Investigation of Catalytic Properties of Manganese Peroxidase (MnP) Produced from *Agaricus bisporus A21* and its Potential Application in the Biotransformation of Xenobiotic Compound

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(Received on 17th October 2014, accepted in revised form 27th April 2015)

Summary: In this study Manganese peroxidase, from novel fungus *Agaricus bisporus A21* was purified, thermally characterized and its catalytic properties were investigated. The four step purification procedure i.e., ammonium sulphate precipitation, dialysis, ion exchange and gel filtration chromatography yielded 6.9 % activity with a purification factor of 8.48. The optimum temperature and pH of Manganese peroxidase for the oxidation of Manganese peroxidase were 40˚C and pH 6.0 respectively and remain active within the pH range of 3-10 after 24 h.The catalysis of $MnSO_4$ by Manganese peroxidase was expressed by the Michaelis-Menten equation, suggesting that the maximum velocity (V_{max}) was 231 U/mL and the Michaelis constant was 3.33mM revealing a binding site with higher substrate affinity. Enthalpy of activation decreased where Free energy of activation for thermal denaturation increased at higher temperatures. The partially purified manganese peroxidase shows excellent decolorization potential for Orange G (from 19.32 to 96 %) by the addition of natural mediator of wheat bran. UV–Vis spectrum and HPLC chromatogram clearly revealed that orange G transformed into different products. Thermostability and efficient decolorization suggest that this enzyme could be receiving substantial attention for its potential application in the biotransformation of organo-pollutants.

Key words: MnP, Catalytic properties, Orange G, *Agaricus bisporus A21*, Kinetic and Thermo-stability.

Introduction

Lignin modifying enzymes are involved in the biotransformation of various xenobiotic compounds, and dyes. This group of enzymes is highly versatile in nature and they find application in a wide variety of industries [1] Their capacities to remove xenobiotic substances and produce polymeric products make them a useful tool for bioremediation purposes [2].

Manganese Peroxidase (EC 1.11.1.13) is an extracellular enzyme, belongs to the family of oxidoreductases, produced by various micro-organism. MnP seem to be most extensive among white rot fungi than lignin-peroxidase [3] manganese peroxidase (MnP) oxidizes Mn^{2+} to Mn^{3+}, which turn the structure of phenolic to phenoxyl radicals [4]. Mn^{3+} is extremely reactive and complex with chelating organic acid, as oxalate or malate, which is synthesized by the fungus [5].Manganese peroxidases have lower redox potential then that of LiPs and preferably oxidize in vitro phenolic substrates. It can nonspecifically oxidize a variety of phenolic and non-phenolic substances including lignin and various other xenobiotic pollutants [6].Many researchers focused on producing the enzyme using biotechnology due to its important degradative potential [7]. In the last years, MnP has surprising attention for its potential application in the transformation of xenobiotic compounds [8].

MnP is a biotechnologically important enzyme having a broad range of industrial applications; (i) Potential to produce natural aromatic flavours in food industries [9].ii) efficient in transformation of kraft pulp mill effluents [10] iii) Textile dye transformation and bleaching. iv) Mineralization of many environmental contaminants are used for bioremediation process [11].v) It has excellent electron transfer capability with electrodes. It has many functions like the synthesis of biosensors supported on direct electron transfer (DET), efficient bio-fuel unit, and perceptive bio-organic production [12].

Orange G is a class of Azo dyes, differentiated by the presence of one or more azo bonds (–N=N–) in association with aromatic systems and auxo-chromes (–OH, $–SO_3$, etc.). Because of

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their chemical stability and versatility, and most of them (azo dye) are non-biodegradable, toxic and potentially carcinogenic in nature [13]. The widespread utilization of azo dyes has caused an important environmental problem. Prior to the disposal of these industrial effluents, it is necessary to detoxify the polluted water. Many wastewater treatment technologies have been applied for the removal of azo dyes from aqueous solutions including physical and chemical processes [14].However, these treatments are unproductive and expensive for elimination of industrial dyes effluents [15]. Interest is therefore now focused on the microbial degradation of dyes as a better. In the early 1980s, researchers for the first time developed an idea of using oxidoreductase for treating wastewater contaminated with aromatic pollutants [16]. Per-oxidases have high potential in targeting a wide variety of xenobiotic pollutants.

These enzymes can act on a wide range of substrates and convert them into less toxic insoluble compounds, which can be easily took away from waste [1]. Sometime these enzymes cannot act on noxious waste, owing to the intractable nature of such compounds. But these toxic compounds get converted into non detrimental in the existence of certain low-molecular weight compounds that are known as redox mediators. A redox mediator increased the rate of enzyme-catalyzed reaction and enhanced the range of selection of their substrates [17].

Worth of these lignin modifying enzymes has led to a drastic boost in the demand of MnP in the recent time. Therefore the objective of this study was to, purify and characterize a thermally stable manganese peroxidase, furthermore its catalytic properties were investigated and explore the capability of this extracellular enzyme of *Agaricus bisporus A21* for biotransformation of orange G dye.

This study will enhance our knowledge for the ligninolytic system of this edible mushroom, and show the potential applications of the manganese peroxidase of *Agaricus bisporus A21* in industry and biotechnology. To our knowledge, it is first report on isolation, purification, thermal characterization and catalytic properties, of manganese peroxidase from *Agaricus bisporus A21* and its potential application in the biotransformation of textile dye as well.

Experimental

Chemicals

The chemicals used were of analytical grade and highest purity, mainly procured from Sigma-Aldrich Chemical Co, USA. The orange G dye used this study was in purchased from Sigma-Aldrich Chemical Co, USA for research purposes.

