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EXTRACTION OF EXTRACELLULAR LACCASE FROM WILD, MUTANTS AND HYBRID STRAINS OF TWO WHITE-ROTS FUNGUS AND ITS APPLICATIONS IN DECOLOURIZATION AND LIGNINOLYSIS

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ABSTRACT

Extracellular laccases were extracted from a 5-day old submerge cultures of the wild, mutants and hybrid of Lentinus subnudus. Mutants were generated by exposure of the wild strain of L. subnudus to ultraviolet radiation (ג = 280 nm) at specific time intervals while the hybrid was produced by cross-breeding L. subnudus with L. edodes. The crude enzyme was fractionated with 80% ammonium sulphate and further purified on DEAE column. The laccase has a molecular weight of about 45KDa. Purification yield on DEAE column gave the highest purification yield of 23.25% in SWT and least in SHT (5.29%). Its potentials in decolourization of 2, 6-dichlorophenol-indophenol dye at different pH conditions were investigated. Five out of the six fungal strains tested gave significant ($P<0.05$) percentage decolourization (≥43.94%) at pH 8. The fungus was further studied for their ability in degrading wheat and paddy straws. The solid substrate fermentation was inoculated with two pieces (0.6cm diameter) mycelial agar blocks of each of the fungal strains, supplemented with 30mg/100g sucrose, 24mg/100g KNO_3 and 60mg/100g $CaCO_3$. The periodic reduction in weight of the solid substrate medium and enzymatic activity of laccase for each of the fungal strains was assessed. Therefore, the ability of the wild, mutants and hybrid of L subnudus strains to produce laccase enzyme shows their significant potential in textile industry, especially in decolourization of dye and bioconversion of lignocellulosic wastes.

Keywords: *Lentinus subnudus*, laccase, decolourization, degradation

Introduction

Laccase is one of the enzymes that have gained great importance in industries and are widely present in the nature. Laccases are the oldest and most studied enzymatic systems (Williamson, 1994). Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston, 1994; Levine, 1965). In 1896 laccase was demonstrated to be present in fungi for the first time by both Bertrand and Laborde. Since then, laccases have been found in Ascomycetes, Deuteromycetes and Basidiomycetes; being particularly abundant in many white-rot fungi that are involved in lignin metabolism

(Bourbonnais *et al*., 1995; Leontievsky *et al*., 1997). Laccases are widely distributed in higher plants, fungi and insects, but recently it was found in some bacteria such as *S.lavendulae*, *S.cyaneus*, and *Marinomonas mediterranea* (Arias *et al*., 2003; Jimenez-Juarez *et al*., 2005; Thakker *et al*., 1992). They are the enzymes which are secreted out in the medium extracellulary by several fungi (Agematu *et al*., 1993) during the secondary metabolism but not all fungal species produce laccase such as Zygomycetes and Chytridiomycetes (Morozova *et al*., 2007). They have been isolated from Ascomyceteous, Deuteromycteous and Basidiomycetous fungi to which more than 60 fungal strains belong (Gianfreda *et al*., 1999). The white-rot Basidiomycetes fungi efficiently degrade the lignin in comparison to Ascomycetes and Deuteromycetes which oxidize phenolic compounds to give phenoxy radicals and quinines (Eggert *et al*., 1996). Basidiomycetes such as *Phanerochaete chrysosporium, Theiophora terrestris,* and *Lenzites, betulina* (Viswanath *et al*., 2008), and white-rot fungi (Kiiskinen *et al*., 2004) such as *Phlebia radiate (*Niku-Paavola *et al*., 1988), *Pleurotus ostreatus* (Palmieri *et al*., 2000), and *Trametes versicolour* (Bourbonnais et al., 1995) also produce laccase. Many *Trichoderma* species such as *T. atroviride, T. harzianum* (Olker *et al*., 2002), and *T. longibrachiatum* (Vel´azquez-Cede˜no *et al*., 2004) are the sources of laccases. Laccase from the *Monocillium indicum* was the first laccase to be characterized from Ascomycetes which shows peroxidase activity (Thakker *et al*., 1992) *Pycnoporus cinnabarinus* produces laccase as ligninolytic enzyme while *Pycnoporus sanguineus* produces laccase as phenol oxidase (Eggert *et al.*, 1996; Pointing and Vrijmoed, 2000). In plants, laccase plays a role in lignifications whereas in fungi it has been implicated in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis (Thurston, 1994; Yaver *et al*., 2001). Laccases play an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors (Couto and Toca Herrera, 2006). These enzymes are used for pulp delignification, pesticide or insecticide degradation, organic synthesis (Faccelo and Cruz, 2008), waste detoxification, textile dye transformation, food technological uses, and biosensor and analytical applications. Recently laccases have been efficiently applied to nanobiotechnology due to their ability to catalyse electron transfer reactions without additional cofactor. When substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or

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non-enzymatic reactions including hydration, disproportionation, and polymerization (Faccelo and Cruz, 2008).

