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Hyper-production of manganese peroxidase by mutant *Pleurotus ostreatus* MTCC 142 and its applications in biodegradation of textile azo dyes

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ABSTRACT

Enhanced production of manganese peroxidase (MnP) by the mutant *Pleurotus ostreatus* MTCC 142 was carried out in a solid and submerged fermentation by using an agro-industrial waste (rice bran and wheat bran) under optimized conditions. Among the various substrate combinations examined, rice bran shows the highest MnP activity (273 U/L), when compared with the wheat bran (218 U/L). Maximum MnP (368 U/L) was produced when rice bran (5 g) at 75% moisture (w/v) was used with 1 mL inoculum at pH 4 and 25°C in the presence of 2% (w/v) glucose as a carbon source, 0.2% (w/v) sodium nitrate as a nitrogen source and 4% of (v/v) glycerol as an inducer for MnP production under submerged fermentation. MnP was purified from the culture fluid by using microfiltration (1.2 μm) and ammonium sulphate precipitation followed by ion exchange chromatography. Additionally, the protein content of the recovered supernatant was also noted. The purified MnP was a monomer, which showed a molecular mass of 58 ± kDa as determined by SDS-PAGE. Furthermore, the purified MnP was exploited for decolourization of structurally different azo dyes. Among the selected azo dyes, Ponceau S, Tartrazine and Trypan Blue were decolourized rapidly by the purified enzyme extracts under optimized condition and it was found to be 78, 56 and 59%, respectively.

Keywords: Azo dyes; MnP; *Pleurotus ostreatus*; Biodegradation; Random mutagenesis

1. Introduction

Control of environmental pollution from human activities is one of the prime concerns of science today. Wastewater from textile industries creates a serious pollution problem due to the discharge of synthetic dyes. Among all the dyes, azo dyes are predominantly utilized as dyes and analytic reagents; they are also used in substrates such as textile fibers, leathers, plastics, papers, foodstuff and cosmetics [1–3]. However, azo dyes are recalcitrant to biodegradation in nature due to the stability, structural variation and the presence of different auxochromes such as NH_2, NR_2 and OH groups. Generally, the azo dyes degradation metabolites are highly toxic, carcinogenic and mutagenic amines, which pose a serious environmental pollution in aquatic system [4]. Currently, there are many physico-chemicals methods available to treat coloured effluent but they are quite expensive and also cause a secondary disposal problem in the reactor due to the formation of concentrated sludge [5,6]. Recently, many

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researchers have developed new biotechnological strategies to eradicate this pollution source in an eco-efficient manner [7]. Current technologies for the degradation of azo dyes by microbial cultures and the enzymes derived from them have been recently reviewed [8]. Several micro-organisms belonging to different taxonomic groups of bacteria, fungi and yeast have been used for the treatment of various dye effluents. However, aerobic degradation of azo dyes by bacteria leads to the production of colourless carcinogenic and toxic aromatic amine compounds [9,10]. To overcome this problem, a number of white-rot fungi have been explored for the decolourization of various industrial dyes due to the presence of ligninolytic enzyme system, especially manganese dependent peroxidases (MnP) [11–13]. MnP (EC. 1.11.1.13) is an extracellular ligninolytic enzyme produced by most white-rot fungi, especially during secondary metabolism [14–16]. MnP is a glycosylated hemeprotein with a molecular weight of approximately 46,000 kDa and occurs as a family of isozymes. This enzyme catalyzes the H_2O_2-dependent oxidation of Mn(II) to Mn(III), which then binds to an appropriate ligand, diffuses from the enzyme, and in turn oxidizes phenolic substrates [17–19]. Uses of ligninolytic enzymes, particularly MnP, for degradation of industrial dyes have been a great concern today. To date, there is no efficient technique for the accelerated production of MnP from bacteria and fungi. Keeping this in view, the present study was carried out to investigate the potential of a mutant strain for hyper production of MnP and it also focused to access the degradation ability of purified enzyme on textile azo dyes.

2. Materials and methods

2.1. Chemicals

All microbiology reagents and other chemicals like Ethidium bromide (EtBr), 2,6-dimethoxyphenol and azo dyes such as Acid Blue 113, Naphthol Blue Black, Congo Red, Ponceau S, Tartrazine, Reactive Orange 16, Reactive Black 5, Trypan Blue and Disperse Blue 106 were purchased from Sigma–Aldrich, India.

2.2. Fungal strain

White-rot fungal strain *Pleurotus ostreatus* MTCC 142 (slant culture) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. This fungal strain was maintained in glucose yeast agar medium at 4˚C and periodically sub-cultured every two weeks for further investigation.

2.3. Screening of MnP producing fungi

Screening of MnP producing species and their variants is important for selecting suitable MnP producing strains. Rapid determination of MnP production in the extracellular fluid was performed by using plate assay method. About 20 mL of sterile minimal medium containing (g/L): glucose, 20; $(NH_4)_2HPO_4$, 1.5; KH_2PO_4, 0.46; $MgSO_4{\cdot}7H_2O$, 0.5; thiamine hydrochloride, 120 μg amended with 0.2% of Congo Red was prepared and inoculated with mutant fungal strain at 25˚C for 7 d. The development of a zone around the mycelium was considered as a positive test for MnP activity.

2.4. Random mutagenesis and mutant selection

In order to obtain a highly mutated stable fungal strain for the hyper-production of MnP, random mutagenesis was carried out by exposing the wild strain *P. ostreatus* MTCC 142 to various mutagens like UV and EtBr. For the mutation study, *P. ostreatus* MTCC 142 young culture was grown for 7 d in potato dextrose broth and the mycelium was collected, washed twice with a salt solution (NaCl, 0.8% w/v) homogenized for 1 min with sterile distilled water (1:5 v/v, mycelium:water). About 10 mL of homogenate suspension was aseptically transferred into sterile Petri dish and exposed to UV-A mutagen at 320 nm at regular intervals of 5, 10, 15, 20, 25 and 30 min. Immediately after the UV radiation, the plates were kept at the dark room to avoid photoreactivation. The mutated strain possessing a higher rate of MnP production was screened out. The selected mutated strain was again exposed to the chemical mutagen and highly-stable double-mutated strain was obtained. The selected stable double-mutated strain was used for the biomass production.

2.5. Mutant strain cultivation

1 mL of double mutated suspension aseptically transferred to freshly prepared Sabouraud Dextrose broth and incubated on a rotary shaker (150 rev^{-1}) at 30˚C for 7–10 d. After the period of incubation, the mycelium was transferred to 50 mL of yeast malt medium prepared with distilled water in a 250 mL flask and aerobically cultivated for 24 h. The cell culture will be centrifuged at 6,000 rpm at 4˚C for 5 min and washed three times with sterile saline water. The washed cells were transferred to 50 mL of the basal medium, and grown with shaking at 170 rpm, at 28˚C for 8 d [20].