Preparation of Partially Purified MnP

Fungal strain *Agaricus bisporus A21* obtained from Institute of Horticultural Sciences University of Agriculture Faisalabad, Pakistan, was maintained on nutrient agar medium. The decolorization medium (Kirk basal medium) [18] was optimized for enhance production of MnP by dissolving following composition (g/L): glucose 10; ammonium sulphate, 0.22; KH2PO4, 0.21: MgSO4·7H2O 0.05; CaCl2.2H2O, 0.01, Thiamine, 0.001.Along with modified amounts: 3 ml /100 mL of 10% solution of Tween-80; 1 mL /100mL of 1 mM solution of $MnSO_4$; 10 ml/L of trace elements solution and 5 mL of 24 h old vegetative inoculum was used to inoculate sterile fermentation medium under aseptic conditions at pH 4.5. The fermentation medium was incubated at 30 °C and 200 rpm in temperature controlled shaker (Weiss-Gallenkamp, Loughborough, UK) to produce high titre of MnP during decolorization of NOVACRON Reactive dye [19].The fermented broth was harvested periodically and centrifuged at 10,000 ×g for 15 min to remove the fermented particles. All the centrifuged were pooled and analyzed for activity, thermal constancy as well as catalytic properties of MnP.

*Manganese Peroxidase Assay***:** The manganese peroxidase (MnP) activity was determined by the oxidation of 1.0 mM Manganese sulphate in 50 mM sodium malonate buffer having pH 4.5, in the presence of H_2O_2 [20], a complex is formed between maganic ion and malonate, which absorbs at 270 nm (ϵ_{270} =11590 $M^{-1}cm^{-1}$). One unit was defined as the amount of enzyme that oxidized 1 μmol $Mn2^{+}$ per minute under reaction conditions. Total protein contents were estimated using the Bradford method with crystalline bovine serum albumin (BSA) as standard [21] .The concentrations of protein in the different fractions from the chromatography were determined at 280 nm.

Purification of Manganese Peroxidase (MnP)

Fractional precipitation of protein was achieved using ammonium sulfate method. The concentration of ammonium sulfate was first optimized, after that crude enzyme was kept in chilled water and crystals of $(NH_4)_2SO_4$ were added to get 85 percent saturation at zero temperature and placed at 4^oC for overnight. Then it was centrifuged at 10,000 × g for 20 min. at 4^oC.

The resulting pellets were collected, dissolved in minimum amount of phosphate buffer (pH 6.0) and dialyzed (membrane with a molecular weight of 8000 Da) several times against distilled water to remove salts by continue stirring. The dialyzed sample was filtered through 0.4 µm Millipore filter and the resulting solution was loaded onto a DEAE-sepharose (3 x100 centimeter) column with a flow rate of 10 mL/min, equilibrated with 4 mM phosphate buffer (pH 6.0). The active fractions from ion-exchange chromatography were collected and pooled collectively, loaded onto gel filtration column (2.6 x 100 cm) filled with Sephadex G-75 and equilibrated with the same buffer. A fraction with high MnP activity were pooled and after each purification step, the total protein content and enzyme activity was measured to calculate specific activity and purification factor.

Enzyme Catalytic Activity and Enzyme Stability

The enzyme catalytic activity of manganese peroxidase at different pH levels was measured for manganese sulphate using 1 mM of buffers in the pH range of 3-10 at room temperature. To examine the effect of pH on the stability of enzyme, the purified enzyme was incubated in the buffer with different pH levels at room temperature for 1h. The samples were then withdrawn after interval and tested for activity at room temperature using manganese sulphate as the substrate. The thermo-stability was investigated by pre-incubating the enzyme in phosphate buffer, at particular temperatures ranging from 25 to 70 °C for 5 min at its optimum pH, the remaining activity values were determined under the standard assay conditions. The experiments were carried out in duplicate.

Irreversible Thermal Denaturation

Kinetics and thermodynamics for irreversible thermal denaturation of MnP was determined by incubating the enzyme in phosphate buffer (pH 6.0) at different temperatures in the absence of substrate. Fractions were withdrawn at specific time period, chilled on ice coldwater for 3 h [22] and assayed for residual enzyme activity as described above. This procedure was repeated at various temperatures ranging from 50 to 65 °C. The data was fixed to 1st order plots. From a semi-logarithmic plot of residual activity vs. time, the denaturation constants (kd) were calculated and perceptible half-lives were estimated. The temperature dependence of kd was investigated from the Arrhenius plot, the activation energy (Ea) for thermal denaturation was calculated from the slope of the plot as described previously [23]. Activation enthalpy (ΔH*) for thermal denaturation was calculated according to the following equation:

$$\Delta H^* = Ea - RT \quad \text{i}$$

$$\Delta G^* = - RT \ln (kd,h/kb.T) \quad \text{ii}$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad \text{iii}$$

Extraction of Phenolic Compounds

Five gram of wheat bran was mixed with 100 ml of 65% (v/v) ethanol (methanol and acetone) and then sonicated for 30 min at 50˚C in an ultrasonic water bath independently. The mixture was immediately filtered under vacuum, and then evaporated the solvent by incubating at 50˚C in an oven [24] .Then subjected to quantify the Phenolic contents by Folin-Ciocalteu method [25].

Estimation of Phenolic Contents

1.5 mL of 10 fold diluted Folin-Reagent was added into 0.2 mL phenolics extract, Shake well and allowed for 5 minutes then Add 1.5 mL Sodium carbonate solution (60 g/L). Incubated at room temperature for 90 min and recorded absorbance at 725 nm.Used distilled water in blank sample instead of phenolics extract. Exploited different FA concentration (10, 20, 30----50 µg FA/reaction) in standard as an alternative of sample and then added other reagent as shown in phenolic estimation.