Submerged and solid-state modes of fermentation have been used intensely for the production of laccase (Shraddha *et al*., 2011). Wild-type filamentous fungi are used for large-scale production of laccase in different cultivation techniques. This work describes the production of laccase from the wild, mutants and hybrid strains of *Lentinus subnudus and L. edodes* and its applications in decolourization of 2,6-dichlorophenol-indophenol and ligninolysis.

MATERIALS AND METHODS

Organism and culture conditions

L. subnudus^{wt} wild type (SWT) was collected from green vegetation environment growing on a decaying mango log of wood of six months. The fruiting body of the plugged fungus was washed with 90% ethanol, and then dissected longitudinally through the gill with a sharp and sterile razor blade. The mycelium was picked and transferred aseptically onto a freshly prepared Potato dextrose agar (PDA) slants. It was maintained on slants by sub-culturing in every one month interval. The ambient temperature for culturing the fungus is 25-28^{o}C and it takes a minimum of 72 hours for optimum mycelia elongation. The fully grown fungus was maintained at 4^{0}C until when needed for use.

Lentinula edodes^{wt} wild type (EWT) was collected from Mushroom Research Centre, Himachal Pradesh Solan, India.

Production of Mutants

Lentinus subnudus^{mt} mutant types (SMT**)** were produced by exposing an actively growing culture (5days old) of the fungus on Potato Dextrose Agar (PDA) plate to an ultraviolet-radiation (ג = 280nm) at different time durations of 60 mins, 120mins and 135 mins. Mycelia plugs obtained from the cultures were transferred onto the centre of a fresh PDA plates, incubated at 25^{o}C and the fungal strains were labelled as SMT-060, SMT-120 and SMT-135 respectively.

Production of Hybrid by crossbreeding

L. subnudus^{ht} hybrid type (SHT) was produced by cross-breeding *L. subnudus*^{wt} (SWT) and *L. edodes*^{wt} (EWT) as follows: EWT was cultured on PDA plate and incubated at 25^{o}C. On the fourth day after inoculation, SWT was inoculated onto the same plate at a distance of 8mm apart with the culture conditions remaining the same. The junction where the two fungal strains intersect was picked with a sterilized cutter and then inoculated onto a freshly prepared PDA media. The new fungal strain obtained was used as the hybrid type (SHT).

Culture of fungal strains by submerge fermentation

According to Majolagbe, *et al* (2012), all the six (6) fungal strains were cultured in the optimized culture conditions as follows Glucose (10g/l), Yeast Extract (10g/l), K_2HPO_4 (0.5 g/l), $CaCO_3$ (1.0 g/l), $MgSO_4.7H_2O$ (0.2g/l) and NaCl (0.1g/l). The initial pH of the medium was adjusted to 5.5 using 2N NaOH and 2N HCl. Equal size of each fungal agar plugs kept on PDA slants was inoculated into 100 ml substrate volume, cultured for 5 days at 25^{o}C.

Extraction and quantification of total protein from culture broth

Cultures were harvested on the fifth day of inoculation and extracellular protein extracted. Total protein was estimated by Folin-Ciocalteau's method as modified by Lowry *et al.,* (1951) using bovine serum albumin as standard. The blue colour developed was measured at 660 nm against the blank.

Enzyme purification and characterization

The crude enzyme-protein solution was saturated by 80% ammonium sulphate fractionation. The protein was recovered by centrifugation at 10,000rpm for 15min and the pellet dissolved in 10 mM Phosphate buffer (pH 6.5). The sample was dialyzed against a large volume of 10mM phosphate buffer (pH 6.5) using dialysis membrane -60 as supplied by Hi-Media Laboratories, India. Dialyzed product was kept in the refrigerator at 4^{o}C. The samples were further purified on DEAE liquid column chromatography unit at room temperature eluting with 0.1 M NaCl in 10 mM phosphate buffer (pH 6.5) at a flow rate of 0.5ml min^{-1}. The purity of the enzyme protein was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which shows the molecular weight to be about 45kDa.

Enzyme Activity assay

Determination of Laccase Activity assay:

Laccase activity assay was determined according to the modified method of Shin and Lee, 2000; Saranyu and Rakrudee, 2007 as follows:

The routine assay for laccase was based on the oxidation of [(2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) diammonium salt] (ABTS) (Sigma) ($\varepsilon 420 = 3.6 \times 10^4$ $mM^{-1}cm^{-1}$). 50µl of the enzyme was incubated in 940µl of 0.1 M Sodium acetate buffer (pH 4.5) containing 10µl of 10mM ABTS incubated at 30^{o}C in Water-bath B-480 for 10mins. The reaction mixture was stopped by adding 50µl of 50% (w/v) Trichloroacetic acid (TCA). Oxidation of ABTS was monitored by spectrophotometer at 420nm. The inhibitory effects of Ethylene DiamineTetraacetic Acid (EDTA) on laccase from submerge culture of each of the fungal strains was investigated at 30^{o}C using standard phosphate buffer (pH 6.5) for 10 mins. 2-10 µg/ml inhibitor concentrations were used while assay without inhibitor was taken as the control.