2.6. MnP production by solid-state and submerged fermentation

In solid fermentation, lignocellulosic substrates like wheat bran and rice bran were collected from the local market and dried in oven at 80˚C. All the substrates were ground in an electrical blender and the powder was stored in airtight container to avoid moisture. Production medium was prepared for 100 mL containing (g/L): Glucose—10.0; KH_2PO_4—1.0; $MgSO_4{\cdot}7H_2O$—0.5; $CaCl_2{\cdot}2H_2O$—0.1; $FeSO_4{\cdot}7H_2O$—0.005; $(NH4)_2SO_4$—0.3; $ZnSO_4{\cdot}7H_2O$—0.005; KCl—0.5 and L-Glutamine—0.5. MnP production was carried out in 500 mL Erlenmeyer flasks containing 150 mL production medium, 50 g of lignocellulosic substrate and 30 mL basal medium containing mutant strain of mycelium. The flasks were adjusted to 60% moisture (w/w) with 0.1 M acetate buffer (pH 6.0) and incubated at 28˚C for 21 d.

The submerged fermentation was performed in 500 mL Erlenmeyer flasks containing 150 mL of the above-mentioned production medium, 50 g substrate and 30 mL basal medium containing the mycelium of mutant strain. The flask was adjusted to 72% moisture (w/w) with 0.1 M acetate buffer (pH 6.0) and incubated at 28˚C for 21 d.

2.7. Optimization parameters for the accelerated production of MnP

Hyper-production of MnP was carried out with various process parameters for a period of 14–28 d.

2.7.1. Effect of pH on MnP production

The effect of pH on MnP production was examined in both solid and submerged fermentation by optimizing the production medium with different pH of 3, 4, 5, 6 and 7. The enzyme activity was determined from the production medium with different pH.

2.7.2. Effect of carbon sources on MnP production

The four different carbon sources like lactose, maltose, glucose and sucrose were amended at the concentration of 2% in the production medium. The mutant fungal strains were incubated to Erlenmeyer flasks (500 mL) containing 15 mL (solid fermentation) and 100 mL (submerged fermentation) production medium, which is inoculated with mutant fungal strains and were incubated at 25˚C for a period of 14–28 d.

2.7.3. Effect of nitrogen sources on MnP production

In order to find out the suitable nitrogen source for the hyper-production of MnP, the following nitrogen sources, namely sodium nitrate, peptone, beef extract and yeast extract were amended at a concentration of 0.2% along with the production medium in both solid and submerged fermentation. The flasks were incubated at 27˚C for a period of 14–28 d.

2.7.4. Effects of temperature on MnP production

Effects of various temperatures 25, 30, 35 and 40˚C were studied for the MnP production in both solid and submerged fermentation. The production media was distributed in different flasks with mutant fungal strain and incubated for a period of 14–28 d.

2.7.5. Effect of inducers on MnP production

The effects of different inducers like copper sulphate, Tween 80 and glycerol were amended at a concentration of 4% in the production medium. The mutant fungal strains were inoculated to 500 mL Erlenmeyer flasks containing 15 mL of solid fermentation media and 100 mL submerged fermentation media and incubated at 27˚C for a period of 14–28 d.

2.8. MnP purification

MnP was purified by a modified procedure based on a method of [21]. The culture supernatant was separated from the mycelium by filtration through a 1.2 μm-pore-size filter. All purification steps were carried out at 4˚C. The extract was further precipitated with 60–80% ammonium sulphate and precipitated proteins were collected by centrifugation at 8,000 *g* for 30 min at 4˚C. The resulting pellets were dialyzed against 100 mM sodium phosphate buffer, pH 7.0. The enzyme collected was applied to an ion-exchange chromatography (DEAE cellulose) (Bio-Rad) equilibrated with 100 mM sodium phosphate buffer, pH 7.0, at a flow rate of 0.4 mL/min. The column was washed with 200 mM NaCl in 100 mM sodium phosphate buffer, pH 7.0. The retained proteins were eluted with 600 mM NaCl in 100 mM sodium phosphate buffer, pH 7.0. Fractions with MnP activity were pooled, concentrated and then stored at 4˚C.

2.9. MnP activity

MnP activity was assayed spectrophotometrically by measuring optical density at 465 nm

($\varepsilon = 12{,}100\ mM^{-1}\ cm^{-1}$) on the basis of the oxidation of guaiacol (2-methoxyphenol) [22]. One unit (U) of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate per min under assay conditions [23].

2.9.1. Protein assay

The extracted enzyme protein was estimated by Lowry's method using BSA as standard [24]. The absorbance was read at 750 nm using a Perkin–Elmer LAMBDA 45 UV/vis spectrophotometer. All sample measurements were done at least in triplicate.

2.9.2. Decolourization assay

In order to examine the decolourization potentiality of purified MnP from the mutant strain, the selected azo dyes were first solubilized in the sodium acetate buffer, pH 5. The reaction was then carried out stepwise for each azo dye in a separate flask containing 2 mL of azo dye (50 mg/L) and 368 units of MnP (contained in the concentrated culture filtrate) in 20 mM sodium acetate buffer, pH 5. Enzymatic decolourization reaction was performed with an addition of an enzyme and incubated at 30°C. Enzyme dosing was optimized such that dye was degraded by 25% after a cuvette was placed in the spectrophotometer. Dye decolourization was determined by measuring residual absorption at the appropriate wavelength for each azo dye. An UV/vis spectrophotometer was used for the absorbance measurement. A control test containing the same amount of dyes without an enzyme was performed simultaneously. Decolourization activity was expressed in terms of percent decolourization as described by [25].

Decolourization was calculated according to Eq. (1):

$$D\% = 100 \times \frac{(A_{ini} - A_{fin})}{A_{ini}} \quad (1)$$

where D is the decolourization, A_{ini} is the initial absorbance and A_{fin} is the final absorbance of dye after incubation time.

2.10. HPLC analysis of decolourization metabolites

The products formed during the biodegradation of azo dyes were periodically monitored at the regular intervals of time. Among all the dyes, Ponceau S, Tartrazine and Trypan Blue were decolourized rapidly by the partially purified enzyme extract under optimized condition. The equal volume of organic solvent, such as dichloromethane (DCM), was added to the decolourized mixture to extract the degraded products. The DCM extracts were pooled and evaporated at 40°C in a rotary evaporator. HPLC analysis was performed in an isocratic system (Shimadzu with HPLC system with LC-10AT vp Pump) equipped with dual absorbance detector using C18 column with HPLC grade ACN:water:methanol (80:10:10) as mobile phase at a flow rate of 1.2 mL min^{-1} for 7 min at a range of 510, 470 and 422 nm for the Ponceau S, Tartrazine and Trypan Blue, respectively.

2.11. Molecular mass determination through SDS-PAGE

To examine the molecular mass of purified MnP, the enzyme was resolved on 12% polyacrylamide gel electrophoresis using standard molecular weight protein markers as described by [26]. Protein was visualized by staining the gel with silver staining.

