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Peroxidases of *Aspergillus niger* for Decolorization of Azo and Anthraquinone and Heterocyclic Dyes

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ABSTRACT

Aspergillus niger SA1, was screened for the production of Peroxidase enzymes (Laccase, Lignin peroxidase "LiP" and Manganese peroxidase i.e., MnP), on agar plates and in liquid cultures. Production media supplemented with AR 151 dye (10 ppm) was the best among all tested media for all the three enzymes (Laccase, LiP and MnP). Maximum production of Laccase was 1784.38 IU/ml by *Aspergillus niger* SA1. Effect of incubation time for the production of all the three enzymes increased as the fermentation progressed from 24 hr to 168 hrs. Characterization of crude lignin peroxidase for substrate concentration, pH and temperature with the selected fungal strain gave significant rise in the activity of LiP with an elevated concentration of substrate from 2.5 to 10 mm. Optimum pH of 7 was found for LiP activity for the strain. Significant interaction was observed at different temperatures with LiP activity from fungal strain, highest LiP activity (63.32 IU/ml) at 80°C and reduced activity (13.36 IU/ml) at 10°C. Molecular weight of Laccase, Manganese peroxidase and Lignin peroxidase after pretreatment of crude enzyme was found 66.2, 43 and 38Kda, respectively. Peroxidase was purified by column chromatography (Sphadex G-75 and DEAE cellulose column cartridge with a linear gradient of 0.1-0.5 M NaCl in Tris buffer pH 7.5). Over all a coexistence of higher absorbance Vs activity was observed in the elevated peaks with the fungal strain. Maximum fold purification was observed with the greater yield of the enzyme activity originally present in the crude enzyme. It was found that minimal time of 10 sec was sufficient for decreasing the absorbance of initial color for all four textiles dyes (AR 151, DBK$_2$RL, Orange II and Sulphur black) with crude and purified enzyme.

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INTRODUCTION

Dyes are synthetic chemicals, widely used in the industry. Percentage of the lost dye in the effluents can reach up to 50% with a high toxicity towards living organisms and can affect man through the food chain (Muzariri *et al*., 2001). Fungi have been proved to be able to decolorize dyes with long lasting benefits and for having almost no harmful effects on environment. In addition, it would be cheaper as compared to other techniques tested (Unyayar *et al*., 2005). In order to be biodegraded, contaminants must interact with enzymatic systems in the degrading organisms. The development processes based on enzyme system, seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Ryan *et al*., 2003), including synthetic dyes currently employed in the

industry leads to the detoxification of azo dyes since no aromatic amines are formed (Rashid *et al.*, 2008). Different studies propose several degradation mechanisms for phenolic and non-phenolic azo dyes (Shin *et al.*, 1997). In the proposed model azo dyes are degraded without direct cleavage of the azo bond through a highly non-specific free radical mechanism forming phenolic type compounds, thereby avoiding the formation of toxic aromatic amines, which might be useful to control environmental pollution (Wesenberg *et al.*, 2003).

And the unspecific nature of the catalysis by fungal peroxidases makes the possibilities for their biotechnological applications numerous and include several fields of industry (Rodríguez *et al.*, 2004). In industrial scale laccase is not only utilized for the treatment of dye-containing wastewaters as well as for denim bleaching (Wallace, 2001). For industrial application, peroxidase production can be increased by different physicochemical cultural conditions (pH, temperature, fermenting period/time) and nutritional levels (carbon, nitrogen etc.) of the fermenting media.

In order to trigger LiP expression in liquid cultures of *Phanerochaete chrysosporium* there is need to be concomitantly starved and exposed to pure oxygen (Dosoretz and Grethlien, 1991).The Mn^{2+} is a specific effector that induces MnP and represses LiP. Addition of manganese has a stimulating effect on the expression of MnP in several fungi such as *P. chrysosporium* (Brown *et al.*, 1993), *Pleurotus ostreatus* (Cohen *et al.*, 2001), *T. versicolor* (Johansson *et al.*, 2002), and *C. subvermispora* (Lowry *et al.*, 1951). Manganese has been suggested to have a role also in the post-translational modification steps as the observed transcript levels and extracellular MnP activity do not correlate and Mn^{2+} is required for the production of active MnP by *C. subvermispora* (Manubens *et al.*, 2003). Addition of manganese has been observed to repress the transcription of veratryl peroxidase (VP) in *P. ostreatus* (Cohen *et al.*, 2001) and the production of LiP in *P. chrysosporium* (Pearce *et al.*, 2003). Instead, addition of veratryl alcohol or lignin induces LiP production in *P. chrysosporium* (Cancel *et al.*, 1993). Other factors influencing the expression of lignin degrading peroxidases include oxidative stress (Bonnarme *et al.*, 1991), heat shock (Brown *et al.*, 1993) and the availability of nutrients, such as nitrogen (Johansson *et al.*, 2002).

The production of LiP and MnP by white rot fungi occurs at the onset of the secondary growth phase, when utilizable nutrients are depleted and primary fungal growth ceases. These enzymes play a central role in lignin degradation. Carbon, nitrogen and maganese are critical nutritional variables in the production of LiP and MnP and indeed other lignolytic enzymes by *Phanerochaete chrysosporium* and other white rot fungi (Bonnarme *et al.*, 1991). The advantages of replacing chemical treatments with enzymes are often compelling from a societal and environmental view point; enzymes must compete economically with often entrenched and extremely inexpensive traditional chemical processes. Therefore, in the present study enzyme production was carried out by indigenous fungal strains with optimized conditions and the crude enzymes were characterized and purified for their wide application in textile and other industries.

MATERIALS AND METHODS

The solid screening of lignolytic enzymes production was carried out with *Aspergillus niger* SA1*,* according to the method described earlier (Munoz *et al.*, 1997) with some modifications (Table1).

Measurement of enzyme activity

Laccases and Manganese peroxidase (MnP) activity was measured by using 5mM 2, 6-dimethoxyphenol (DMP) as substrate in 100mM tartarate buffer 4.5 pH (Munoz *et al.*, 1997; Gold *et al.*, 1988). MnP activity was estimated by the method described earlier (Manubens *et al.*, 2003). One unit of MnP activity represents 1 m mole of Mn^{2+} oxidized per min. Whereas, Lignin peroxidase was assayed by using 10mM veratryl alcohol as a substrate (Glenn *et al.*, 1983). LiP activity was determined by the rate of oxidation of 10mM Veratryl alcohol, 250mM Sodium tartarate buffer pH 3.0 with 4mM H_2O_2.

Table1. Peroxidase enzyme assay.