Biodegradation of lignocellulosic materials

Wheat and paddy straws were purchased at Boruah Cherali Market, J.B. Road, Jorhat, Assam, and India and per-boiled in water for 30 mins. The substrates were supplemented with 30mg/100g sucrose, 24mg/100g KNO_3 and 60mg/100g $CaCO_3$, packed in double-jacketed heat resistant polypropylene bag and then sterilized. The substrates were inoculated aseptically with mycelial agar blocks of each of the wild, mutants and hybrid strains of *L. subnudus.* Weight of the substrate before and after inoculation with the fungus was taken and recorded at 7 days intervals. Crude enzymes from solid cultures were extracted by adding 20 ml of sterile distilled water, shaked for 30 min and then filtered with 0.45µm Whatman Filter paper. The samples were centrifuged at 12,000 rpm at 4^{o}C for 20 min. The supernatant were assayed for laccase activity using ABTS as substrate as aforementioned.

Decolourization of 2, 6-Dichlorophenol indophenol, sodium salt dihydrate.

Dye decolourization potential of the laccase from each of the wild, mutant and hybrid strains were monitored with 66.67 μgml^{-1} dye concentration at different pH values at 30^{o}C. The modified method according to Saranyu and Rakrudee, 2007 was used. The reaction mixtures were monitored by following the decrease in the absorbance of the dye (λmax = 592 nm) using

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UV-VIS Spectrophotometer (Manufactured by Analytikjena) and fading away of the dye colour. Experiments were monitored after the addition of the enzyme. Control set-up was without any enzyme solution. The percentage dye decolourization was calculated as follows:

$$\% \text{ Decolourization} = \frac{Ao - At}{}$$

There are many reports dealing with extracellular laccases produced by white-rot basidiomycetes (Leonowicz *et al.*, 2001; Viswanath *et al*., 2008) and few studies reported for intracellular laccases by these fungi (Burke and Cairney, 2002; Schlosser et al., 1997; Roy-Arcand and Archibald, 1991). Both submerge and solid state fermentations of wild, mutants and hybrids strains of *L. subnudus* contains ligninolytic enzymes, laccase which has vital applications in biotechnology. In this study, we first develop a new hybrid of *L. subnudus* (SHT) by cross-breeding wild strain of *L. subnudus* (from Nigeria) with *L. edodes* (from India) as shown in plate 1. Three mutants obtained by exposure of the wild to ultraviolet radiation (ג = 280nm) were also generated and extracellular laccase enzyme-protein was extracted, purified and activity compared among the six fungal strains investigated. The laccase was purified to 80% $(NH_4)_2SO_4$ fractionation and DEAE-cellulose anion-exchange chromatography column. The laccase was adsorbed on DEAE cellulose column and eluted out with 0.1M NaCl in 10mM phosphate buffer (pH 6.5) at flow rate of $0.5 ml min^{-1}$. The active fractions collected for each of the fungal strains had the characteristics blue-green colour after reaction with ABTS substrate, these were pooled and concentrated (Figs. 1 and 2). The laccase has a molecular weight of about 45KDa (Plate 2); this appears to be lower than that of other reported laccases from white rot basidiomycetes which are in the range of 55-65KDa (Eggert *et al*., 1996; Munoz *et al.*, 1997; Cambria *et al.*, 2000; Shin and Lee, 2000; Garzillo *et al.*, 2001), although higher than that reported for laccase of *L. Polychrous* which was about 32KDa (Saranyu and Rakrudeen, 2007). Purification yield on

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DEAE column gave the highest purification yield of 23.25% in SWT and least in SHT (5.29%) as shown in table 1. The relative inhibitory effects of Ethylenediamine tetra-acetic acid (EDTA) on laccase activity obtained from the submerge cultures of all the fungal strains were determined using 2-10 μg EDTA concentrations. Fig. 3a shows that maximum relative inhibition of 70.48%, 56.08% and 64.72% were recorded in SWT, EWT and SHT respectively. In the same pattern, maximum inhibitory concentrations of 66.54%, 67.49% and 73.36% were observed in SMT-060, SMT-120 and SMT-135 respectively as shown in Fig. 3b. Our results show that there was gradual degradation of ligninolytic wastes as observed in complete ramification of the husk and periodic reduction in weight of the straws inoculated with the fungus. Figures 4 (a-d) shows the linear relationship between the substrate degradation over time. This shows the potential of fungus in reducing environmental pollution being caused by ligninolytic agricultural wastes and also the bioconversion of such wastes into the mushroom fruiting body. This result is similar to the report of Pukahuta *et al* (2005) who reported the degradation of rice husk and rice straw by *Lentinus polychrous.* The presence of the laccases enzyme could have enhanced the degradation abilities of the fungal strains. Tables 2 and 3 show the percentage decolourization of 2,6-dichlorophenol indophenol dye by the partially purified laccase of the wilds, mutants and hybrid strains of the fungus. The experiment was done at different pH at 30^{o}C. Among the five pH tested, pH 8 gave >40% decolourization in all the fungal strains with the highest in SHT (56.84%), although SMT-120 gave optimum decolourization of 45.29% at pH 2 as shown in table 3. Plates 2 and 3 shows the resultant fading of the dye colour at 2 hours and 72 hours of incubation respectively. Saranyu (2007) had earlier reported the decolourization of Remazol Brilliant Blue R (RBBR) with partially purified laccase of *Lentinus polychrous* where 65.8% decolourization was achieved at pH 4 within 3.5 hours. Most currently existing processes to treat dye wastewater are ineffective and not economical (Cooper, 1995; Stephen, 1995). Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Abadulla *et al*., 2000; Blánquez *et al*., 2004; Hou *et al*., 2004), including synthetic dyes currently employed in the industry (Rodríguez Couto *et al*., 2004a, 2005). The ability of the wild, mutants and hybrid of *L subnudus* to produce laccase enzyme shows their significant potential in textile industry, especially in decolourization of dye.