2.12. Phytotoxicity assay

Phytotoxicity assay was performed to measure the toxicity effect of Ponceau, Tartrazine and Trypan Blue and their metabolites formed after decolourization by mutant strain. Tests were carried out on two kinds of plants which are commonly used in agriculture: *Sesamum indicum* and *Vigna mungo*. Fifteen seeds of each plant were sowed in a plastic sand pot. The sand pot was prepared by adding 20 g of washed sand into the plastic pot. The selected dyes and ethyl acetate extracted metabolites (dry) of each dye were dissolved separately in distilled water and the final concentration made was about 500 ppm. The filtered textile effluent was directly used to assess its toxicity. Toxicity study was done by watering (5 mL) the seeds of each plant with dye samples and extracted metabolites sample. The control was run by watering the seeds with distilled water. The watering of plants was done twice a day. Germination (%), length of plumule (shoot) and radical root was recorded after 10 d. The experiments were carried out at room temperature.

2.13. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) with Turkey–Kramer multiple comparison test. All the experiments were carried out in triplicate.

3. Results and discussion

In this study, the primary screening was conducted to differentiate the production rate of MnP from the parental and mutant strain. Our result revealed that mutant strain predominantly produces a clear visible zone around the colonies in plate assay when compared to the parental strain (Fig. 1). MnP plate assay confirmed that MnP production occurred rapidly in the mutant strain of *P. ostreatus* due to the over expression of mnp1 gene, which was not detected in the wild-type strain. Furthermore, our result indicates that the plate assay could be used as the simple rapid assay for visual demonstration in the presence of MnP [27].

Extended level of the zone by the mutant strain in plate assay indicates that the double mutated strain capable of producing higher amounts of extracellular enzyme. In order to achieve the hyper-production of MnP, the mycelial suspension of wild strain *P. ostreatus* was exposed to UV radiation (1.2×10^2 J/m^2/S) for different incubation periods (0–45 min) with 6 cm distance from the source. After 42 min of exposure, culture was amended with 0.2% of EtBr in a basal medium; nearly 90% kill rate was observed (Figs. 2 and 3) and the maximum MnP activity (368.18 U/L) was found in double-mutated stable strain under optimized conditions when compared to parental strain.

Double-mutated stable strain showed 80% increase MnP activity when compared with the parental strain (Table 1). Mostly, strain improvement for the hyper production of enzymes using filamentous fungi is achieved by inducing mutagenesis with various mutagens, such as UV light, N-methyl-N-nitro-N nitrosoguanidine and HNO_2, applied separately or in combination [28–33]. Many researchers have reported

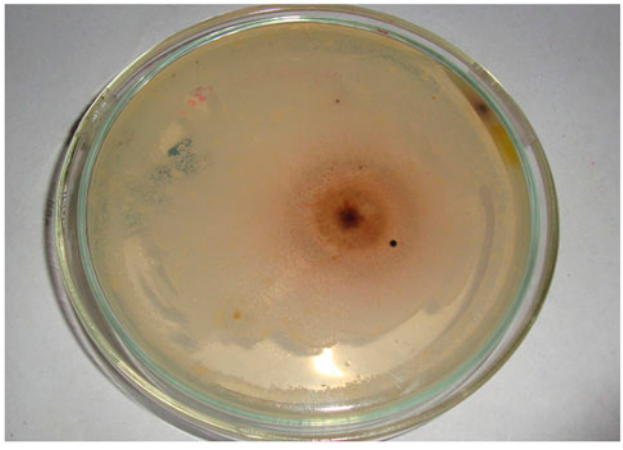

Fig. 1. Plate assay for mutant strain.

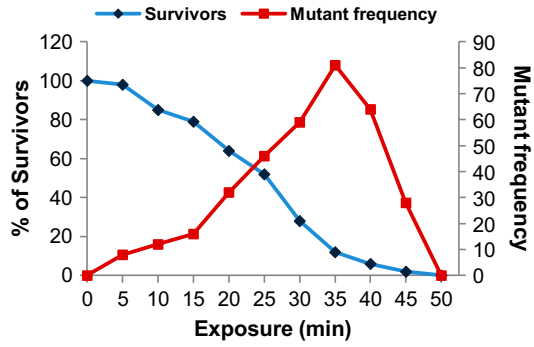

Fig. 2. Effect of UV radiation doses on survival rate and mutant frequency of *P. ostreatus*.

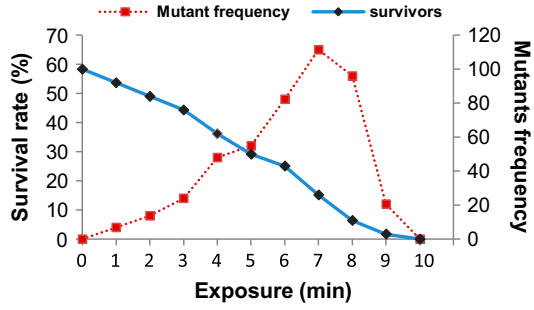

Fig. 3. Effect of EtBr doses on survival rate and mutant frequency of *P. ostreatus*.

that extracellular enzyme production increased when fungi were exposed to various mutagens in sub lethal concentrations. The effectiveness of the induced mutagenesis depended on the type of mutagen, concentration of mutagenic agent and the duration of exposition [34].

Our study revealed that the pH and temperature significantly influenced the extra cellular MnP activity in mutant *P. ostreatus*. The fungus was able to release a maximum MnP activity of 318 U/L at pH 4 and 335 U/L at 25°C at a period of 21 d of incubation (Figs. 4 and 5). Generally, the enzymes are very sensitive to environmental parameters, *P. chrysosporium* produced maximum MnP activity (52.60 U/L) in solid-state fermentation using arecanut husk as a substrate at pH 6.0 [35]. The effect of pH on purified enzyme activity

Table 1
MnP activity assay for wild and mutant strain

S. no.	Sample	Enzyme unit (U/L)
1.	Control	–
2.	Wild strain/parental strain	86.8
3.	Double-mutated strain	368.18

was studied with various buffers. It was reported that the MnP activities were very low in acidic medium (pH 3.5) when compared to the slightly acidic medium (pH 4–6). This result clearly indicated the important role of organic buffers in MnP activity determination [36]. Moreover, similar results were obtained for the determination of MnP activity at the optimum pH 4.0 [37]. It was clearly proved that MnP activities decreased when the temperature reached above or below 25˚C, which is due to the fact that the temperature may inhibit the fungal biomass and enzyme activities. Due to deficiency of moisture content, the fungal cells took more time to resist the stress condition. May be this stress condition induced the fungal cells to enter into the synchronous growth; thereby, it has directly influence on the enzyme production. The effect of temperature on purified ligninolytic enzyme was examined in the range from 25 to 60˚C with 5˚C increments at (specify time intervals here, 5–10 min) intervals. Temperature optimum for MnP production was observed at 30˚C [38]. Most literature surveys revealed that when the temperature was higher than 35˚C, enzymes lost their activity.