Enzymatic Dye Decolorization

Dye decolorization capability of the purified enzyme, obtained from *Agaricus bisporus A21* SmF culture, was accessed using orange G. Reaction mixture contained, 50 ppm dye concentration, 0.5mL of 1mM $MnSO_4$, varying concentration of enzyme (100 uL-300uL per 1.3 mL) 250µL of 0.1mM H_2O_2 in 50 mM sodium malonate buffer (pH 4.5), in a total volume of 1.35 ml in an Eppendorf tube. The reaction mixture was placed at 30 °C in dark and the percent removal was determined by monitoring the decrease in lambda max of orange G (475 nm) dye in a UV–Vis Spectrophotometer and expressed in terms of percentage. Whereas control contained heat inactivated enzyme. Wheat bran extract was used as a natural redox mediator for the biotransformation of dye.

UV–Vis Spectral Analysis

Orange G decolorization was determined by computing the decrease in optical density of the dye at the wavelength of maximum absorbance (λmax 475 nm) in a UV–vis spectrophotometer (Cary 3 Bio, Varian, UK) and expressed in percentage. Control

samples were also run parallel and contained the reaction mixture without enzyme. All experiments were conducted in triplicate and data were presented as mean ± SD.The percentage decolorization was calculated as follows:

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

HPLC analysis

The biotransformation product of Orange G was monitored through Reversed phase-HPLC analysis (Agilent 1100 series, Agilent, Waldbronn, Germany). Enzyme treated and control samples were vortex well and filtered through 0.45 µm syringe filter then injected (10 µL) into ZORBAX SB C-18 (Applied Biosystems) column and eluted using 60% acetonitrile at the flow rate of 1 mL min−1. The eluate was monitored at 475 and 450 nm and scanned at the range of 200–700 nm in a diode array detector.

Results and Discussion

Purification of Manganese Peroxidase

The biosynthesis of MnP from *A. bisporus A21* was carried out in 250 mL flasks under optimal condition during the decolorization of NOVACRON reactive dye [18].Crude MnP was purified using ammonium sulphate precipitation, DEAE-Sepharose column chromatography and gel filtration (Sephadex-G 75). The purification results in Table-1 show that the culture broth contains a high proportion of contaminating peptides and protein (with 1067 mg of total proteins having specific activity 73.66 U/mg, which clearly demonstrated that ammonium sulphate precipitation reduces this amount considerably and also removes peptides which interfere with the manganese peroxidase assay [26]. After ammonium sulphate precipitation the specific activity of MnP increased. The partially purified MnP sample was fractionated on a DEAE-Sepharose column chromatography and gel filtration with % age recovery of 9.39 and 6.9 respectively.

The specific activity of purified MnP was found to be 624.94 U/mg which is greater that the specific activity reported for MnP from *Pleurotus ostreatus* [27].

The eluted enzyme showed a single symmetrical peak from gel filtration column. MnP yield obtained by this procedure was low (6.9 %), nevertheless, our goal was to purify and characterize the enzyme.[28] purified manganese peroxidase from *Lenzites betulinus* using ammonium sulphate precipitation, ion-exchange chromatography and gel filtration on Sephadex G-75.The specific activity of purified MnP was 125 U/mg having 625 purification factor.

$$\% \text{ yield} = \frac{\text{Total enzyme activity of each step}}{\text{Specific activity of crude enzyme}} \times 100$$

$$\text{Purification fold} = \frac{\text{Specific activity of each step}}{\text{Total enzyme activity of crude}}$$

Temperature Stability and pH Optimum

The effect of pH on enzyme activity and stability was examined after 1 h of incubation. As can be seen in Fig. 1, the maximum rate of manganese sulphate oxidation was observed at around pH 6.0, a further rise in pH was found to deactivate the MnP protein, pH appear to be crucial parameter regarding MnP activity, it is worth mentioning that manganese peroxidase remains active within the pH range of 3-10 which may be interesting for wide range of applications. Similar to our findings, a pH optimum 6.0 has been reported for MnP isolated from *Irpex lacteus* [29]. Previously the optimum pH for manganese peroxidase isolated from different white rot fungi has been reported in the range of 4-7 [30-34]. Whereas the MnP produced by *S. commune* grown on banana stalks solid state culture was optimally active at pH 5 [35].

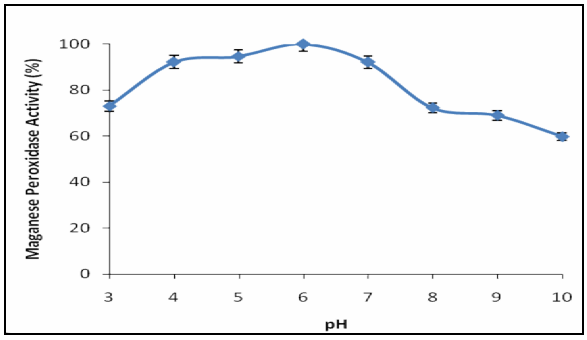

Fig. 1: Effect of pH on the activity of manganese peroxidase.

Table-1: Purification of manganese peroxidase (MnP) producing while the decolorization of Reactive black dye by A*garicus bisporus A21* through different techniques

Purification Steps	Total volume(mL)	Total enzyme activity(IU)	Total protein content (mg)	Specific activity (U/mg)	Yield(%)	Purification(fold)
Crude Enzyme	1000	78600	1067	73.66	100	1
$(NH4)_2SO_4$Precipitation	100	53980	93	580.43	68.60	7.88
Dialyzed enzyme	100	49560	83.23	595.45	63.0	8.08
DEAE-Sepharose	15	7320	12.13	610	9.30	8.28
Sephadex-G 75	10	5487	8.78	624.94	6.9	8.48

While the results presented in Fig. 2 showed that a preliminary increase in temperature increased the enzyme activity, might be due to by increasing the kinetic energy of the molecules and increasing the interaction between enzyme active site and interacting groups of the substrate. It was found that the optimum temperature of Manganese peroxidase was 40˚C above and below this temperature activity was dramatically decreased. Our outcomes are complete agreement with, the optimum temperature of purified MnP secluded from *Phanerochaete chrysosporium* was 40°C [36]. Optimum temperatures of Manganese peroxidase have been reported in the range of 40-60°C, isolated from various white rot fungi [30-34]. According to the [29] MnP from *Irpex lacteus* was stable in the range of 30 to 40˚ C, whereas MnP from another WRF strain, *Rhizoctonia* sp. SYBC-M3 was stable at 55°C [37]. While the isozyme MnP2 produced by *Lentinula edodes* in SSF of corn cobs had 40˚ C optimum temperature [38].