$$x \quad 100$$
$$Z_o$$

Where, A_o is the absorbance at 592nm without the enzyme,
A_t is the absorbance at 592nm with the enzyme after each time intervals.

RESULTS AND DISCUSSION

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FIGURES AND TABLES

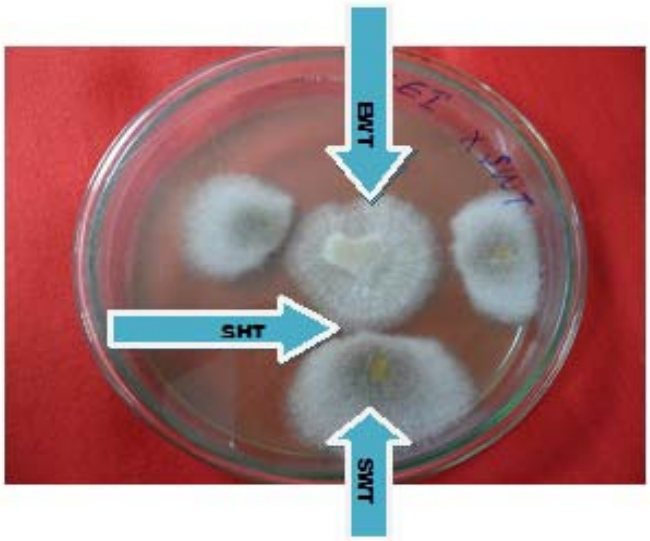

Plate 1 Production of *L. subnudus* hybrid type (SHT) by crossbreeding *L. subnudus*^{wt} (SWT) with *L. edodes*^{wt} (EWT)

Table 1 Summary table for the purification of laccase enzyme from all the fungal strains.

Fungal Strain	Total Vol. (ml)	Total Act. (U)	Total Prot. (mg)	Specific Act. (Umg^{-1})	Purification fold	Yields (%)

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SWT	1.00	21.94	1.06	20.54	22.35	23.25
EWT	1.00	23.03	1.55	14.85	6.52	9.19
SHT	1.00	10.83	0.34	31.85	14.88	5.29
SMT-060	1.00	15.80	0.38	41.58	39.35	13.92
SMT-120	1.00	9.76	0.32	30.50	15.12	6.86
SMT-135	1.00	15.87	0.46	34.50	28.63	11.53