Four different carbon sources such as, maltose, sucrose, glucose and lactose, were tested at 2% for MnP production in mutant *P. ostreatus*. Among all the carbon sources, glucose supported a maximum MnP activity of 327 U/L (Fig. 6) and also the highest MnP production (320 U/L) was observed in the culture media containing sodium nitrate as a nitrogen source (Fig. 7). The influences of four different inducers like copper sulphate, Tween 80, glycerol and Glycerol + Tween 80 were also investigated for the hyper-production of MnP from the mutant *P. ostreatus*. Among them, glycerol supported the maximum MnP production of 342 U/L (Fig. 8). It was noted that all other supplements increased the production of MnP activity, but the highest yield of MnP was observed only on glucose as a substrate. Furthermore, the ligninolytic

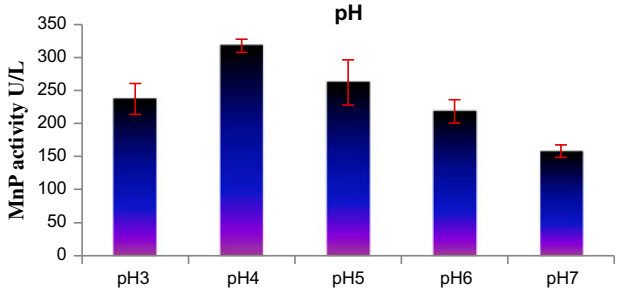

Fig. 4. Effect of pH on submerged fermentation using rice bran by the mutant *P. ostreatus* after 21 d of incubation at 25˚C.

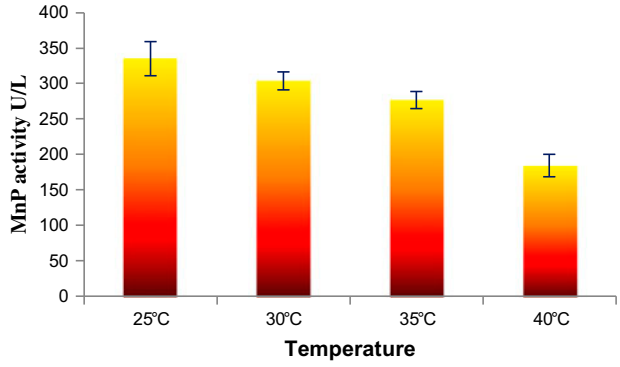

Fig. 5. Effect of temperature on submerged fermentation using rice bran by the mutant *P. ostreatus* after 21 d of incubation at pH 4.

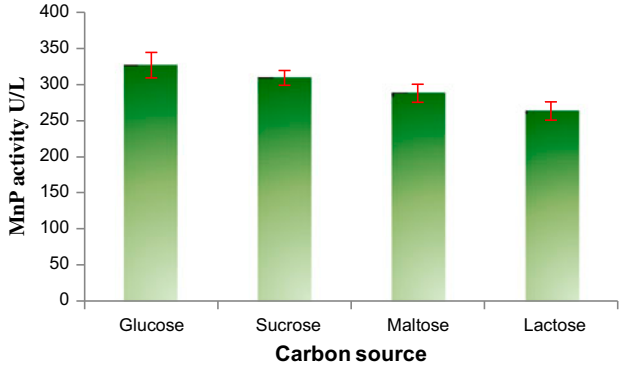

Fig. 6. Effect of carbon source on submerged fermentation using rice bran by the mutant *P. ostreatus* after 21 d of incubation.

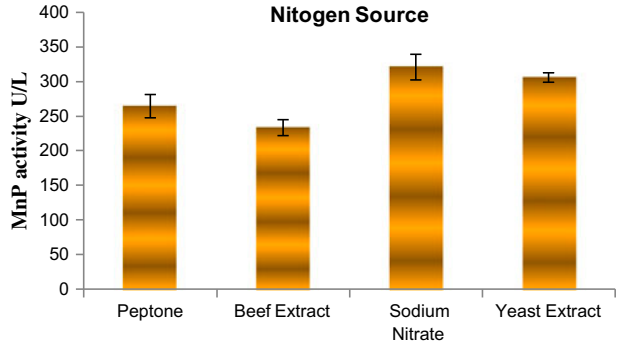

Fig. 7. Effect of nitrogen source on submerged fermentation using rice bran by the mutant *P. ostreatus* after 21 d of incubation.

gene expression was triggered in *P. chrysosporium*, only during the depletion of the nutrient carbon source [39]. It was noted that the maximum enzyme production was obtained in *Trametes pubescens* when

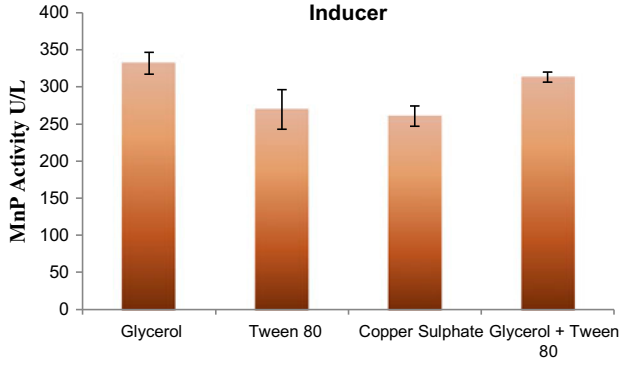

Fig. 8. Effect of inducer on submerged fermentation using rice bran by the mutant *P. ostreatus* after 21 d of incubation.

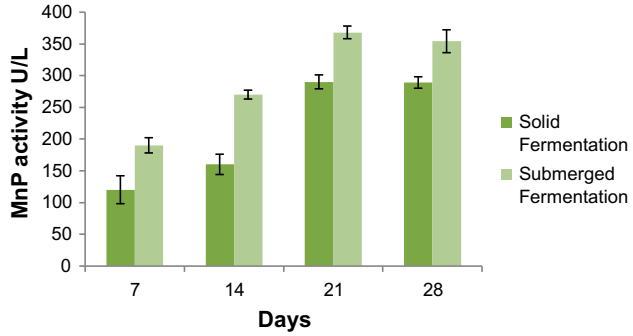

Fig. 9. Production of MnP on solid and submerged fermentation under optimized condition by mutant *P. ostreatus*.

the glucose concentration was very low in production medium [40]. But *P. ostreatus* MTCC 142 showed higher production of MnP when glucose was used as a supplement in culture medium. The positive effect of glucose on MnP production was probably due to the presence of simple sugar, which can be readily utilized by the fungal culture. Therefore, the screening of appropriate carbon source for the cultivation of fungal biomass and target enzyme synthesis plays a vital role in the development of a modern technology of enzyme production. In fact, most literature surveys reported that nitrogen sources greatly increase the ligninolytic enzyme production, but there is contradictory evidence reported on the effects of nitrogen sources (nature and concentration) on ligninolytic enzyme production. *T. pubescens* produced maximum MnP activity when amended with high nitrogen source in the media, while *P. cinnabarinus* produced the highest MnP activity in the nitrogen-limited condition [41,42]. In general, sodium nitrate showed stimulating effects on MnP Production when compared with the other nitrogen sources. Generally, glycerol influences the growth morphology of fungi, whereas surfactant Tween 80 improves the enzyme excretion in culture of *P. chrysosporium*. However, Tween 80 increased the porosity of cell membrane structure and promoted the permeation of MnP from the cell into the medium. Moreover, the actual mechanism by which surfactants (Tween 80) and glycerol mediated extracellular enzyme production in fungi has not been clearly elucidated. The possible mechanism might be due to the activation of MnP gene in fungal mass or by modification of the cytoplasmic membrane or a change in lipid metabolism of the fungus [43].