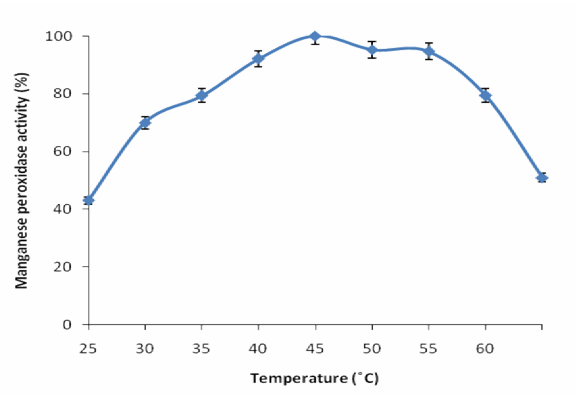

Fig. 2: Effect temperature on MnP activity.

The kinetics constants (*Km* and *Vmax*,) values for the enzyme, using manganese sulphate as substrate, were determined by Lineweaver-Burk plot.This was achieved by incubating specific amount of enzyme with different concentrations of manganese sulphate as a substrate (1-35 mM). The maximum rate achieved during this was 231 U/mL while Michaelis–Menten constants (K_m) of MnP for Mn^{+2} was relatively low (3.33 mM) revealing a binding site with higher substrate affinity. The mode of action of MnP is reported to involve Mn^{+3} diffusing out of the active site which in turn acts as a nonspecific oxidant that attacks phenolic lignin structures by one-electron abstraction [39]. [40] reported that the *Km* value of fungus *Irpex lacteus* MnP using manganese peroxidase as substrate was 21.4 µmolL^{-1}.

Irreversible Thermal Denaturation of MnP

Denaturation is a process in which proteins lose the tertiary structure and secondary structure which is present in their native state, Thermodynamics and activation parameters provide a detailed mechanism for many chemical and biological reactions [41]. Thermal denaturation of enzymes occurs as:

Native enzyme ↔ Unfolded inactive enzyme → Inactivated enzyme

Unfolding is studied to determine the conformational stability of the enzyme. Kinetics and thermodynamics of irreversible thermal denaturation of manganese peroxidase was studied at different temperature ranging from 50 to 65 °C using phosphate buffer (pH 6.0) in the absence of substrate.

The thermodynamic parameters were determined from Arrhenius plot Fig. 3. The half life (t1/2) of MnP was decrease and inactivation constant (kd) was increase as we increase the incubation temperature, the value of enthalpy of denaturation and free energy of denaturation were 39.78 kJmol-1 and 286.41 kJmol-1 respectively. The opening up of enzyme structure is accompanied by an increase in disorder in entropy (*ΔS**) [42] but converse to this we found that the entropy of deactivation (*ΔS**) of MnP was decrease as we increased the temperature (Table-2).

Table: 2. Kinetics and thermodynamic parameters for irreversible thermal denaturation of manganese peroxidase from *Agaricus bisporus A21*

Temp.(K)	K_d (min^{-1})	$t_{1/2}$(min^{-1})	ΔH* (kJmol^{-1})	ΔG* (kJmol^{-1})	ΔS* J$mol^{-1}K^{-1)}$
323	0.0089	77.65	39.91	272.12	-707.94
328	0.0134	51.86	39.87	277.35	-713.18
333	0.0148	46.67	39.82	281.75	-715.77
338	0.0178	38.86	39.78	286.41	-719.04

Activation energy (Ea) of denaturation is 42.64 kJmol^{-1} calculated from Fig.1. 4.
Half life ($t_{1/2}$) = 0.693/k_d
ΔS* = (ΔH*- ΔG*)/T
ΔG* = - RT ln (kd,h/kb.T)

Thermal denaturation of enzymes is escorted by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (*ΔH**) [43].The activation energy (*Ea*) calculated from Arrhenius plots for MnP isolated from *Agaricus bisporus A21* was 42.64 kJmol^{-1} (Fig. 3).

A decrease in *ΔH** was also observed with increase of temperature which indicated that less energy is required to denature the MnP at higher temperature. Moreover, a high value of free energy

change (*ΔG**) showed that MnP exhibited a resistance against thermal unfolding at higher temperature. These results are analogous to the reported outcome in which a decrease in entropy of activation (*ΔS**) and increase in free energy change (*ΔG**) for thermal inactivation has been investigated for various enzymes [44].

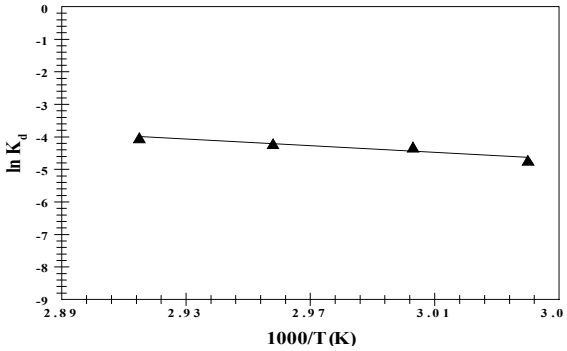

Fig. 3: Arrhenius plot for the activation energy of thermal denaturation of MnP.

At low temperatures and in the presence of urea, several protein like lacto- globulin are denatured [45] and shows decrease in entropy which might be due to water ordering increases in the vicinity of non-polar amino acids which are exposed during unfolding [46].This ordering of water around hydrophobic residues is disrupted at higher temperatures; so as to concord to our findings.