**1ml ammonium sulphate fractionation was used on DEAE column for each of the fungal strain in the table*

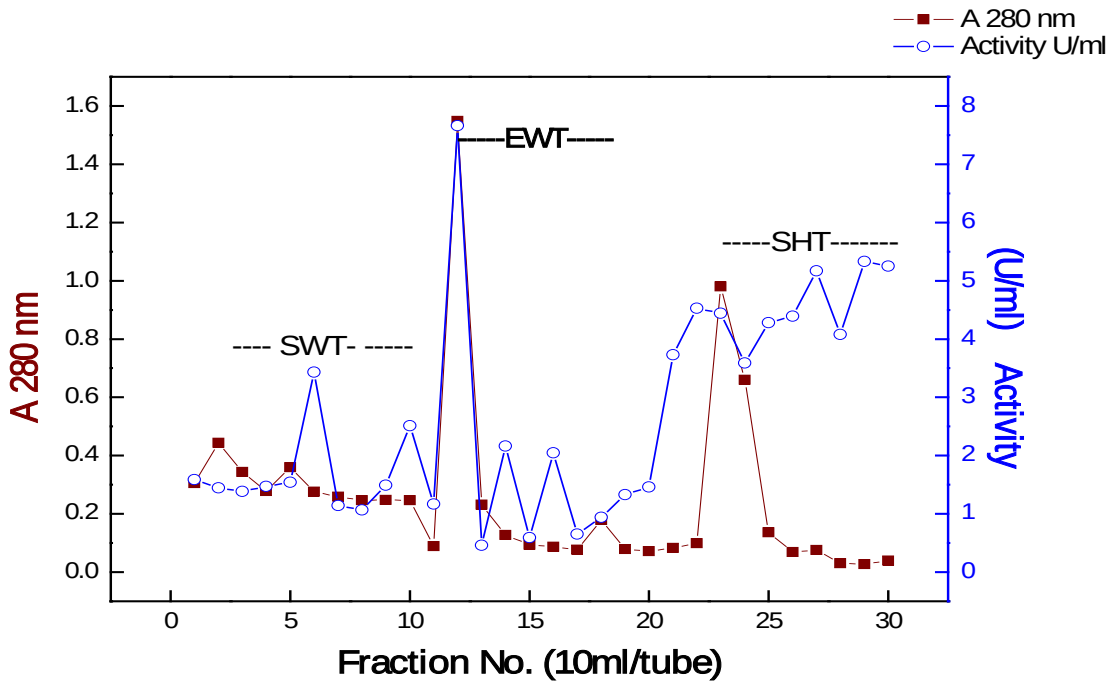

Fig. 1 Anion exchange column chromatography of 80% ammonium sulphate Laccase precipitate of SWT, EWT and SHT on DEAE- cellulose eluting with 0.1 M NaCl in 10 mM phosphate buffer (pH 6.5) at flow rate of 0.5ml min^{-1}.

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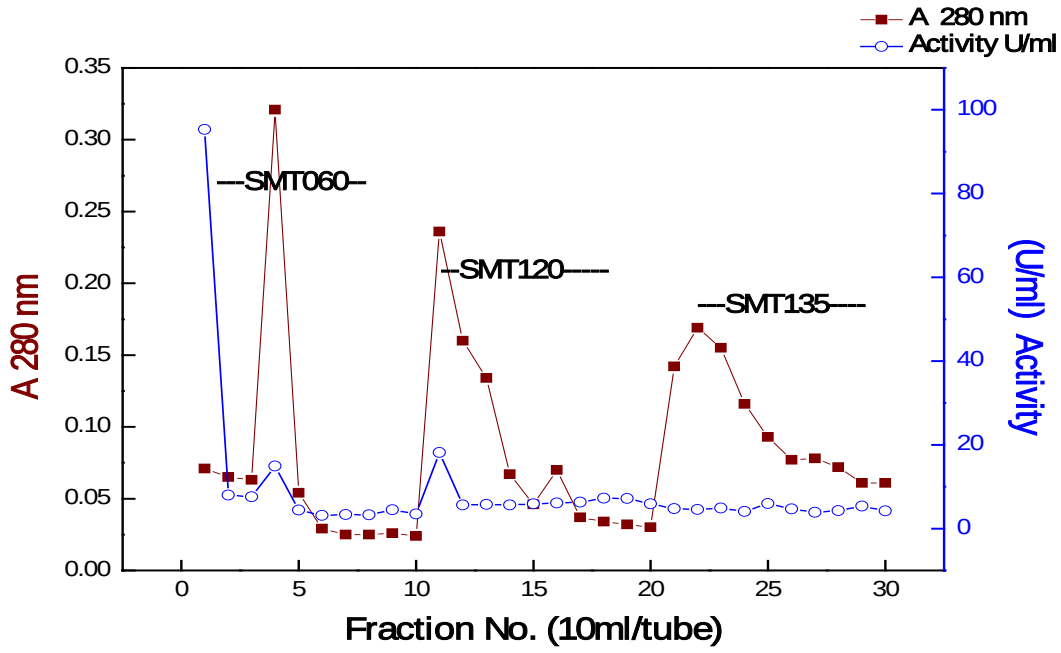

Fig. 2 Anion exchange column chromatography of 80% ammonium sulphate Laccase precipitate of SMT-060, SMT-120 and SMT-135 on DEAE- cellulose eluting with 0.1 M NaCl in 10 mM phosphate buffer (pH 6.5) at flow rate of 0.5ml min^{-1}.