The hyper-production of MnP by mutant *P. ostreatus* was investigated in both solid and submerged fermentation. Among the fermentation methodology, the mutant *P. ostreatus* shows maximal MnP activity (368 U/L) under submerged fermentation containing rice bran as the main lignocellulosic substrate (Fig. 9). Maximum production of MnP was reached on day 21 under optimized conditions such as pH 4.0, temperature 25°C, 75% moisture content (w/w), glucose 2%, sodium nitrate 0.2% and glycerol 4%.

The protein concentration was higher than the control in the presence of wheat grain, reaching 115 mg/mL for glucose as carbon source, 89 mg/mL

Table 2
Assay of protein content under optimized condition

S. no.	Optimization parameters		Protein content (mg/100 mL)
1.	Carbon source	Glucose	115 ± 3
		Sucrose	80.3 ± 8
		Maltose	74 ± 7.8
		Lactose	65.3 ± 7.5
2.	Nitrogen source	Peptone	89.6 ± 4.1
		Beef extract	78.3 ± 7.5
		Sodium nitrate	79 ± 11.1
		Sodium carbonate	54 ± 4.5
3.	pH	3.0	33 ± 2.6
		4.0	56.3 ± 6.4
		5.0	74.3 ± 4.7
		6.0	65.6 ± 4.1
4.	Temperature	25°C	52 ± 4.5
		35°C	43 ± 4
		45°C	41 ± 3.8
		55°C	36.7 ± 3
5.	Inducers	Glycerol	84.6 ± 5.6
		Tween 80	73.6 ± 6.5
		Copper sulphate	60 ± 6.5
		Glycerol + Tween 80	39 ± 2.6

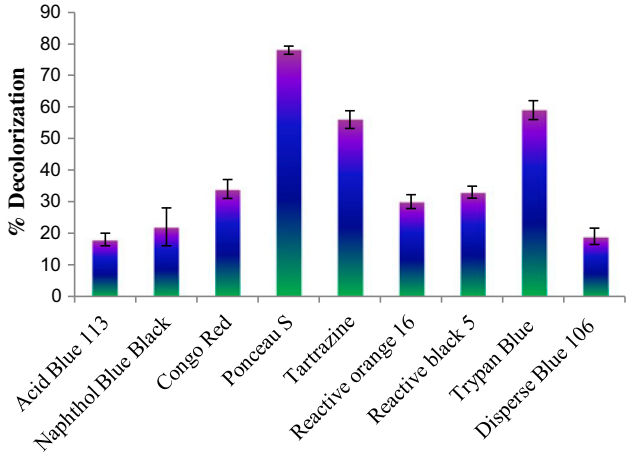

Fig. 10. Decolourization of azo dyes by the purified MnP (368 U/L) under pH 5.0 for 20 min incubation at 30°C.

for peptone as nitrogen source, 75 mg/mL at pH 3, 52 mg/mL at temperature 25°C and 84 mg/mL glycerol as an inducer (Table 2). The concentration of protein increases to the maximum on day 21 under optimized condition. Increase in the concentration of protein in the medium depends upon the optimal parameter, nature of the medium and choice of micro-organism. Wheat bran, as lignocellulosic substrates was inoculated with *P. ostreatus* and *P. chrysosporium* individually and in various combinations; the *P. ostreatus* showed maximum MnP (254 U/L) activities on neem hull waste on the day 20 of incubation. However, very low MnP activity was observed during neem hull degradation with maximum activity (20 U/L) on day 25 of incubation [6]. Interestingly, maximum enzyme activity was observed much earlier in the liquid cultivation. This could have been due to the presence of more carbon and nitrogen source in the basal medium that stimulated the growth and enzyme production of *P. ostreatus*.

The purified MnP extracellular enzyme was exploited for decolourizing the selected azo dyes under the optimized condition for 20 min. Among all the azo dyes, Ponceau S, Tartrazine and Trypan Blue were rapidly decolourized as 78, 56 and 59%, respectively, with the purified MnP enzyme concentration of (368 U/L), other five dyes (Acid Blue 113, Naphthol Blue Black, Congo Red, Reactive Orange 16, Reactive Black 5 and Disperse Blue 106) were decolourized up to 15–37% (Fig. 10).

HPLC elution profile indicated that purified MnP from the mutant strain was able to achieve higher degradation of three dyes, such as Tartrazine, Ponceau S and Trypan Blue, at a concentration rate of 50 ppm. HPLC analysis of three different dye samples extracted at 0 h incubation showed a peak at retention time 2.6, 1.8 and 2.4 min, respectively, and sample extracted after 21 d of incubation showed major metabolites at a different retention time (Figs. 11–13). Detection of two or more peaks in each HPLC of the sample as compared with a single peak in control

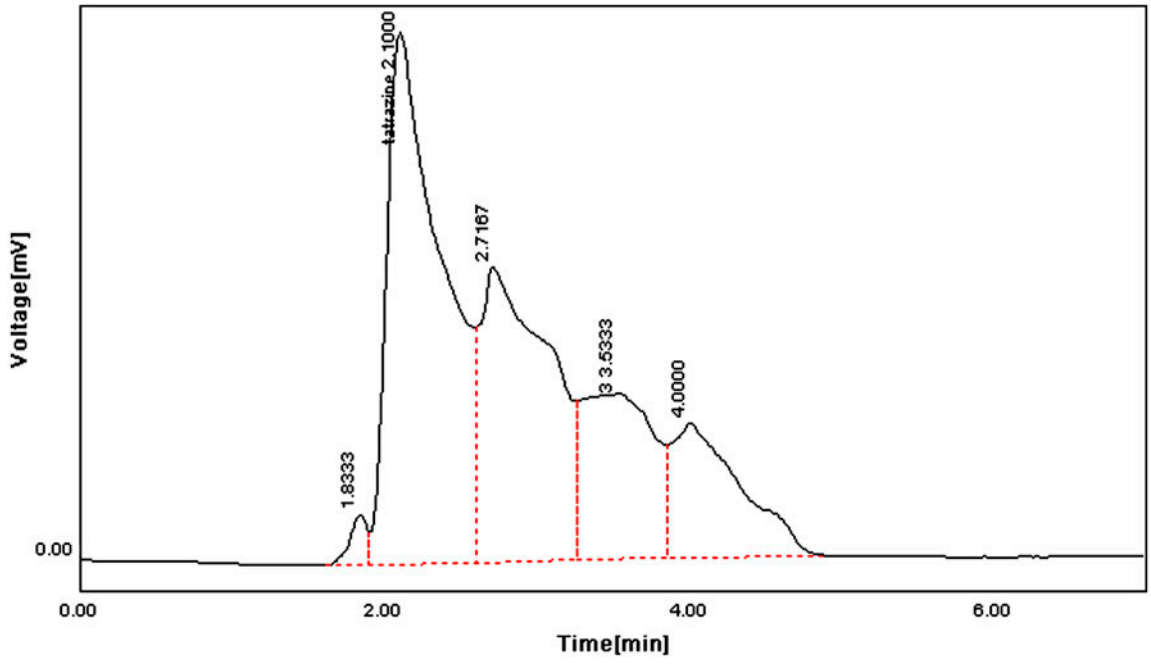

Fig. 11. HPLC elution profile for the degradation metabolites of Tartrazine with a concentration of 50 ppm.