Lignin Peroxidase	Manganese Peroxidase	Laccase
Reagents: 250mM Sodium Tartrate Buffer (ST), 10mM Veratryl Alcohol (VA), 4mM H_2O_2	Reagents: 250mM sodium malonate (SM), 20mM 2,6-dimethoxdy phenol (DMP), 20mM $MnSO_4$. H_2O	Reagents: 250mM sodium malonate, 20mM $MnSO_4$. H_2O, 5mM DMP, 100mM sodium tartarate buffer (pH: 4.5)

Assay: Only samples and H_2O_2 were kept in water bath at 30 ºC for 30 min while other reagents were used without incubation. Reaction volume was 1ml (ST: 200 µl; VA: 200 µl; d.d.H_2O: 400 µl; H_2O_2:100 µl; sample:100 µl). Absorbance was monitored at 0 time and after every 15 second, the total time of incubation was 120 seconds at ambient condition. The assay for enzyme was carried out in silica cuvette and absorbance was measured at 310 nm by Shimadzu UV visible Spectrophotometer.	Assay: DMP was wrapped in aluminum foil and incubated all the reagents at 30 ºC for half an hour. Reaction volume was about 3ml (SM: 600 µl; DMP: 150µl; d.d.H_2O:1650 µl; H_2O_2:100 µl; sample: 150 µl). Absorbance was monitored at 0 time and subsequently after every 5sec for 15 seconds. Enzyme assay was carried out in silica cuvette and absorbance was measured at 270nm by Shimadzu UV visible Spectrophotometer.	Assay: DMP was wrapped in aluminum foil and incubated all the reagents at 30 ºC for 30 minutes. Reaction volume was 2ml containing Sodium malonate, 400 µl; DMP, 100 µl; Distilled Water, 900 µl; Sodium tartarate buffer, 500 µl; 300 µl of sample. Absorbance at λ 469 nm, ε = 46,900 $M^{-1}cm^{-1}$ was monitor at 0 time, and after every 5 second total time of incubation was 15 seconds and absorbance was measured at 469nm.

Production of enzymes in liquid culture

Batch cultures for enzyme production were set up in shake flasks under sterilized conditions. Various parameters were optimized for enzyme production including media, incubation time, pH and temperature. The inoculum was allowed to grow in Saboraud dextrose broth at 30 °C on rotary shaker (120 rpm) for 5 to 7 days. After that whole mass was mixed in a blender to make a homogenous mixture. This homogenized fungal culture was then used as a stock inoculum. The inoculum was stored at 4 °C that was further used in different broth experimentation.

Media optimization for growth and enzyme production was carried out by screening four media having different compositions. Mineral Salt Media (MSM) (Heinzkill *et al*., 1998), Malt extract agar media (MEM), Saboraud dextrose agar media (SDM), Production media (PRM) for Laccase (Bumpus and Aust, 1987), Production media for MnP &LiP (Heinzkill *et al*., 1998). All media were autoclave at 121°C, at 15 psi for 20 minutes.

Effect of pH was monitored by changing the pH of the productive medium (3 to 6.0). Incubation period was optimized by monitoring the fermentation for ten days (24-240hrs) and sample collected after every 24 hrs and analyzed for enzymes. For studying the effect of temperature on enzyme production, extra cellular enzyme production was carried out at a range of temperatures 25, 30, 37 and 55 °C on shaker (120rpm). The samples were collected after the completion of incubation period (168 hrs). The crude enzyme extract was filtered and used for enzyme assay.

Determination of copper in crude enzyme

Bulk production of crude enzyme was carried out under optimized condition by *Aspergillus niger* SA 1, in one litre productive media with the amendment of 10ppm AR 151 dye in Erlenmeyer flasks. The copper concentration in crude enzyme was detected by Atomic Absorption Spectrophotometer through flame method (A Analyst 700) against standard copper solution ($CuNO_3$ in HNO_3 0.5 mol/L of Merck company).

Determination of toxin in crude enzyme

Aflatoxin detection in crude extra cellular Peroxidase enzyme with 10ppm of AR 151 dye, was tested by using Neogen ELISA kit (Veratox for Aflatoxin HS Product No. 8031).

Protein estimation of crude enzyme

The estimation of protein was carried out by taking Bovine Serum Albumin (BSA) as a standard by adopting the procedure of (Kirk *et al*., 1986).

Characterization of crude enzyme (Lip)

Effect of temperature, pH, substrate concentration

The activity of crude enzyme was determined by incubating the enzyme at various temperatures ranging from 10 to 100 °C for half an hour and then enzyme assay was performed for LiP at ambient temperature. The effect of different pH on crude enzyme was analyzed by using buffer of different pH from 3-10 and enzyme activity (LiP) was measured accordingly. The substrate concentration (Veratryl alcohol) ranging from 2.5, 5, 10, 20 and 40 mM was made and used for the enzyme activity of LiP to observe the effect of substrate concentration on enzyme activity.

Molecular weight determination

Crude Peroxidase enzyme was analyzed for molecular weight determination from *Aspergillus niger SA*1*,* by SDS PAGE.

Protein extraction

Crude enzyme (1 ml) was suspended in 1.0 ml of 0.5M NaCl in pre-weighed sterilized eppendorf tube and centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded leaving the pellet at the bottom of eppendorf tube. The pellet was washed with 1.0 ml of distilled water repeatedly. Fifty micro-liters (50 µl) of gel sample buffer were added to the pellet, mixed thoroughly and kept at 100 °C for 10 minutes in water bath. Thereafter, the eppendorf was cooled by placing in ice for 10 minutes. Then the sample was spun at 13,000 rpm for one minute to settle the insoluble residue.

Electrophoresis

Enzyme protein was analyzed through slab type SDS-PAGE following the method of Laemmli (1970) using 12.25% polyacrylamide gel. The molecular weight and variation among the samples were recorded by comparing the bands with the standard marker protein (Code # SM0431).

Purification of peroxidase enzyme

Fermentation media was incubated with all determined optimized conditions in shake flask for the production of bulk enzyme extract. After the maximum production of the enzymes, the following steps were carried out for the purification of Peroxidase (LiP). All the purification steps were carried out at 4 °C in cold room. Fungal hyphae were separated from fermentation medium by centrifugation at 10,000 rpm for 30 minutes at 4°C and the supernatant was further filtered through 0.2µ millipore filter paper. The filtrate was kept at 4 °C in refrigerator.

Optimization of $(NH_4)_2SO_4$ and salt precipitation

Different concentration (30, 40, 50 and 60%) of ammonium sulfate $(NH_4)_2SO_4$ was added in 100 ml aliquots of crude enzyme. The suspension was stirred on magnetic stirrer for half an hour at 4 °C in a cold room. Then precipitates were collected by centrifugation at 10,000 rpm for 30 minutes at 4°C in a Kokusan Model H-251 centrifuge. The peroxidase activity and protein content of the supernatant was checked. The optimum concentration for $(NH_4)_2SO_4$ was selected on the basis of peroxidase activity and used for further precipitation.

Dialysis

The precipitates were suspended in small volume of 10mM Tris buffer (pH 7.5) and dialyzed by using 1200 MW cut off dialyzing bag, and the bag was placed in 2 liters of 10mM Tris buffer (pH 7.5) for overnight at 4 °C with three changes of 2 liters of the same buffer. After dialysis, the sample was used for gel filtration.