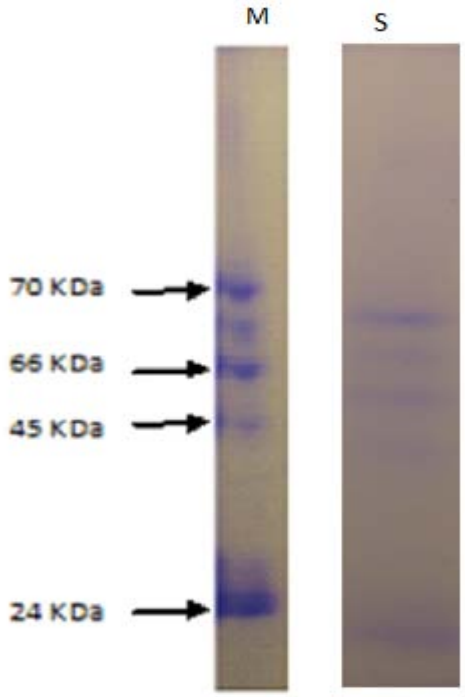

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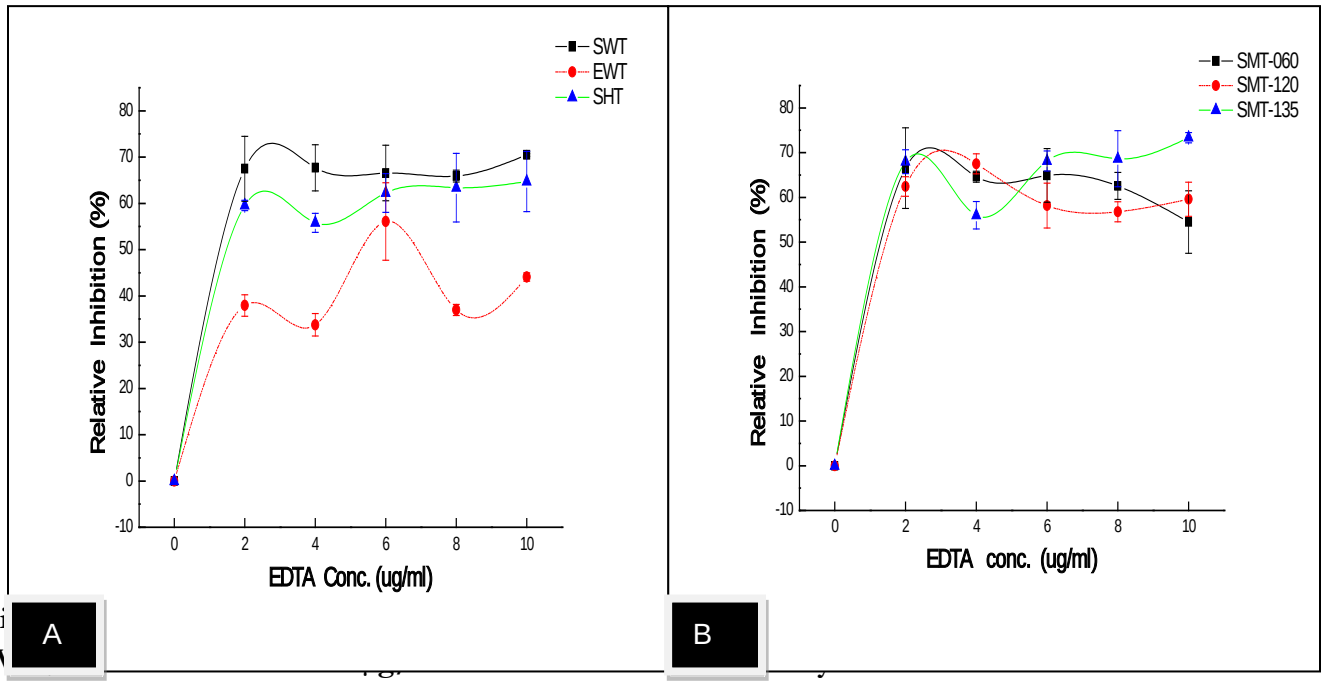
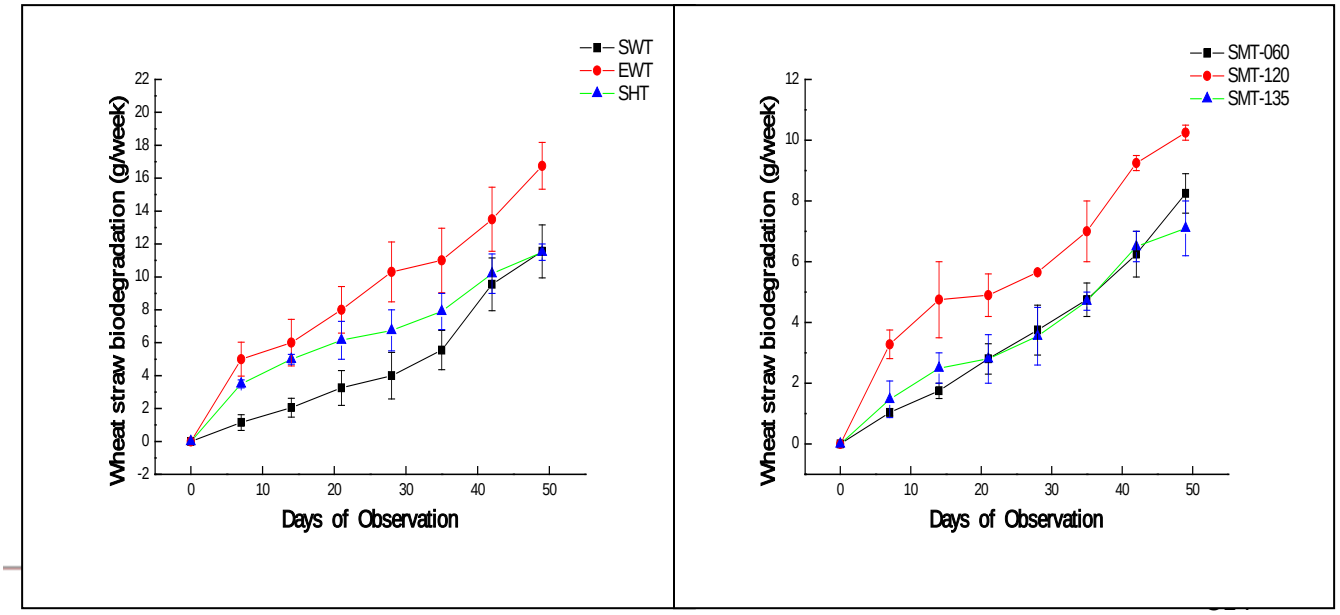
Plate 2 *Polyacrylamide Gel Electrophoresis (SDS-PAGE) of DEAE purified protein samples using 10% SDS. Lane M is the protein standard marker while Lane 'S' shows bands of laccase enzyme protein of SWT*



