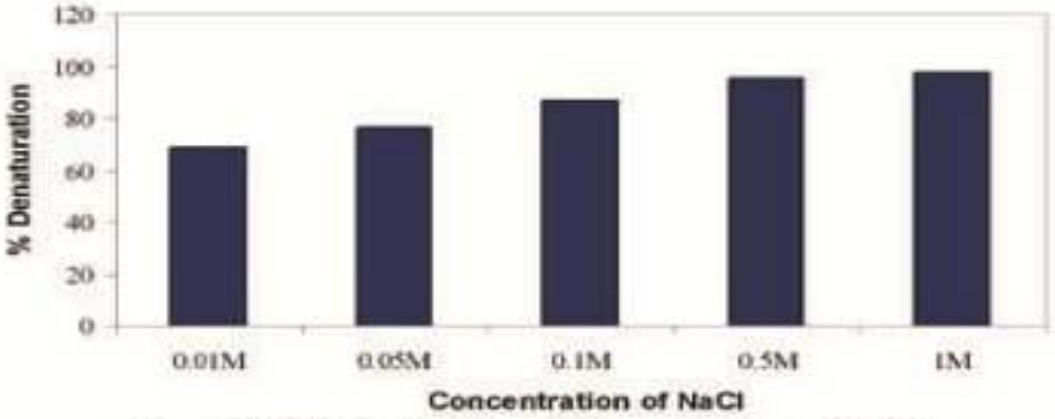
Graph 6. Determination of Molecular Weight of Laccase

Effect of Metal Ions and Inhibitors

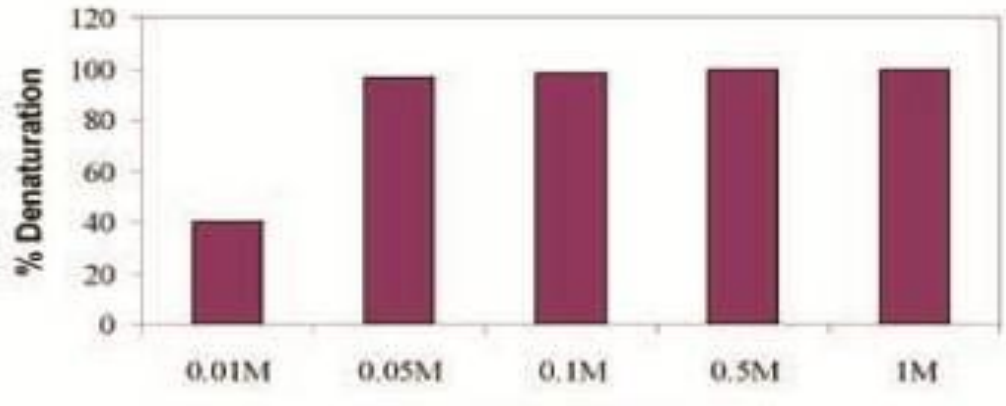
The enzyme was strongly inhibitted in 25 mM EDTA (100% Inhibition) but slightly stimulated by 1 mM and 5 mM EDTA.(Graph 7) The enzyme was essentially unaffected by 0.01 M NaCl, 0.01 M NaOH and in 0.01 N HCl and enzyme was inhibitted completely when concentration of chemical increases. These result were also very similar to those previously reported for the laccase from chaemotium thermophilum (Benny Cheeftz et al., 1998). (Graph 8,9 &10)



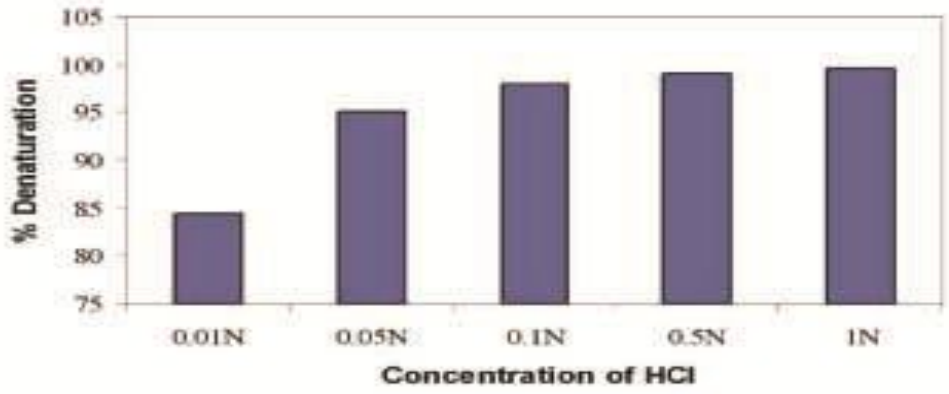
Graph 7. Effect of EDTA on Purified Laccase