Gel permeation chromatography using Sephadex G-75

Sephadex G-75 was used for gel filtration. 10.0 g of Sephadex G-75 was soaked in 500ml of 10mM Tris buffer (pH 7.5) containing 0.1g of sodium azide as bacteriostatic agent and was kept at room temperature for 48-72 hrs, for maximum swelling of beads in gel. The gel was deaerated in sonicator for 20 minutes, and then the gel was poured and packed in a 0.9×60 cm column. The packed column was washed with 10mM Tris buffer (pH 7.5) until fully packed. Dextran blue solution was used for the determination of its void volume. 2 ml of dialyzed enzyme extract was applied on the column and eluted with excess volume of 10mM Tris buffer (pH 7.5) and fractions of each 3 ml were collected by automatic fraction collector Advantec SF-100. The absorbance of each fraction was then recorded at 280nm. The fractions showing high absorbance were further analyzed for enzyme activity and protein estimation.

Ion-exchange chromatography using DEAE cellulose

Purification of enzyme from the crude precipitates was also done by ion exchange chromatography. Diethyl amino ethyl (DEAE) cellulose was used as gel. The DEAE-cellulose was pretreated with acid to swell the exchanger so that it becomes fully accessible to the charged macromolecules in solution. The 15 g weighed exchanger was suspended in 15 volumes (w/v) in the acid 0.5M HCl as the first treatment and was allowed to stand at least 30 minutes but not more than two hours. The supernatant was decanted and the exchanger was washed until the effluent was at pH 4.0 (intermediate pH). The exchanger was stirred in to 15 volumes of the 0.5M NaOH as the second treatment and allowed to stand for additional 30 minutes. The second treatment was repeated and the exchanger was washed with distilled water until the effluent is at neutral pH. The treated exchanger is placed in the acid component of the buffer (the pH should be less than 4.5) and sonicated for at least 30 minutes. The exchanger was then titrated with the basic component of the same buffer, filtered and suspended in a fresh buffer to complete the pretreatment.

The exchanger was allowed to settle and "fines" above

the settled exchanger were removed by decantation. Buffer was added to the exchanger so that the final volume of slurry was 150% of the settled wet volume of the exchanger. Pharmacia Column K (15/30) was packed with the slurry of the exchanger. The packed column was then equilibrated over night with 10mM Tris buffer (pH 7.5) at the operating flow rate of 18 ml/hr using automatic microtube pump (EYELA-MP-3). The column was packed with reconstituted enzyme which was first eluted with 50 ml of 10mM Tris buffer (pH 7.5) and then with NaCl gradient (0.1-0.5M). Fractions each of 3 ml aliquots were collected using the automatic fraction collector. Optical density at λ 280 nm and protein contents was determined of each fraction. Protein containing fractions were assayed for peroxidase activity and samples showing activity were pooled, decanted and lyophilized.

Single step identification and isolation procedure for Laccase

Protein sample (purified enzyme) was loaded on 12.25% gel. After electrophoresis, the gel was stained with Drimarene Blue X3LR and incubated at 30 °C for 30 min to obtain colorless zone (Soares *et al*., 2002).

Application of peroxidase enzyme

Decolorization assay for different textile dyes

All decolorization assays by the crude filtrate and lypholized peroxidases enzymes were performed at pH 7.5 at 30 °C with different textile dyes (AR 151, DBK_2RL, Orange II and Sulphur Black) of 10 ppm. Decolorization was assayed by measuring decrease in the absorbance at specific wavelength for each dye (AR 151 at 512nm, DBK_2RL at 620nm, Orange II at 480 nm and Sulphur Black at 530nm). Crude filtrate (10 µL) or purified enzyme/lypholized and 1990 µL dye solution (containing 10 ppm dye) were used in all analyses. Changes in the absorbance were detected during two minutes incubation period by spectrophtometric measurements, which were done at every 10 seconds; absorbance change was given as percentage change of color intensity (Soares *et al*., 2002).

RESULTS AND DISCUSSION

TIn the present study Peroxidase (particularly laccase, lignin peroxidase and manganese peroxidase) were produced and optimized by selected fungal strain. Besides it has revitalized the role of Peroxidase for textile dye removal.

Screening of *Aspergillus niger* SA1 for peroxidase production

Aspergillus niger SA1 was screened for the production of Peroxidase enzymes (Laccase, Lignin peroxidase and Manganese peroxidase). Tested strain showed positive results when screened on agar plates and in liquid cultures (Table 2). The most widely researched fungi in regard to dye degradation were the ligninolytic fungi which produced enzymes as lignin peroxidase, manganese peroxidase and laccase that degrade many aromatic compounds and oxidize dyes (Wallace, 2001; Ehlers and Rose, 2005).

Table 2. *Aspergillus niger* SA1 screened for lignolytic enzyme detection in solid and broth malt extract medium with the incorporation of AR 151 dye (10ppm).

Strain	Solid	Liquid
Aspergillus niger SA1	+ + +	+ + +

Where, +++ indicate the presence of enzyme activity.

Production of enzymes in liquid culture

Effect of media, incubation time, pH, temperature

Media PRM, promoted the enzyme production significantly, as it produced the continuous best result proved by treatment for all the three enzymes i. e., Laccase (1784.38 IU/ml), LiP (2741.43 IU/ml) and MnP (2719.49 IU/ml) respectively (Figure 1a). Growth media enhance the adsorption capacities of fungus. Laccase production by *Phlebia fascicularia, P. floridensis* and *Dichomitus squalens* in mineral salts broth, malt extract broth and in the presence of various supplements has shown maximum activities (Arora and Gill, 2001). Production of all the three enzymes laccase (482.66 IU/ml), LiP (732.45 IU/ml) and MnP (854.70 IU/ml) respectively, increased as the fermentation progress from 24 hr to 168 hrs. Where after the decrease in activities of enzyme was observed (Figure 1b). Previously it is reported that excellent correlation between the decolorization speed and additional nutrient concentration reached upto 92.9% in a short time (Chen *et al*., 2002).