Fi
SV
control; Error bar = standard error of observed values.

Fig. 3(b) Relative inhibitory effects of EDTA on Laccase activity of SMT-060, SMT-120 and SMT-135. 10 µg/ml EDTA was used and assay without inhibitor was set as control; Error bar = standard error of observed values.



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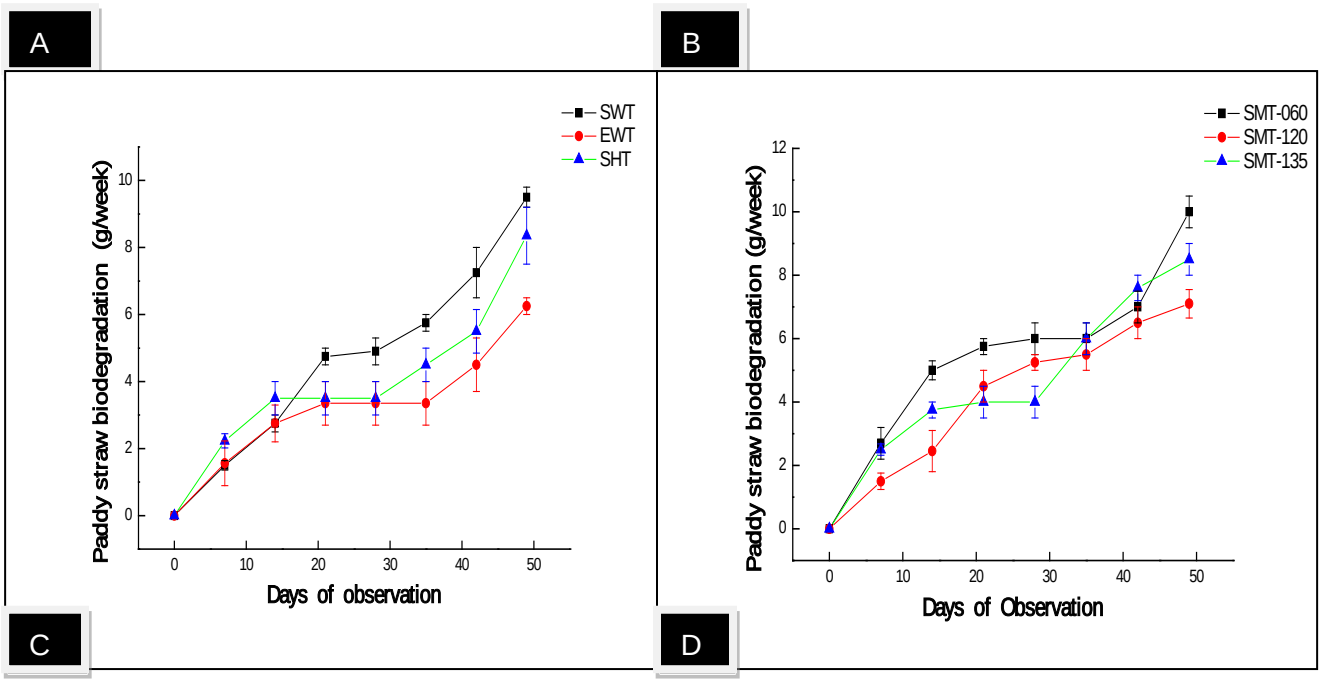
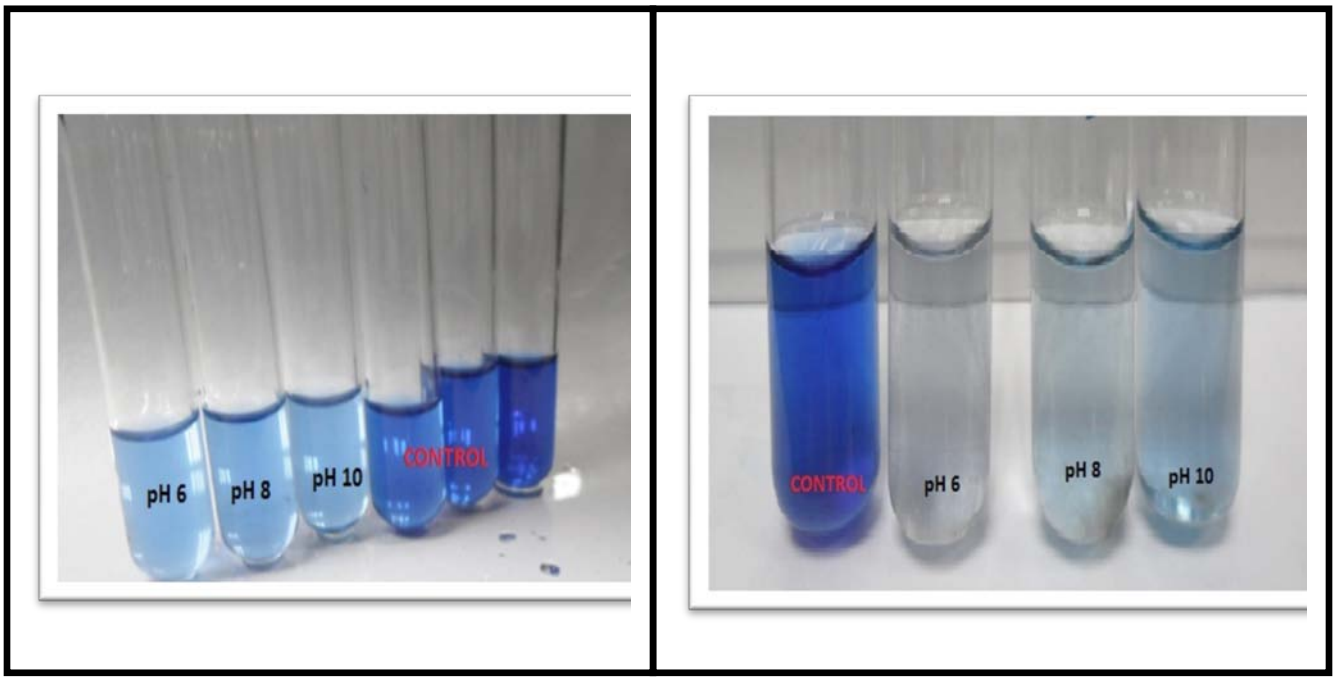