clearly indicated the degradation of the dyes. The degradation of azo dyes normally started with an initial reduction or cleavage of azo bond by an enzyme azoreductase or MnP, which resulted into colourless compounds. Moreover, a complete degradation of aromatic amines occurs strictly under aerobic conditions. It was evident that appearance of five new peaks and disappearance of the single peak in the chromatogram

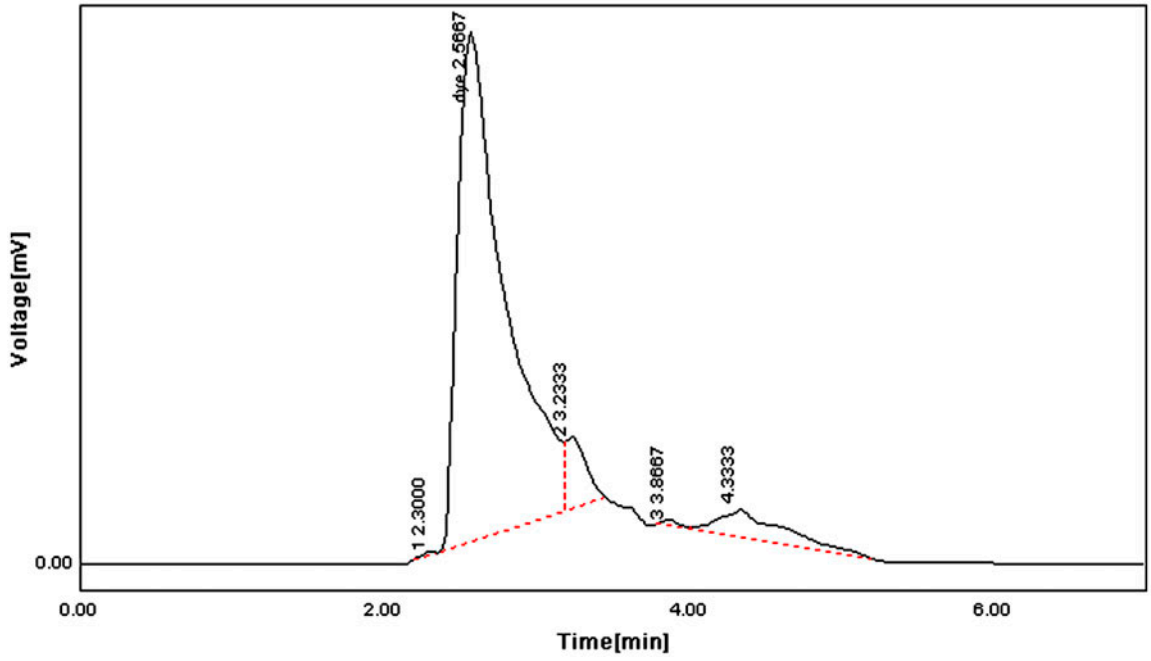

Fig. 12. HPLC elution profile for the degradation metabolites of Ponceau with a concentration of 50 ppm.

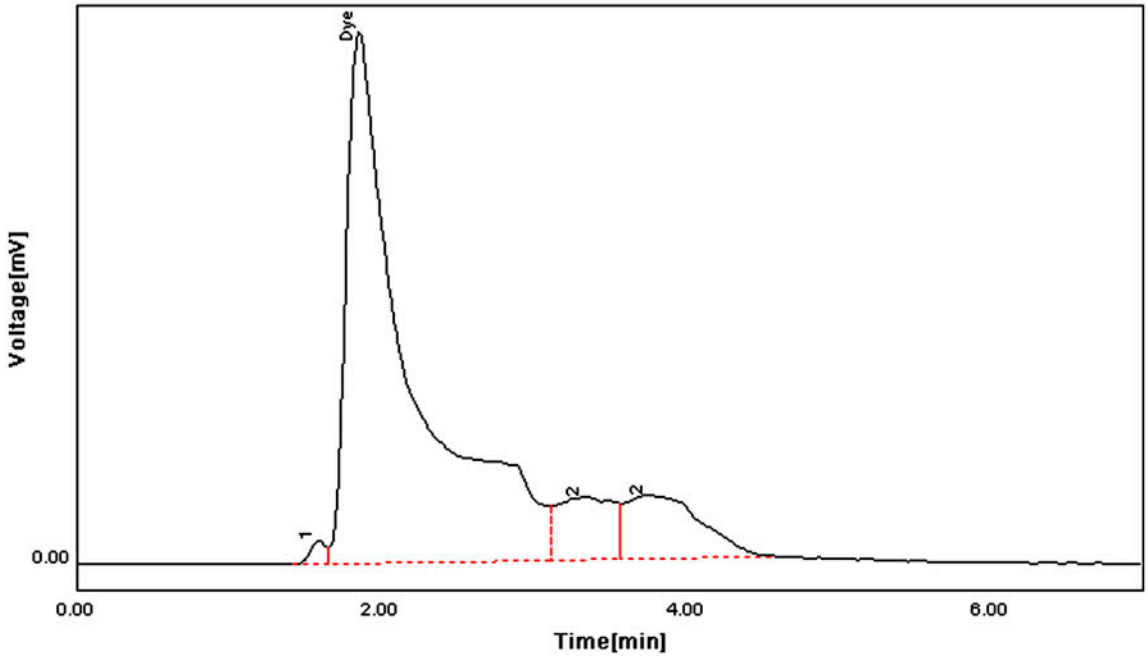

Fig. 13. HPLC elution profile for the degradation metabolites of Trypan Blue with a concentration of 50 ppm.

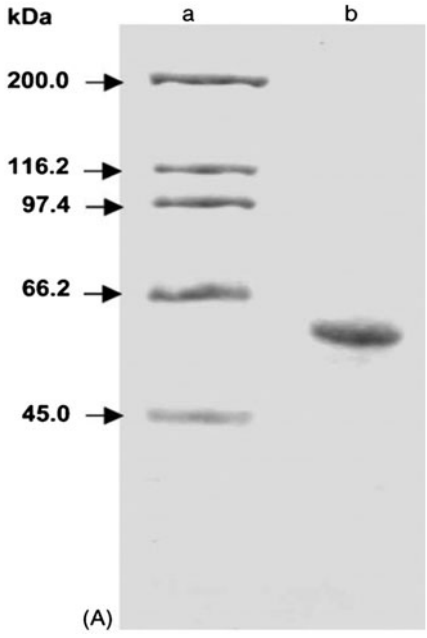

Fig. 14. SDS-PAGE of the purified MnP from mutant *P. ostreatus* MTCC 142. Lane a: high standard markers; Lane b: purified MnP enzyme.

indicated the decolourization parent dye, Rubine GFL, by consortium-AP [10].