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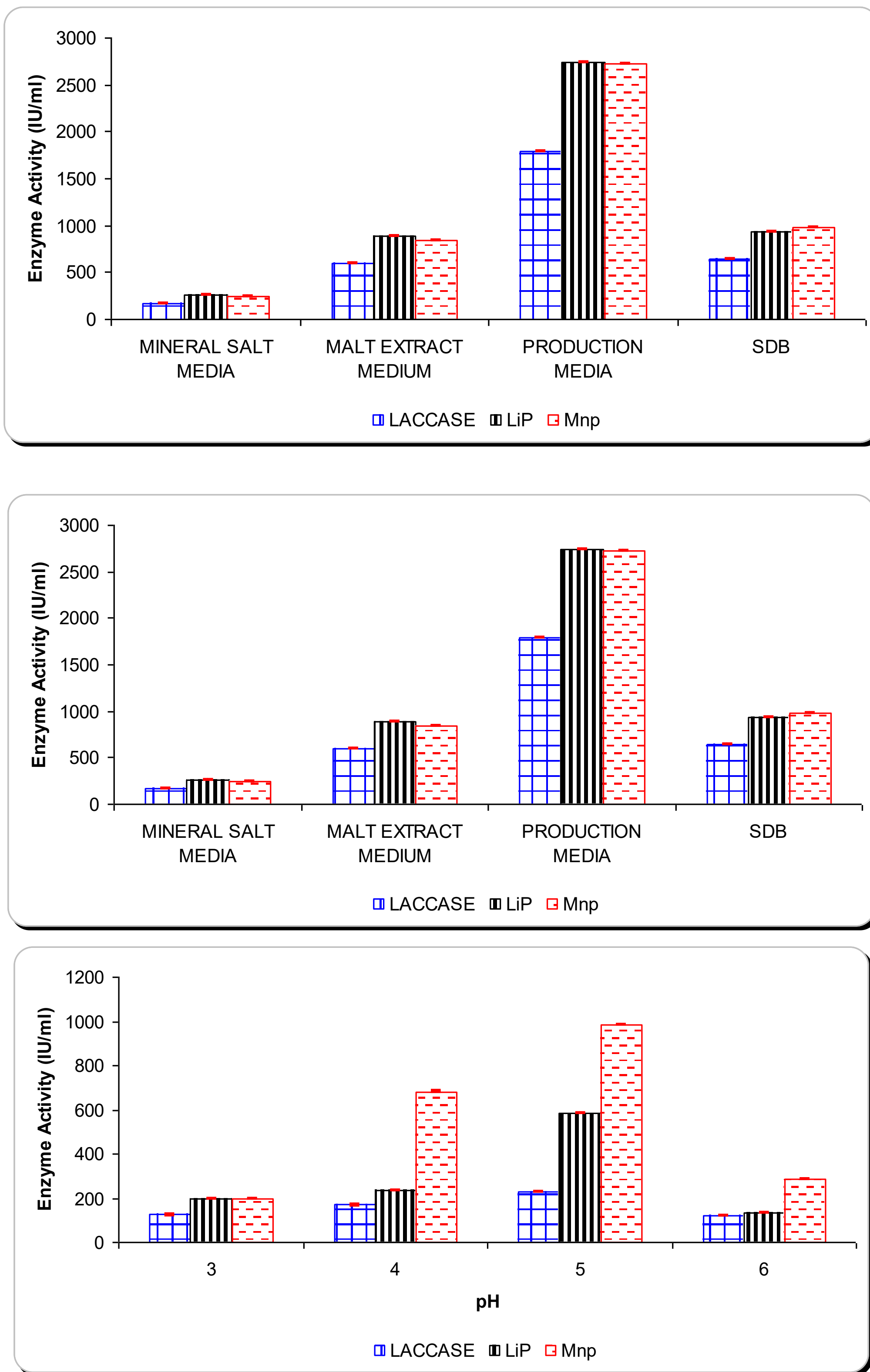

Figure 1 (a). Effect of different media (a), incubation time (b), pH (c) and temperature by *Aspergillus niger* SA1.

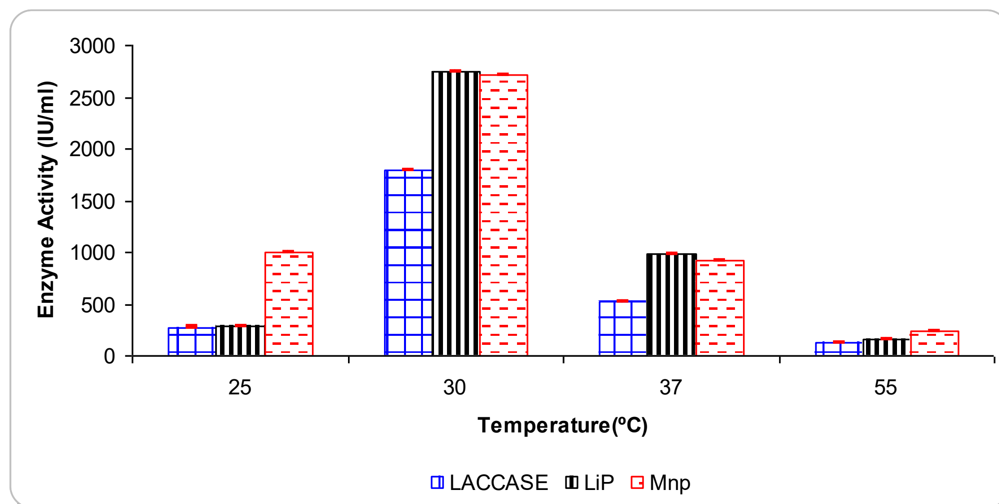

Figure 1 (b). Effect of different media on (d) the peroxidase production (laccases, lignin peroxidase and manganese peroxidase) by *Aspergillus niger* SA1.

It was observed that at pH 5, fungus give the enzyme production with the robust outcome of 228.4IU/ml (laccase), 583.60 IU/ml (LiP) and 983.59IU/ml (MnP). Within treatment there was a general trend of an increase in enzymatic activity (IU/ml) for all three enzymes by increasing the pH. On the other hand a decline in the enzymatic activity was observed after pH 5 (Figure 1c). Call and Mucke (Call and Mucke, 1997) mentioned optimum pH range of laccases 3.0 to 7.5 but 3.6-5.3 in *Trametes* laccase. Where as the initial pH of the culture medium did not significantly affect the MnP production (Ruiz *et al*., 2002). When production of enzyme was carried out at different temperature it was observed that the optimum temperature of 30 °C was found with a superb Peroxidase activity of 1786.59 IU/ml, 2743.21 IU/ml and 2719.48 IU/ml for laccases,LiP and MnP respectively (Figure 1d).

Copper detection in crude peroxidase

Laccases are multicopper oxidases mainly secreted by filamentous fungi (Christian *et al*., 2003). Total concentration of copper in the crude enzyme was 0.334 ppm (and control -0.005). This is permissible concentration of Cu (mg/l) according to WHO guidelines (1-2), PCRWR (1.5), PSI (1), IBWA (1), FA (1), EPA (1).

Toxin detection in crude peroxidase

Aflatoxin detection in crude Peroxidase by ELISA showed that the concentration of aflatoxin produced by *Aspergillus niger* SA1 was found 1.00 ppb, to be under permissible limits (<20 ppb). Similar evidence provided that *Aspergillus flavus* and *A. parasiticus* strains population invading wheat grains were all non aflatoxigenic ones except one *A.parasiicus* AP4 strain which was toxinogenic out of 157 *A. flavus* and 36 *A. flavus* isolates (Perez and Jeffries, 1992).

Characterization of peroxidases

Peroxidase was tested for the effect of different conditions on enzyme activity.

Effect of substrate concentration, pH, temperature

There was a steady rise in the activity of Peroxidase with an elevated concentration of substrate from 2.5 to 10 mM which on further increase in substrate levels up to 40mM, shows a decline (Figure 2a). However, a predominant interaction was given by 10mM concentration with *Aspergillus niger* SA1 exhibiting an activity of 89.44IU/ml respectively. It is also noticeable, that lower substrate level had a negative effect on peroxidase activity with the fungal strain (SA1). In contrast maximum decolorization efficiency was reported at 0.2 and 0.4 mmol/L hydrogen peroxide, 2.5 mmol/L veratryl alcohol (17, 35). Effect of different pH on the activity of crude enzyme, ranging from 3 to 10 was monitored. It is evident from the Fig 2b that high acidic (at pH 3 was 7.43 IU/ml) and basic pH (25.21 IU/ml) has no predominant effect on the enzyme activity. However, highest enzyme activity (69.66 IU/ml) was worked out at pH 7 with *Aspergillus niger* SA1 which is quite discernible than the bearing of other pH. Peroxidase enzyme was shown to be active at pH 3.0 to 3.5 and at 25 °C but enzyme was stable at 30°C in the neutral pH ranging from 4.0 to 6.0 (Shin and Lee, 2000).