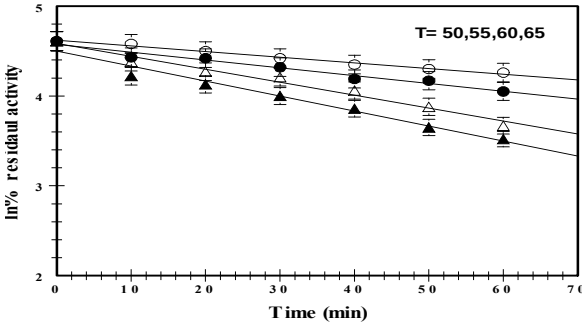

Fig. 4: Irreversible thermal denaturation of Manganese peroxidase.

Dye decolorization by partially purified Manganese Peroxidase

The partially purified enzyme was utilized for enzymatic decolorization activity using orange G dye. A spectrophotometric scanning (300–900 nm) of the orange G dye showed a single peak at 475nm. The dye decolorization was represented by a decrease in absorbance and the absorbance spectral pattern of each recovered dye solution was similar to that of the initial one (Fig. 5) indicated that there was no any biotransformation of parent dye while in case of dye treated by enzyme in the presence of phenolic extract the absorbance spectral pattern of each recovered dye solution was not alike to that of the original one. Common decline of this kind is usually attributed to dye degradation rather than adsorption [47].

The results clearly indicated that there was only 20% transformation of orange dye by 300 uL of partially purified enzyme alone (Fig. 5). However when 100 uL enzyme was mediated by 300 uL of natural redox mediators of wheat bran then the rate of removal was more than threefold (96.84%) then the MnP alone within 1h Fig. 6. Indicate that manganese peroxidase exhibit highly efficient decolorization potential in the presence of natural mediator of wheat bran (Fig. 7).

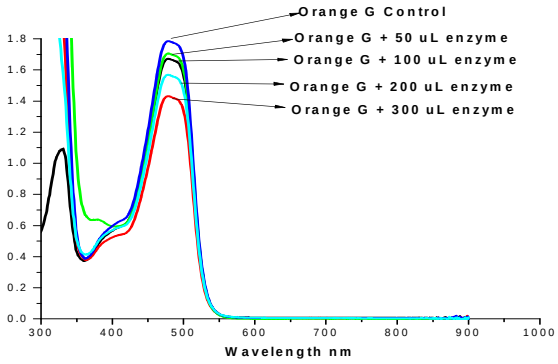

Fig. 5: UV-visible spectra (300–900 nm) of enzymatic (50-300 uL of partially purified MnP) transformation of dye.

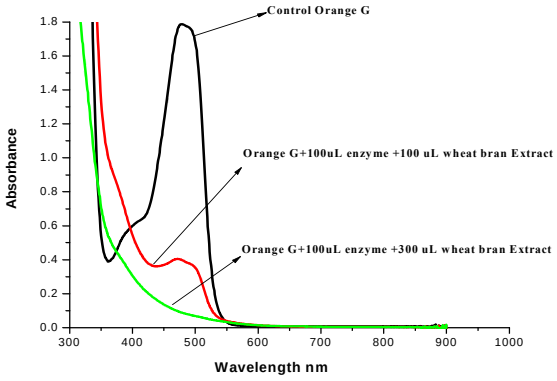

Fig. 6: UV-visible spectra (300–900 nm) of enzymatic transformation of dye in the presence of 100 and 300 uL phenolic extract of wheat bran.

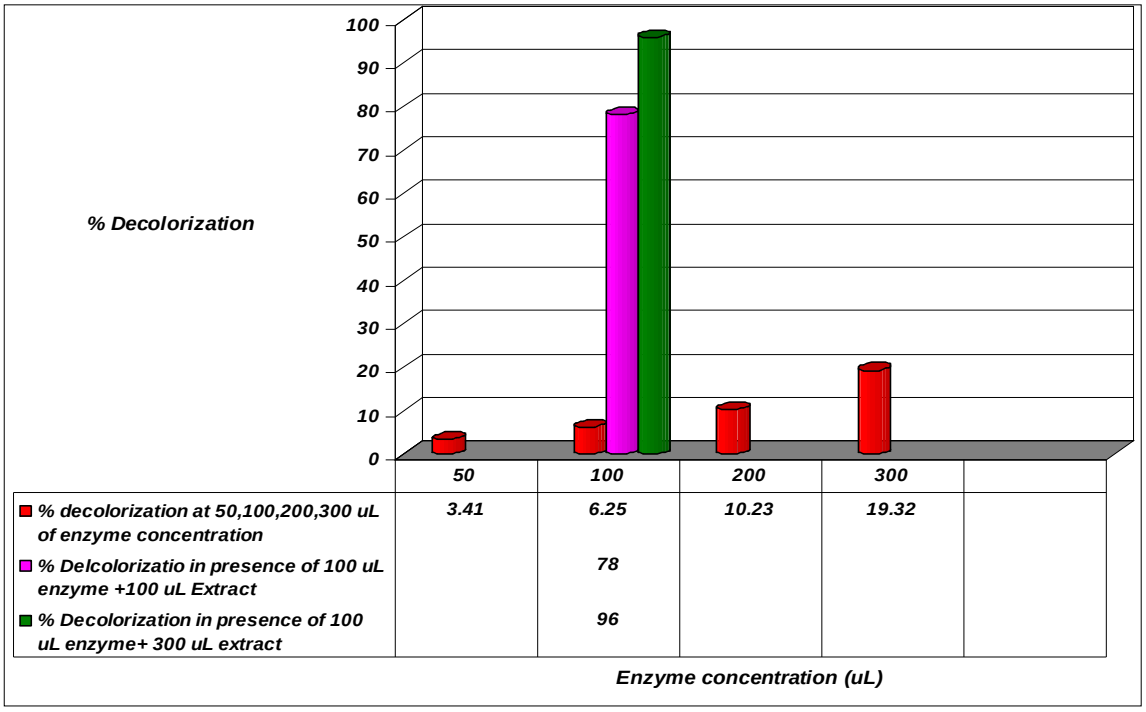

Fig. 7: Decolorization of dye by MnP alone and in the presence of phenolic extract of wheat bran.