Graph 8. Effect of Different Concentrations of NaCl on Purified Laccase Enzyme



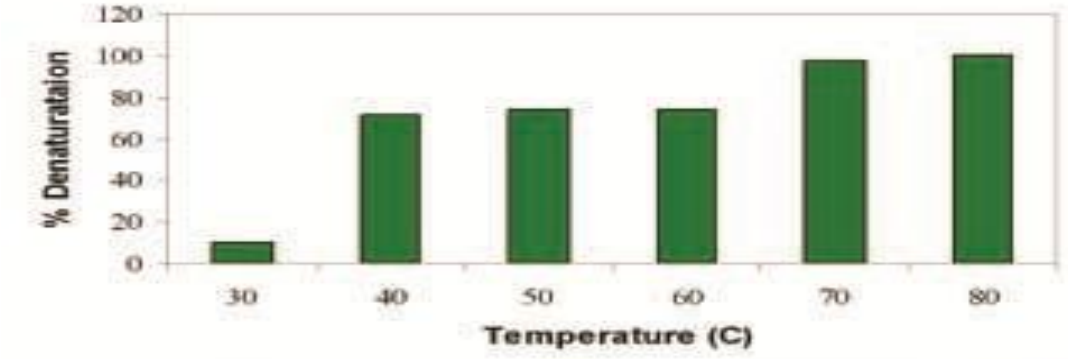
Graph 9. Effect of Different Concentrations of NaOH on Purified Laccase Enzyme



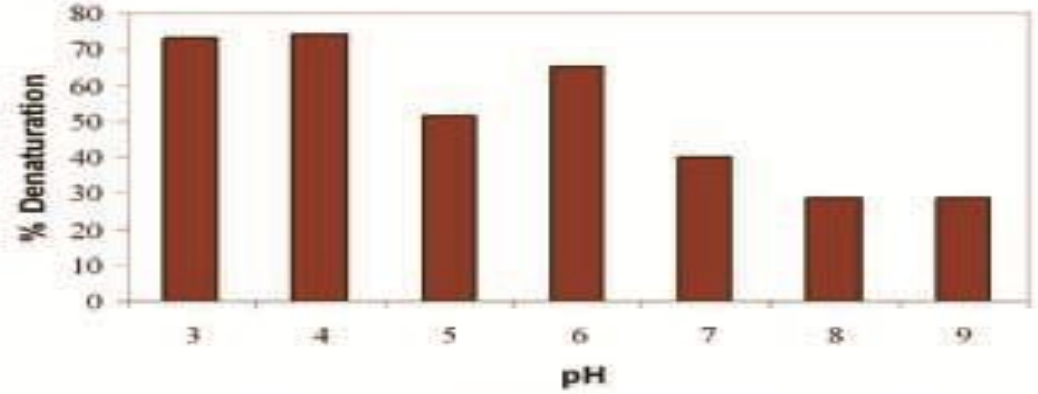
Graph 10. Effect of Different Concentrations of HCl on Purified Laccase Enzyme

Effect of pH and temperature on enzyme activity and stability

When the effect of pH on the enzyme stability was examined at 4°C for 1 hr, the enzyme remained stable with alkaline pH range from 7.0 to 9.0.(Graph12). The enzyme was completely stable at 30°C, Yet the stability decreases rapidly above 4°C and this was also similar to the results previously reported for the laccase from Trametes trogii and from chaemotium thermophilum . (Zouari et al., 2006, Benny Chefetez et al., 1998). (Graph 11) Thus the enzyme from the strain can be used in various biotechnological applications where stability at alkaline pH is demanded



Graph 11. Effect of Different Temperatures on the Purified Enzyme



Graph 12. Effect of Different pH on Purified Laccase Enzyme

Summary and Conclusions

- The Ascomycete strain was grown on substrate supplemented media (ABTS,Guaiacol and Catechol) and oxidation of the substrates was observed on the modified VMM plates.
- The activity was increased to 20 fold when Rice bran was supplemented as inducer to modified VMM production media.
- On purification and study of isoenzyme profile, it was observed that there was a change in the isoenzyme pattern when Rice bran was supplemented.
- One of the isoenzyme was highly enhanced by the inducing effect of Rice bran.
- After Gel Permeation Chromatography, all the contaminating proteins were removed from the active enzyme.
- The purified protein had a molecular weight of approximately 74 KDa.
- On characterization of the enzyme it was observed that the enzyme was stable at an alkaline pH and had good stability at room temperature.
- 31% activity was retained after incubation for 1 hour at 4ºC in 0.01 M NaCl, 60% activity was retained after 1 hour in 0.01M NaOH and 16% activity was retained after 1 hour in 0.01N HCl.
- The purified laccase was strongly inhibited by 25mM EDTA concentration after incubation for 15 minutes at 4ºC.

Thus the enzyme from the strain can be used in various biotechnological applications where stability at alkaline pH is demanded

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