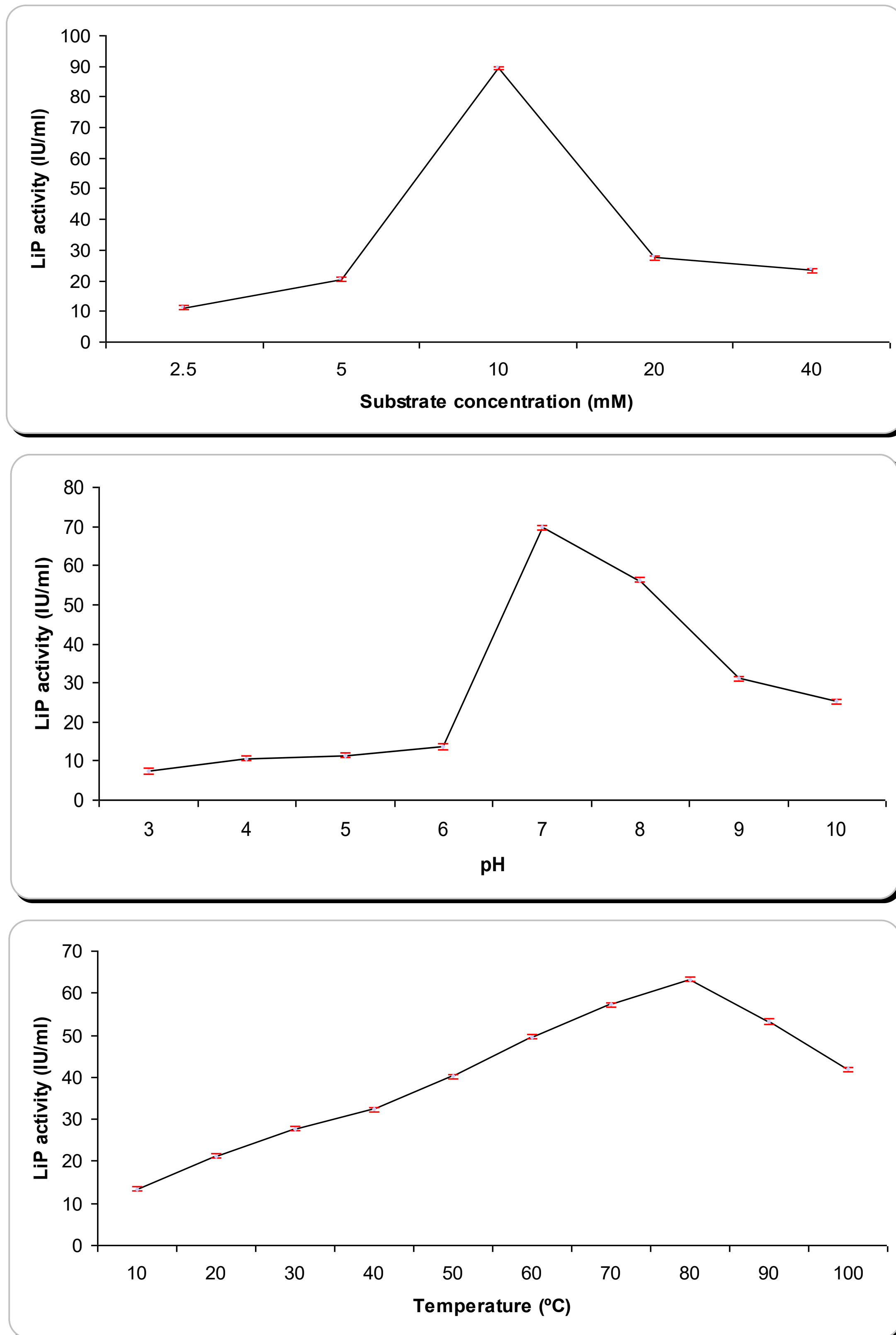

Figure 2. Effect of substrate concentration (a) pH (b) and stability of enzyme to different temperature (c) on enzyme activity of peroxidases.

Crude samples were assayed for the characterization of temperature. As far as the treatments are concerned, 80°C was dominant to other treatments (Figure 2c) and resulted in an exception peroxidase activity (63.32 IU/ml) at that temperature. In contrast at low temperature (10°C), enzyme had much impecunious outcome in terms of their activity (13.36 IU/ml). Previously it was found that decolorizing enzyme activity was not losing at high temperatures 50-60 °C. Optimum temperature range of laccases 40-80°C but 60°C in *Trametes laccase* (Soares *et al*., 2002).

Molecular weight determination by SDS-page

Molecular weight of crude Peroxidase after heat/cold treatment produced by *Aspergillus niger* SA1 was analyzed by SDS PAGE. Three bands were clearly visible in the samples of individual strains (Figure 3). The molecular weight of these bands was estimated 66.2, 43 and 38Kda by comparing the bands of Marker (#SM 0431). The molecular weights of the appeared bands were found of Laccase, Manganese peroxidase and Lignin peroxidase respectively. It was found similar for laccase, manganese peroxidase and lignin peroxidase as reported in literature (Soares *et al*., 2002; Hublik and Schinner, 2000; Rodriguez *et al*., 2006).

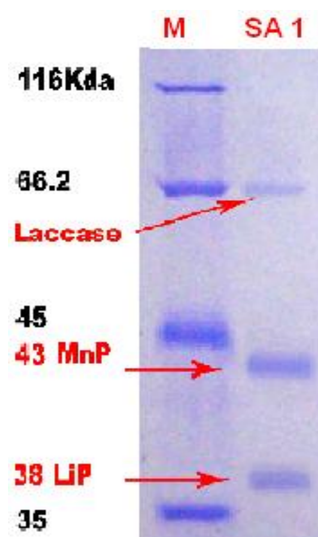

Figure 3. SDS PAGE of crude Peroxidase after heat/cold shock treatment on 12.25 % separating gel and 4.5 % stacking gel. Where in figure, lane 1 was unstained marker (code#SM0431) and lane 2 was the enzyme from *Aspergillus niger* SA1.

Purification of crude peroxidase

Initially, partial purification of crude enzyme was carried out with Ammonium sulphate. It is clear from the Figure 4 that the maximum percentage of protein can be precipitated by the addition of 40-50% $(NH_4)_2SO_4$ to the culture filtrate. Although highest enzyme activity was observed at 40% Ammonium Sulphate concentration. Moreover, gel filtration effectively removed the considerable amounts of impurities present in crude extract obtained from *Aspergillus niger* SA1 as the enzyme activity was raised and purification fold was 304.27 fold purification with 293.08 % recovery respectively (Table 3). When fractions subjected to DEAE –Cellulose anion exchange chromatography that eluted again active major peaks (Figure 5) which confirms by 494.68 fold purification with 463.82 % recovery by the selected fungal strain (*Aspergillus niger* SA1) (Table 3). The fractions obtained by gel filtration with Peroxidase activity were pooled and later on lyophilized.