The purified MnP from mutant *P. ostreatus* appeared as single band on 10% SDS-PAGE and showed a molecular mass of 58 ± 1 kDa (Fig. 14). MnP-PGY and MnP-GY produced by two different fungi, namely *P. ostreatus* [44] and *Trametes versicolor* [45], which revealed the molecular weight of 42–50 kDa, which is slightly lesser than the molecular weight found by the mutant *P. ostreatus*.

The chemical nature of dye imparts toxicity. Disposal of untreated dyeing effluents in water bodies might cause serious environmental and health hazard problem, which in turn may affect germination rates and biomass of several plant species. Therefore, it is necessary to access the phytotoxicity of the dye before and after degradation. Tables 3 and 4 represent the phytotoxicity analysis of the dyes such as Ponceau S, Tartrazine and Trypan Blue and their metabolites obtained after decolourization. The untreated dye solution of Ponceau S, Tartrazine and Trypan Blue showed 10, 20 and 10% germination, respectively, on *S. indicum* and *V. mungo*, whereas the metabolites solution showed more than 80% germination in all

Table 3
Phytotoxicity assay on *S. indicum*

	S. indicum						
Parameters	Water	Ponceau	Tartrazine	Trypan Blue	Metabolites of Ponceau	Metabolites of Tartrazine	Metabolites of Trypan Blue
Germination (%)	100	10	20	10	100	80	90
Plumule (cm)	10.13 ± 0.98	2.01 ± 0.62	1.50 ± 0.26	1.91 ± 0.23	6.22 ± 0.99	5.73 ± 0.47	3.96 ± 0.09
Radical (cm)	9.11 ± 0.057	5.57 ± 0.06	3.98 ± 0.42	4.6 ± 0.72	7.12 ± 0.058	7.09 ± 0.43	5.32 ± 0.83

Note: Values of mean of three experiments Standard Error Mean (SEM) (±), seeds germinated in Ponceau, Tartrazine, Trypan Blue, and its degraded metabolites are significantly different from the seeds in distilled water at $p < 0.001$ by one-way ANOVA with Tukey–Karmer comparison test.

Table 4
Phytotoxicity assay on *V. mungo*

	V. mungo						
Parameters	Water	Ponceau	Tartrazine	Trypan Blue	Metabolites of Ponceau	Metabolites of Tartrazine	Metabolites of Trypan Blue
Germination (%)	100	10	20	10	90	80	80
Plumule (cm)	12.4 ± 0.98	3.18 ± 0.09	3.8 ± 0.13	2.91 ± 0.38	8.72 ± 0.76	6.31 ± 0.09	4.06 ± 0.18
Radical (cm)	8.09 ± 0.56	3.11 ± 0.31	3.48 ± 0.09	4.13 ± 0.92	7.6 ± 0.53	4.76 ± 0.48	4.84 ± 0.75

Note: Values of mean of three experiments SEM (±), seeds germinated in Ponceau, Tartrazine, Trypan Blue, and its degraded metabolites are significantly different from the seeds in distilled water at $p < 0.001$ by one-way ANOVA with Tukey–Karmer comparison test.

dyes. The length of radical and plumule were drastically affected by the plantlets growth when treated with an untreated dye solution (500 ppm). In contrast, plumule length and radical length in *S. indicum* and *V. mungo* significantly increased in all dye metabolites. This phytotoxicity test suggests the non-toxic nature of the product formed.

In our study, the purified MnP from *P. ostreatus* needs 3–6 h for decolourization of Ponceau S, Tartrazine and Trypan Blue to about complete 50–80% degradation, whereas remaining dyes show only less than 50% degradation. The RBBR dye decolourization by purified laccase from *P. cinnabarinus* needs only 6 h, which often correlates to ligninolytic enzyme activities [46]. Furthermore, the degradation potential of azo dyes depends on the nature of dye, concentration of dyes, amount of enzyme used and the enzyme activity. The amount of dye remaining after 90 min was less than 30% of the original amount.

4. Conclusion

Our result confirmed that random mutagenesis approach may be a potential tool for modern research for the increased production of enzymes in the near future. Moreover, our study also demonstrated successful optimization and production of ligninolytic enzyme MnP, which could be further exploited for decolourization of structurally different textile dyes.

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References

[1] H. Zollinger, Colour Chemistry—Synthesis, Properties and Application of Organic Dyes and Pigment, VCH Publishers, New York, NY, 1987, pp. 92–102.
[2] C.M. Carliell, S.J. Barelay, N. Naidoo, C.A. Buckley, D.A. Mulholland, E. Senior, Microbial decolourization of reactive azo dye under anaerobic condition, Water SA 21 (1995) 61–69.
[3] K.C. Chen, J.Y. Wu, D.J. Liou, S.C. Hwang, Decolorization of the textile dyes by newly isolated bacterial strains, J. Biotechnol. 101 (2003) 57–68.
[4] A. Conneely, W.F. Smyth, G. McMullan, Metabolism of the phthalocyanine textile dye remazol turquoise blue by *Phanerochaete chrysosporium*, FEMS Microbiol. Lett. 179 (1999) 333–337.
[5] R. Krull, M. Hemmi, P. Otto, D.C. Hempel, Combined biological and chemical treatment of highly concentrated residual dyehouse liquors, Water Sci. Technol. 38 (1998) 339–346.
[6] P. Verma, D. Madamwar, Decolourization of synthetic dyes by a newly isolated strain of *Serratia marcescens*, World J. Microbiol. Biotechnol. 19 (2003) 615–618.
[7] M.M. Hassan, M.Z. Alam, M.N. Anwar, Biodegradation of textile azo dyes by bacteria isolated from dyeing industry effluent, Int. Res. J. Biol. Sci. 2(8) (2013) 27–31.
[8] S. Mishra, S. Salony, V.S. Bishra, Decolourization and detoxification of textile dyes and black liquor by laccase of *Cyathus bulleri*, J. Sci. Ind. Res. 66 (2007) 684–688.
[9] T. Joshi, L. Iyengar, K. Singh, S. Garg, Isolation, identification and application of novel bacterial consortium TJ-1 for the decolourization of structurally different azo dyes, Bioresour. Technol. 99 (2008) 7115–7121.
[10] H.S. Lade, T.R. Waghmode, A.A. Kadam, S.P. Govindwar, Enhanced biodegradation and detoxification of disperse azo dye Rubine GFL and textile industry effluent by defined fungal–bacterial consortium, Int. Biodeterior. Biodegrad. 72 (2012) 94–107.
[11] M. Chivukula, V. Renganathan, Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*, Appl. Environ. Microbiol. 61(12) (1995) 4374–4377.
[12] A. Heinfling, M.J. Martinez, A.T. Martinez, M. Bergbauer, U. Szewzyk, Transformation of industrial dyes by manganese peroxidases from Bjerkandera adusta and Pleurotus eryngii in a manganese-independent reaction, Appl. Environ. Microbiol. 64 (1998) 2788–2793.
[13] E. Abadulla, T. Tzanov, S. Costa, K.H. Robra, A. Cavaco-Paulo, G.M. Gubitz, Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta, Appl. Environ. Microbiol. 66 (2000) 3357–3362.
[14] A. Hatakka, Lignin-modifying enzymes from selected white-rot fungi: Production and role from in lignin degradation, FEMS Microbiol. Rev. 13 (1994) 125–135.
[15] I. Schneegab, M. Hofrichter, K. Scheibner, W. Fritsche, Purification of the main MnP isoenzyme MnP2 from the white rot fungus *Nematoloma frowardiib* b19, Appl. Microbiol. Biotechnol. 48 (1997) 602–605.
[16] K.E. Hammel, A.N. Kapich, K.A. Jensen, Z.C. Ryan, Reactive oxygen species as agents of wood decay by fungi, Enzyme Microb. Technol. 30 (2002) 445–453.
[17] J.K. Glenn, L.K. Akileswaran, M.H. Gold, Mn(II) oxidation is principal function of the extracellular Mnp from *Phanerochaete chrysosporium*, Arch. Biochem. Biophys. 251 (1986) 396–688.
[18] H. Wariishi, H.B. Dunford, I.D. Macdonald, M.H. Gold, Manganese peroxidase from the lignin degrading basidiomycete *Phanerochaete chrysosporium*, transient state kinetics and reaction mechanism, J. Biol. Chem. 264 (1989) 3335–3340.
[19] O.U. Raziye, K.P. Nurdan, Enhanced production of manganese peroxidase by *P. chrysosporium*, Braz. Arch. Biol. Technol. 50 (2007) 913–920.
[20] P. Chand, A. Aruna, A.M. Maqsood, L.V. Rao, Novel mutation method for increased cellulase production, J. Appl. Microbiol. 98 (2005) 318–323.
[21] A. Heinfling, M.J. Martínez, A.T. Martínez, M. Bergbauer, U. Szewzyk, Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*, FEMS Microbiol. Lett. 165(1) (1998) 43–50.