HPLC analysis

Then all the treated samples were subjected to RP-HPLC analysis (Reversed-phase chromatography, which separates molecules on the basis of differences in their hydrophobicity. The components of the analyte mixture pass over stationary-phase particles bearing pores large enough for them to enter, where interactions with the hydrophobic surface removes them from the flowing mobile-phase stream to detect orange G transformation products. The HPLC chromatogram profiles of control Orange G shows a single peak at the retention time of 1.5 min (Fig. 8a). In the presence of Manganese Peroxidase, in addition to orange G peak there is a new peak appeared at retention times of 1.8 min (Fig. 8b) whereas as the reaction mixture is mediated by 300 uL natural redox mediators the original dye peak was completely disappeared and two new peak was appeared at 2.0 min and 2.3 min (Fig. 8d) from which we suggest that orange G is completely transformed into different products. HPLC results clearly depicted that as the concentration of natural redox mediators is increased the rate of transformation of xenobiotic compounds was also increased and original compound was completely disappeared when the concentration of natural phenolic compounds was upto 300 uL.

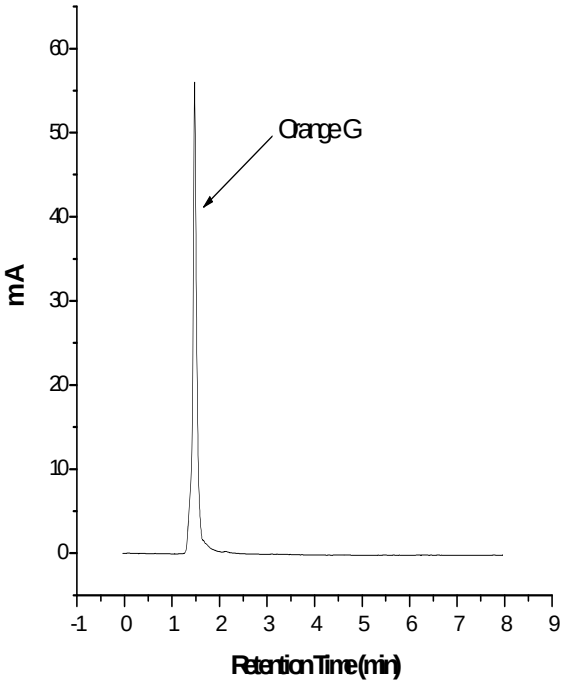

Fig. 8(a): Control dye.

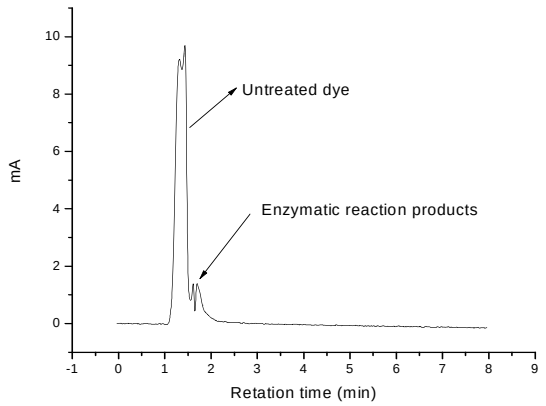

Fig. 8(b): 100ppm dye + 300 uL MnP.

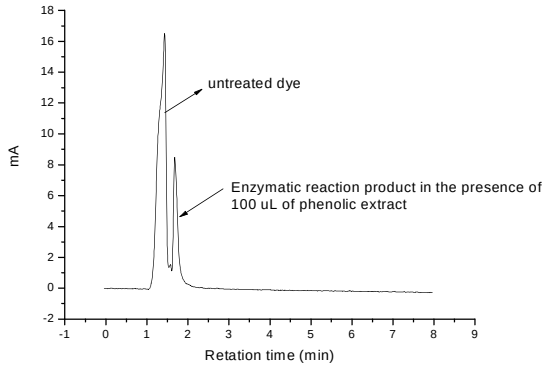

Fig. 8(c): 100 ppm dye + 100uL MnP+100 uL natural redox mediators

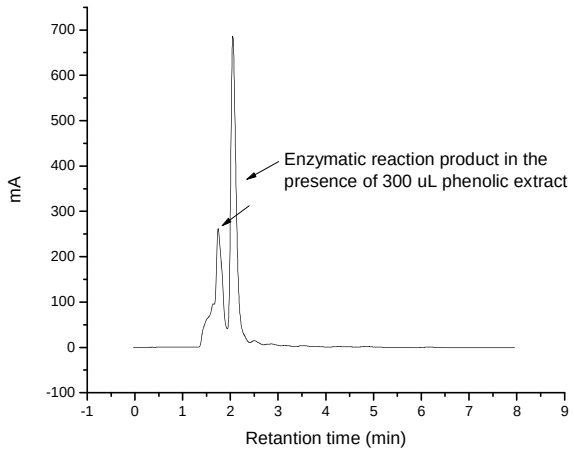

Fig. 8 (d): 100 ppm dye + 100uL MnP+300 uL natural redox mediators.

Fig. 8: HPLC chromatograms of control and treated reactive yellow dye .

Conclusions

In view of the results obtained, it can be concluded that: the newly isolated white rot fungal strain, *Agaricus bisporus A21*, is higher Manganese peroxidase producer under the optimum conditions so as to permits to maintain the culture in the more productive state. From kinetic and thermodynamics studies it was suggested that Manganese peroxidase was quite thermo-stable at higher temperature hence could be use for industrial applications. The remarkable thermo-stable potential of Manganese peroxidase makes this enzyme attractive for biotechnological applications, e.g.in pulping and bleaching of cellulose and in removing of hazardous wastes. All these applications are dependent on the feasible production of the enzyme at high scale and on its long term thermal stabilization. Thus Manganese peroxidase could be use as versatile biocatalyst in biotechnological processes.