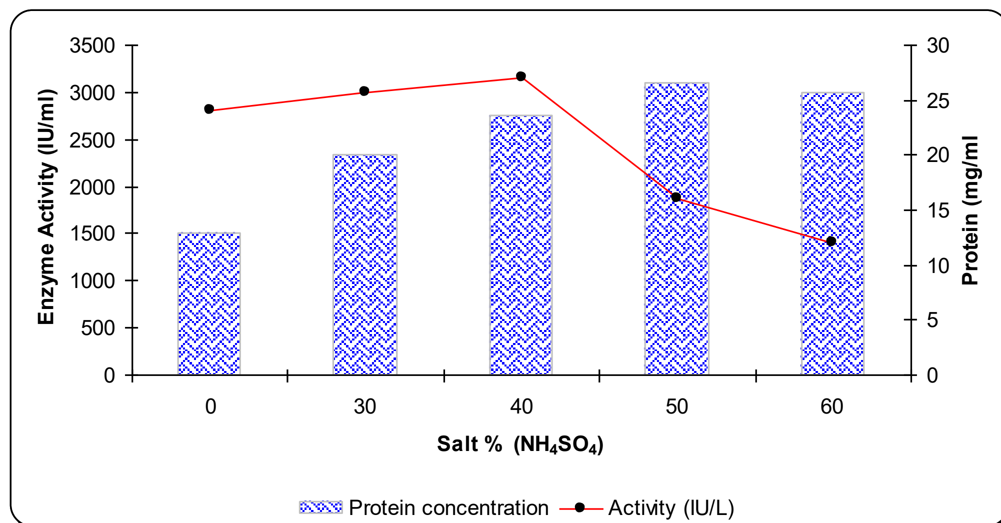

Figure 4. Purification of Peroxidase by Ammonium Sulphate Precipitation Vs activity.

The partially purified peroxidases from *Aspergillus niger* SA1 was further purified by coulmn chromatography (Sphadex G-75 and DEAE cellulose column) during the present study. Extracellular laccases have been purified from numerous fungal sources such as *Pleurotus eryngii* (Martı´nez *et al*., 1996), *Coriolus hirsutus* (Shin and Lee. 2000) and *Pycnoporus cinnabarinus* (Eggert *et al*., 1996). Laccase was also purified by using chromatography (Ruytimann-Johnson *et al*., 1994). Peroxidase was also purified from the culture filtrate of *Pleurotus ostreatus* by an ammonium sulphate fractionation with the outcome of 6.00 U/mg specific activity and was 54.5 fold, with a yield of 9.9% (Shin and Lee, 2000). Our results showed that partial purification by ammonium sulphate and after Sephadex G-75 and DEAE cellulose chromatography respectively was superb with the tested strain.

Purified by Sphadex G-75

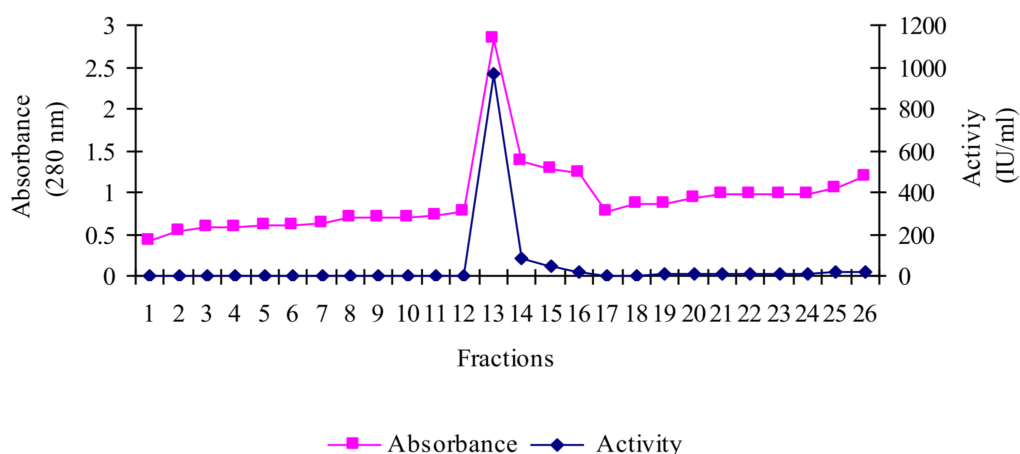

Figure 5(a). Purification of crude peroxidase from *Aspergillus niger* SA1 by column of Sephadex G-75 (a) and DEAE cellulose chromatography (b).

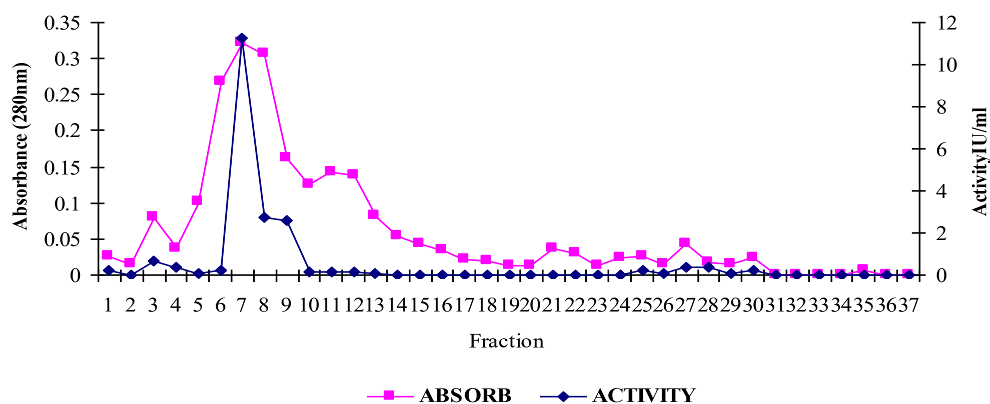

Figure 5 (b). Purification of crude peroxidase from *Aspergillus niger* SA1 by column of Sephadex G-75 (a) and DEAE cellulose chromatography (b).

Table 3. Purification of peroxidase.

Purification steps	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%) (Divide total enzyme activity by starting total activity x 100)	Fold purification (Divide total enzyme activity by total protein)
Crude Extract	5.1787	26.938	0.1922	--	--
Ammonium Sulphate (40%)	11.29	20.938	0.5392	218.00	2.80
Sephadex G-75-120	15.178	0.2598	58.422	293.08	304.27
DEAE Cellulose	24.02	0.2529	94.978	463.82	494.68
Lyophilized	83.56	0.0199	4199	1613.53	21869.79

Single step identification by decolorization zone

SDS PAGE of purified fractions was analyzed for the identification of enzyme activity with *Aspergillus* SA1 without heat/cold treatment of purified fractions of the enzyme. It was observed that a clear decolorization zone (Figure 6) was visible and it was gradually expanding with the passage of time and decolorizes the whole gel within 24hrs that unique behavior of the enzyme can be used for identification of enzyme. That observation was also consistent with the findings, in which it is reported the one step isolation and identification procedure of laccase by obtaining the colorless zone after staining with Drimarene Blue X3 LR dye (Soares *et al*., 2002).