[22] M. Mansur, M.E. Arias, J.L. Copa-Patino, M. Flardh, A.E. Gonzalez, The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities, Mycologia 95 (2003) 1013–1020.
[23] G. Songulashvili, V. Elisashvili, S.P. Wasser, E. Nevo, Y. Hadar, Basidiomycetes laccase and manganese peroxidase activity in submerged fermentation of food industry wastes, Enzyme Microb. Technol. 41 (2007) 57–61.
[24] O.M. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[25] P. Saranraj, V. Sumathi, D. Reetha, D. Stella, Fungal decolourization of direct azo dyes and biodegradation of textile dye effluents, J. Ecobiotechnol. 2 (2010) 12–16.
[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, Nature 227 (1970) 680–685.
[27] L. Xudong, J. Rong, Decolourization and biosorption for Congo Red by system rice hull-*Schizophyllum* sp. F17 under solid-state condition in a continuous flow packed-bed bioreactor, Bioresour. Technol. 99 (2008) 6885–6892.
[28] P. Ellaiah, T. Prabhakar, B. Ramakrishna, A.T. Taleb, K. Adinarayana, Strain improvement of *Aspergillus niger* for the production of lipase, Indian J. Microbiol. 42 (2002) 151–153.
[29] K.V.V.S.N. Bapi Raju, P. Sujatha, P. Ellaiah, T. Ramana, Mutation induced enhanced biosynthesis of lipase, Afr. J. Biotechnol. 3 (2004) 618–621.
[30] V.K. Gochev, A.I. Krastanov, Fungal laccases, Bulg. J. Agric. Sci. 13 (2007) 75–83.
[31] S.K. Karanam, N.R. Medicherla, Enhanced lipase production by mutation induced *Aspergillus japonicas*, Afr. J. Biotechnol. 7 (2008) 2064–2067.
[32] R. Vardanega, D. Remonatto, F. Arbter, A. Polloni, E. Rigo, J.L. Ninow, H. Treichel, D. Oliveira, M. Luccio, A systematic study on extraction of lipase obtained by solid-state fermentation of soybean meal by a newly isolated strain of *Penicillium* sp., Food Bioprocess Technol. 3(3) (2010) 461–465.
[33] L. Toscano, V. Gochev, G. Montero, M. Stoytcheva, Enhanced production of extracellular lipase by novel mutant strain of *Aspergillus niger*, Biotechnol. Biotechnol. Equip. 25 (2011) 2243–2247.
[34] S. Dhawan, R. Lal, R.C. Kuhad, Ethidium bromide stimulated hyper laccase production from bird's nest fungus *Cyathus bulleri*, Lett. Appl. Microbiol. 36 (2003) 64–67.
[35] G. Viniegra-González, E. Favela-Torres, C.N. Aguilar, S.D.J. Rómero-Gomez, G. Díaz-Godínez, C. Augur, Advantages of fungal enzyme production in solid state over liquid fermentation systems, Biochem. Eng. J. 13 (2003) 157–167.
[36] S. Aslam, M. Asgher, Partial purification and characterization of ligninolytic enzymes produced by *Pleurotus ostreatus* during solid state fermentation, Afr. J. Biotechnol. 10 (2011) 17875–17883.
[37] F. Patrick, G. Mtui, A.M. Mshandete, A. Kivaisi, Optimization of laccase and manganese peroxidase production in submerged culture of *Pleurotus sajor-caju*, Afr. J. Biotechnol. 10 (2011) 10166–10177.
[38] R. Akhila, G.K. Jayalakshmi, E.A. Tholath, Solid state production of manganese peroxidases using arecanut husk as substrate, Braz. Arch. Biol. Technol. 53 (2010) 555–562.
[39] P. Wang, X. Hu, S. Cook, M. Begonia, K.S. Lee, H.M. Hwang, Effect of culture conditions on the production of ligninolytic enzymes by white rot fungi *Phanerochaete chrysosporium* (ATCC 20696) and separation of its lignin peroxidase, World J. Microbiol. Biotechnol. 24 (2008) 2205–2212.
[40] C. Galhaup, H. Wagner, B. Hinterstoisser, D. Haltrich, Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*, Enzyme Microb. Technol. 30 (2002) 529–536.
[41] T.A. Mester, J.A. Field, Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera* sp. strain BOS55, FEMS Microbiol. Lett. 155 (1997) 161–168.
[42] L. Gianfreda, F. Xu, J.M. Bollag, Laccases: A useful group of oxidoreductive enzymes, Biorem. J. 3 (1999) 1–26.
[43] M. Asther, G. Corrieu, R. Drapron, E. Odier, Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12, Enzyme Microb. Technol. 9 (1987) 245–249.
[44] H. Kamitsuji, Y. Honda, T. Watanbe, M. Kuwahara, Production and induction of manganese peroxidase isozymas in a white-rot fungus *Pleurotus ostreatus*, Appl. Microbiol. Biotechnol. 65 (2004) 287–294.
[45] P.P. Champagne, J.A. Ramsay, Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*, Appl. Microbiol. Biotechnol. 69 (2005) 276–285.
[46] K. Schliephake, G.T. Lonergan, Laccase variation during dye decolourisation in a 200 L packed-bed bioreactor, Biotechnol. Lett. 18 (1996) 881–886.