Acknowledgements

Authors are thankful to the Higher Education Commission (HEC) of Pakistan for financial assistance.

References

1. I. Bibi, H. N.Bhatti and M. Asgher, Comparative Study of Natural and Synthetic Phenolic Compounds as Efficient Laccase Mediators for the Transformation of Cationic Dye, *Biochem. Eng. J.,* **56**, 225 (2011).
2. M. Asgher, N. Azim and H. N.Bhatti, Decolorization of Practical Textile Industry Effluents by White Rot Fungus *Coriolus versicolor* IBL-04, *Biochem. Eng. J.*, **47**, 61 (2009).
3. K. E. Hammel and D. Cullen, Role of Fungal Peroxidases in Biological Ligninolysis, *Curr. Opin. Plant. Biol*.,**11**, 349 (2008).
4. M. Hofrichter, Lignin Conversion by Manganese Peroxidase (MnP), *Enzyme Microb. Tech.*, **30**, 454 (2002).
5. M. Makela,S. Galkin, A. Hatakka and T. Lundell, Production of Organic Acids and Oxalate Decarboxylase in Lignin-Degrading White Rot Fungi, *Enzyme Microb. Tech.*, **30**, 549 (2002).
6. G. Eibes, M. T. Moreira, G. Feijoo, A. J. Daugulis and J. M. Lema, Operation of a Two-Phase Partitioning Bioreactor for the Oxidation of Anthracene by the Enzyme Manganese Peroxidase, *Chemosphere.,* **66,** 1744 (2007).

7. E. Winquist, U. Moilanen, A. Mettala, M. Leisola and A. Hatakka, Roduction of Lignin Modifying Enzymes on Industrial Waste Material by Solid-State Cultivation of Fungi, *Biochem. Engin.J.*, **42**, 128 (2008).
8. P. Baborova, M. Moder, P. Baldrian, K. Cajthamlova and T. Cajthaml, Purification of a New Manganese Peroxidase of the White-Rot Fungus Irpex lacteus, and degradation of polycyclic aromatic hydrocarbons by the enzyme, *Res. Microbiol.*, **157**, 248 (2006).
9. H. Zorn, S. Langhoff, M. Scheibner, M. Nimtz and R. G. Berger, A. Peroxidase from Lepista irina Cleaves β,β-Carotene to Flavor Compounds, *Biol. Chem.,* **384**,1049 (2003).
10. M. Moreira, G. Feijoo, Canaval, J. Lema, Semipilot-Scale Bleaching of Kraft Pulp with manganese Peroxide., *Wood Sci. Technol*, **37**,117 (2003).
11. Y. Ohtsubo, T. Kudo, M. Tsuda and Y. Nagata, Strategies for Bioremediation of Polychlorinated Biphenyls, *Appl. Microbiol. Biotechnol.,* **65**, 250 (2004).
12. E. E. Ferapontova, S. Shleev, T. Ruzgas, L. Stoica, A. Christenson, J. Tkac, A. I. Yaropolov and L. Gorton, Direct Electrochemistry of Proteins and Enzymes, *Perspect. Bioanal.***, 1**, 517 (2005).
13. S. Bakhshian, H. R. Kariminia and R. Roshandel, Bioelectricity Generation Enhancement in a Dual Chamber Microbial Fuel Cell under Cathodic Enzyme Catalyzed Dye Decolorization, *Bioresour. Technol.*, **102**, 6761 (2011).
14. J. Sun, X. Wang, J. Sun, R. Sun, S. Sun and L. Qiao, Photocatalytic Degradation and Kinetics of Orange G Using Nano-Sized Sn(IV)/TiO2/AC Photocatalyst, *J. Mol.Catal. A: Chem.,* **260,** 246 (2006).
15. Q. Sun, Y. Bai, C. Zhao, Y. Xiao, D. Wen and X. Tang, Aerobic Biodegradation Characteristics and Metabolic Products of Quinoline by a *Pseudomonas* strain, *Bioresour. Technol*., **100,** 5030 (2009).
16. N. Duran and E. Esposito, Potential Applications of Oxidative Enzymes and Phenoloxidase-Like Compounds in Wastewater and Soil Treatment, *Appl. Catal. B. Environ.***, 28,** 83 (2000).
17. Y. Kulshrestha and Q. Husain, Ecolorization and Degradation of Acid Dyes Mediated by Salt Fractionated Turnip (Brassica rapa) Peroxidases, *Toxicol. Environ. Chem*., **89***,* 255 (2007).
18. T. K. Kirk and R. L. Farell, Enzymatic Combustion the Microbial Degradation of Lignin, *Ann. Rev. Microbiol*., **41**, 505 (1990).
19. I. Bibi, H. N.Bhatti and M. A. Asgher, Bioremediation of Wastewater Containing Reactive Dyes by *Agaricus bisporus* A21: Effect of Supplements and Redox Mediators, *Pak. J. Agri. Sci.*, **50**, 445 (2013).
20. H. Wariishi, K. Vali and M. H. Gold, Manganese (II) Oxidation by Manganese Peroxidase from the Basidiomycete *Phanerochaete chrysosporium*. Kinetic Mechanism and Role of Chelators,, *J. Biol. Chem*, **26,** 23688 (1992).
21. M. M. Bradford, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Ana. Biochem.,* **72,** 248 (1976).
22. M. Violet and J. C. Meunier, Kinetic Study of the Irreversible Thermal Denaturation of Bacillus Licheniformis Alpha-Amylase, *Biochem. J.,* **263**, 665 (1989).
23. H. N. Bhatti, M. Asgher, A. Abbas, R. Nawaz and M. A. Sheikh, Studies on Kinetics and Thermostability of a Novel Acid Invertase from Fusarium solani, *J. Agri. Food Chem.*, **54**, 4617 (2006).
24. J. Wang, B. Sun, Y. Cao, Y. Tian and X. Li, Optimisation of Ultrasound-Assisted Extraction of Phenolic Compounds from Wheat Bran, *Food Chem.*, **106**, 804 (2008).
25. V. L. Singleton, R. Orthofer and R. M. Lamuela-Raventós, Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent., *Methods* Enzymol., **299***,* 152 (1999).
26. R. Ten Have, M. C. Franssen and J. A. Field, Lignin Peroxidase Initiates O_2-Dependent Self-Propagating Chemical Reactions which Accelerate the Consumption of 1-(3',4'-dimethoxyphenyl) Propene, *Biochem. J.*, **347**, 585 (2000).
27. S. Sarkar, A. T. Martinez and M. J. Martinez, Biochemical and Molecular Characterization of a Manganese Peroxidase Isoenzyme from *Pleurotus ostreatus*, *Biochim. Biophys. Acta*, **25,** 23 (1997).
28. F. Hoshino, T. Kajino, H. Sugiyama, O. Asami and H. Takahashi, Thermally Stable and Hydrogen Peroxide Tolerant Manganese Peroxidase (MnP) from Lenzites betulinus, *Fed, Eur, Biolog, Soc, Lett*., **530**, 249 (2002).
29. K. Shin, Y. H. Kim and J. Lim, Purification and Characterization of Manganese Peroxidase of the White-Rot Fungus Irpex Lacteus, *J. Microbiol*., **43**, 503 (2005).
30. H. Bermek, H. Yazici, H. Ozturk, C. Tamerler, H. Jung and K. Li, .Purification and Characterization of Manganese Peroxodase from