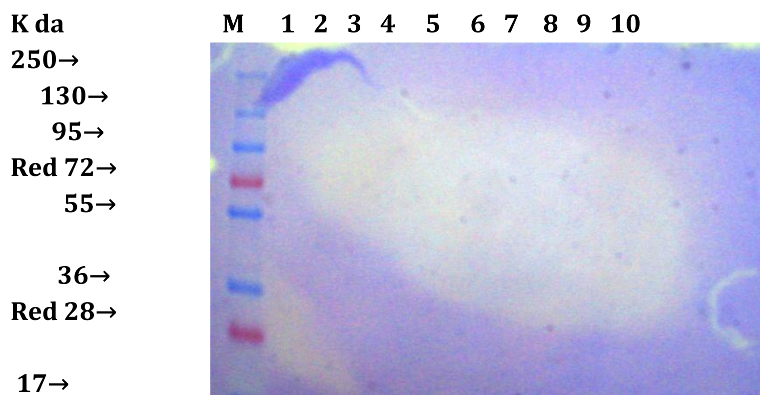

Figure 6. PAGE Analysis of the purified fractions of Peroxidases from Aspergillus niger SA1 showing clearly the decolorization zone developed without destaining the gel that is stained with CBB-R dye with prestained marker (code#SM1811).

Application of peroxidase in textile industry

Peroxidase in the textile industry can be used for bleaching and effluent treatment of the textile industry (Rashid *et al.*, 2008) as it is proved by decolorization experiments on four textile dyes during the study. Different textile dyes (AR 151, DBK_2RL, Orange II and Sulphur black) at specific wavelengths were analyzed for decolorization assay by spectrophotometric method with crude and purified (lyophilized) enzyme. Changes in absorbance of the color were recorded for two minutes of incubation of dye solution with enzyme. Results indicate the decolorization of all four textile dyes was achieved within short period of time (sec) as it evident from the Figure 7 that continuous decrease in absorbance of dye solution with the passage of time due to peroxidase activity. The minimal time of 10 sec was found to be sufficient for decreasing in absorbance of initial color for all four textile dyes (10ppm). Overall, there was a positive decolorization response was observed with crude as well as with purified enzyme (Figure 7). In spite of the fact that rate of decolorization of different textile dyes was higher by purified enzyme as it was achieved within limited time of incubation as compared with crude enzyme (Soares *et al.*, 2002). Decolorization appeared to proceed primarily by enzymatic reduction associated with a minor portion, 13-17%, of biosorption to inactivated microbial cells (Chen *et al.*, 1999). Anyhow dye decolorization efficiency of crude enzyme can be utilized for its large scale production and more economical application in textile industry.

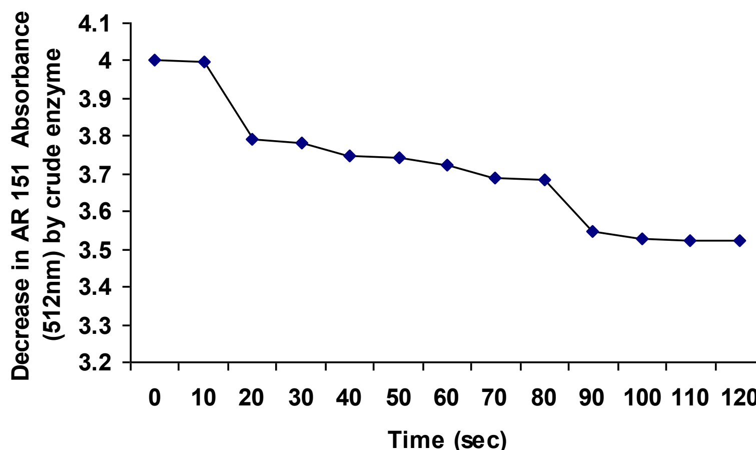

Figure 7 (a). Decolorization assay for different textile dyes by crude (A, C, E, G) and (B, D, F,H) Lyophilized peroxidase shows that perxidase can be applied in textile industry for bleaching and dye removal.

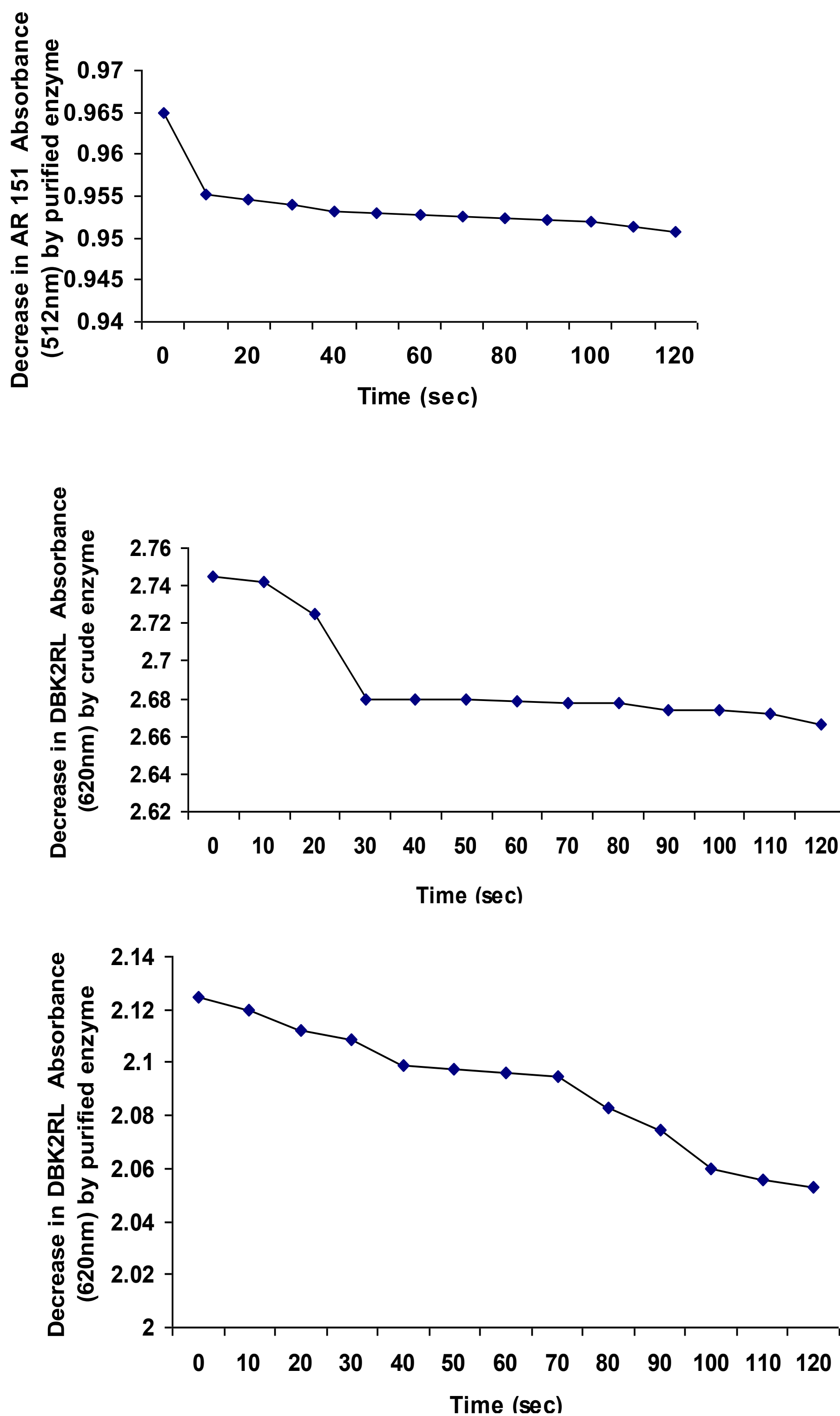

Figure 7 (b). Decolorization assay for different textile dyes by crude (A, C, E, G) and (B, D, F,H) Lyophilized peroxidase shows that perxidase can be applied in textile industry for bleaching and dye removal.