Fig. 4 (a-b) Biodegradation of Wheat straw by wild, hybrid and mutants of *L. subnudus*
Error bar = standard error of observed values

Fig. 4 (c-d) Biodegradation of paddy straw by wild, hybrid and mutants of *L. subnudus*
Error bar = standard error of observed values



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A B

Plate 2a. Decolourization by Laccase enzyme after 2 hours of incubation at 30°C

Plate 2b. Decolourization by Laccase enzyme after 72 hours of incubation at 30°C

Table 2 Percentage Decolourization of 2, 6-dichlorophenol indophenol dye after treatment with the partially purified laccase from wild and hybrid of *L. subnudus* and *L. edodes* at 30°C at different pH

	Dye Decolourization (%)		
pH value	SWT	EWT	SHT
2	6.33±0.45[a]	10.01±1.96[a]	16.78±1.83[a]
4	27.71±2.78[a]	23.04±3.44[a]	7.93±1.86[a]
6	24.72±1.51[a]	28.06±2.75[a]	5.84±0.68[a]
8	43.94±3.89[b]	53.96±5.42[b]	56.84±6.72[b]
10	39.72±2.10[b]	42.27±3.58[b]	41.17±3.96[b]

Data are means of three replicates; ± = standard deviation of three replicate values. Means with different letters in the same column are significantly different (P<0.05) from each other according to Turkey's Multiple Comparison Test.

Table 3 Percentage Decolourization of 2, 6-dichlorophenol indophenol dye after treatment with the partially purified laccase from mutants of *L. subnudus* at 30°C at different pH.

	Dye Decolourization (%)		
pH value	SMT-060	SMT-120	SMT-135
2	37.86±2.64[ac]	45.29±2.49[a]	36.63±3.91[ac]
4	10.81±2.72[bc]	18.35±2.69[b]	8.96±1.59[bc]
6	42.73±1.92[a]	21.09±3.68[b]	51.26±3.67[a]
8	51.51±5.38[a]	23.15±4.82[b]	55.26±3.78[a]

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10	45.75±2.53[a]	21.85±2.59[b]	49.05±4.05[a]

Data are means of three replicates; ± = standard deviation of three replicate values. Means with different letters in the same column are significantly different (P<0.05) from each other according to Turkey's Multiple Comparison Test.