Wood Degrading Fungus *Trichophyton rubrum* LSK-2, *.Enz. Microb. Technol*, **35**, 92 (2004).

31. R. O. Urek, and N. K. Pazarlioglu, Purification and Partial Characterization of Manganese Peroxidase from Immobilized *Phanerochaete chrysosporium, Process Biochem*., **39**, 2068 (2004).
32. T. K. Hakala, T. Lundell, S. Galkin, P. Maijala, S. Kalkkinen and A. Hatakka, .Manganese Peroxidases, Laccase and Oxalic Acid from the Selective White Rot Fungus *Physisporinus rivulosus* Grown on Spruce Wood Chips*, Enz.Microbiol. Technol.,* **36***,* 468 (2005).
33. M. Asgher, H. N. Bhatti, M. Ashraf and R. L. Legge, Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system, *Biodegradation*., **19**: 783 (2008).
34. D. Oliveira, P. L. M. C. T. Duarte, A. N. Ponezi and L. R. Durrant, Purification and Partial Characterization of Manganese Peroxidase from *Bacillus pumilus and Paenibacillus sp,* Braz. *J. Microbiol.,* **40**, 826 (2009).
35. A. Rajan, J. G. Kurup and T. E. Abraham, Solid State Production of Manganese Peroxidase Using Arecanut Husk as Substare, *Braz. Arch. Biol. Technol*., **53**, 562 (2010).
36. W. Boer, L. B. Folman, R. C. Summerbell and L. Boddy, Living in a Fungal World: Impact of Fungi on Soil Bacterial Niche Development, *Fed. Microbiol. Soc.*, **29**,795 (2005).
37. Y. Cai, H. Wu, X. Liao, Y. Ding, J. Sun and D. Zhang, Purification and Characterization of Novel Manganese Peroxidase from *Rhizoctonia* sp.SYBC-M3, *Biotechnol .Bioprocess Eng.,* **15**, 1021 (2010).
38. C. G. Boer, L. Obici, C. G.Marques de Souza and R. M. Peralta, Purification and Some Properties of Mn Peroxidase from *Lentinula edodes, Process Biochem.,* **41***,* 1207 (2006).
39. D. W. Wong, Structure and Action Mechanism of Ligninolytic Enzymes, *Appl. Biochem. Biotechnol*., **157**, 174 (2009).
40. M. L. N. P. Sklenara, P. Santosc, K. Mana, C. Kruusb and Novotnya, Isolation and Characterization of Novel pI 4.8 MnP Isoenzyme from White-Rot Fungus Irpex Lacteus, *Enzyme Microb. Tech.,* **46**, 550 (2010).
41. A. Tanaka and E. Hoshino, Secondary Calcium-Binding Parameter of Bacillus Amyloliquefaciens Alpha-Amylase Obtained from Inhibition Kinetics, *J. Biosci. Bioengin.*, **96**, 262 (2003).
42. C. Vieille and J. Gregory Zeikus, Thermozymes: Identifying Molecular Determinants of Protein Structural and Functional Stability, *Trends Biotechnol*., **14,** 183 (1996).
43. R. M. Daniel, The Upper Limits of Enzyme Thermal Stability, *Enzyme Microbiol, Technol,* **19**, 74 (1996).
44. I. Shaheen, H. N. Bhatti and T. Ashraf, Production, Purification and Thermal Characterisation of Invertase from a newly isolated Fusarium sp. Under Solid-State Fermentation, *Int. J. Food Sci Technol.*, **43**, 1152 (2008).
45. C. A. Nelson and J. P. Hummel, Reversible Denaturation of Pancreatic Ribonuclease by Urea, *J. Biolog. Chem,* **237,** 1567 (1962).
46. P. L. Privalov and S. J. Gill, Stability of Protein Structure and Hydrophobic Interaction, *Adv. Protein Chem*, **39,** 191 (1988).
47. D. Wesenberg, I. Kyriakides and S. N. Agathos, White-Rot Fungi and their Enzymes for the Treatment of Industrial Dye Effluents, *Biotechnol. Adv.,* **22**, 161 (2003).