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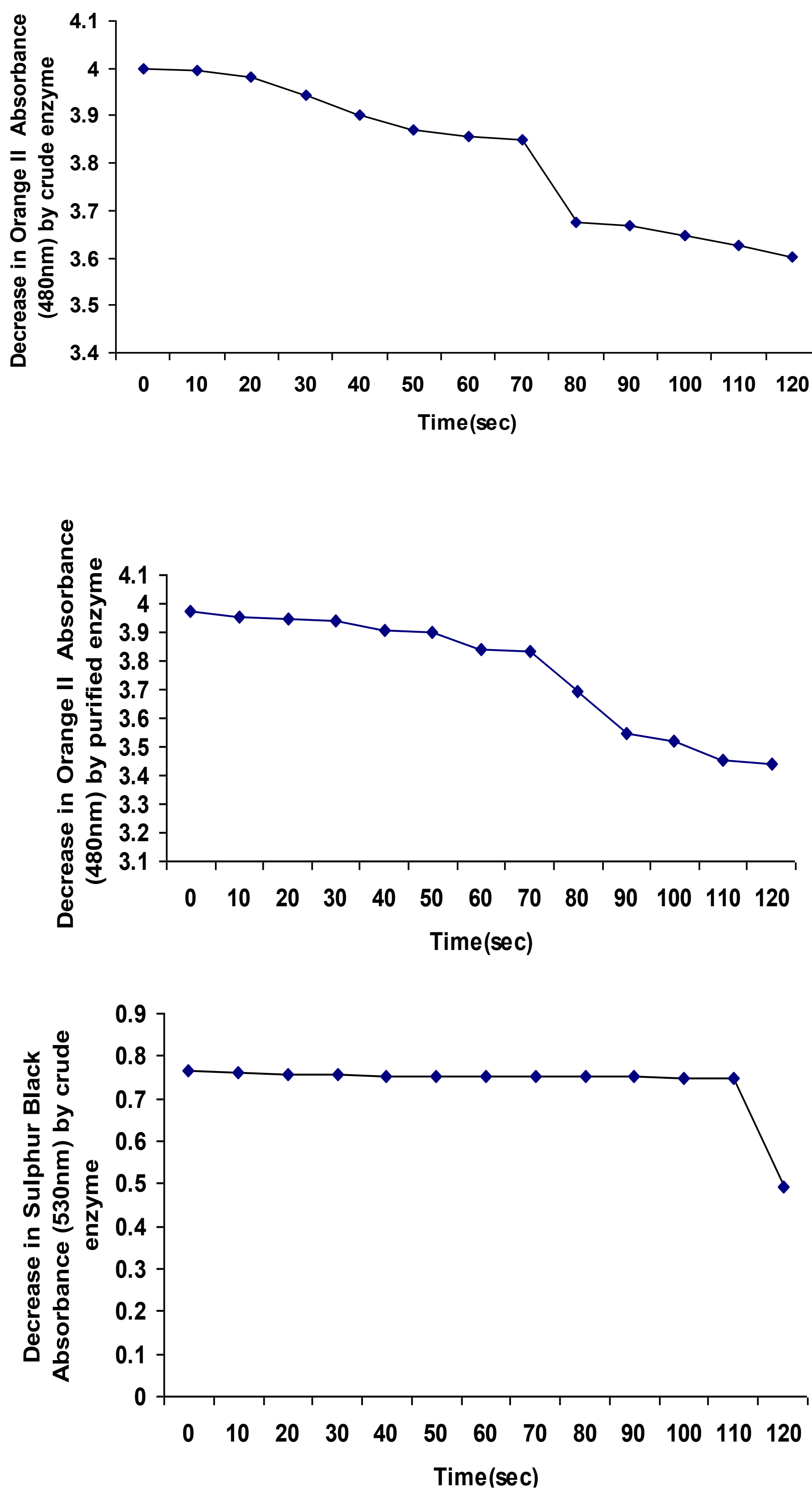

Figure 7 (c). Decolorization assay for different textile dyes by crude (A, C, E, G) and (B, D, F,H) Lyophilized peroxidase shows that perxidase can be applied in textile industry for bleaching and dye removal.

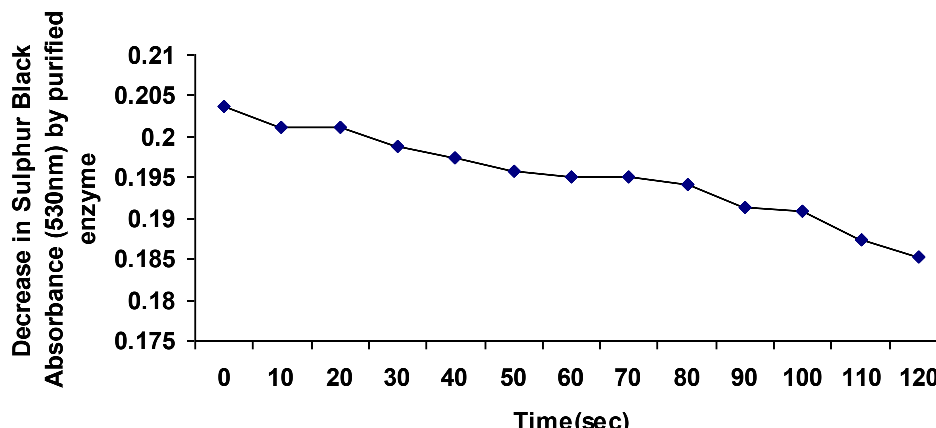

Figure 7 (d). Decolorization assay for different textile dyes by crude (A, C, E, G) and (B, D, F,H) Lyophilized peroxidase shows that perxidase can be applied in textile industry for bleaching and dye removal.

CONCLUSION

Selected fungal strains (*Aspergillus niger* SA 1) can be effectively used for dye removal as they have better adaptability towards bioremediation of dyes. Selected fungal strain was found positive for Peroxidase production in solid and broth medium. For the production of peroxidase enzyme best possible conditions were pH 5, at 30°C for 168 hrs in the productive media. Purification of Peroxidase enzyme by column chromatography offers advantage that enzyme titers produced in the systems can be increase to many folds. Enzyme in crude and purified forms can be applied in both states for the removal of different textile dyes.

CONFLICT OF INTEREST

The authors affirm that the research was conducted without any commercial or financial affiliations that could be perceived as potential conflicts of interest.

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