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Identification of efficient dye decolorizing laccase producing fungi from Kolli Hills

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Laccase is one of the most promising ligninolytic enzymes for the industrial application and ecofriendly bioremediation process. Twenty-five carpophores were collected from different places of Kolli Hills (Namakkal district Tamilnadu, India) and screened on the solid media containing guaiacol, which enabled the detection of laccase secretion. Three positive strains were isolated and the quantitative production of laccase was determined in submerged culture to select hypersecretory strain for further study. Among the three strains, "ST02" the best producer of laccase was selected and was analyzed for the dye decolorization potential using dyes like Poly R-478 and Remazol Brilliant Blue R (RBBR). Identification of the isolated organism was carried out by classical and molecular methods. Approximately 625 bp of the ST02 5.8S rDNA was amplified by polymerase chain reaction (PCR). The phylogenetic relationship of the isolated strain was studied by comparing the internal transcribed spacer (ITS) sequences of ST02 with similar related sequences deposited in the GenBank database. The present study showed that relatively simple plate test screening method and ITS analysis can be used for identification of laccase producing new strain. The isolated organism was designated as *Pleurotus ostreatus* IMI 395545.

Keywords: Laccase, *Pleurotus ostreatus* IMI 395545, internal transcribed spacer, guaiacol, phylogenetic analysis

Introduction

Laccases are interesting enzymes for industrial applications because extensive studies have shown the potential of fungal phenol oxidases as a biological alternative for chemical oxidative processes e.g. pulp delignification in textile industry, food industry, bioremediation, organic synthesis, pharmaceutical sector and nanobiotechnology[1]. Recently, most of the laccase studied are of fungal origin, especially from white rot fungi, *Anthracophyllum discolor*[2], *Pycnoporus sanguineus*[3], *Trichoderma harzianum*[4] etc. Screening of a large number of white-rot fungi is, therefore, necessary to select strains that are able to produce high titers of laccases with novel characters. Such a screening trial should preferably rely on the use of inexpensive, rapid and sensitive testing methods and the screening strategy must be compiled in such a manner as to identify fungal strains and enzymes that will work under industrial conditions[5]. Microbes that produce laccases have been screened for either on solid media containing colored indicator compounds that enable the visual detection of laccase production[6-8] or with liquid cultivation monitored with enzyme activity measurements[9]. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. Recently, Chhaya and Gupte (2010)[10] identified a new strain *Fusarium incarnatum* for the production of laccase using *o*-dianisidine in the plate test. Inadequate experimentation may lead to the misidentification of a laccase for other phenol oxidizing enzymes such as manganese peroxidase. The use of substrates such as syringaldazine and ABTS [2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] should be used with caution since these substrates are catalyzed by both laccase and manganese peroxidase[11]. Plate test screening with laccase indicator compounds has been reported by many groups[12-15].

In fungal taxonomy, sequences from the internal transcribed spacers (ITS) region of the nuclear rDNA are commonly used for the identification of fungi[16-18]. The ITS sequence including both ITS1 and ITS2, which are separated by the conserved short 5.8S rRNA, has been commonly used to infer phylogenetic relationships of closely related species as well as to assess the variability of a population, e.g. of geographically distant isolates (ecotypes). Since the

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ITS region is highly conserved intra-species level but variable between different species it is often used in taxonomy[19]. The evolutionary distance is generally displayed in the form of trees and a wide diversity of algorithms was available to construct them. Innovation of novel laccases with different substrate specificities and improved physical properties such as thermostable or acid tolerant is imperative qualities for industrial applications.

In this study, laccase-producing fungi were isolated from various samples around the Eastern Ghats of Kolli hills. In addition, the production of laccase by the positive strains was confirmed in liquid culture. The isolated hypersecretory strain was identified by phylogenetic analysis.

Materials and Methods

Chemicals

Catalase, lactophenol, cotton blue and potato dextrose agar (PDA) were purchased from HiMedia, (Mumbai, India). Chloramphenicol, Poly R-478 and Remazol Brilliant Blue R (RBBR) were purchased from s d fine-chem Limited, India. Benomyl (Benofit) was purchased from Coromondel Fertilizers Limited (Tamil Nadu, India). SoluteReady® genomic DNA purification kit, PCR reagents, agarose gel electrophoresis consumables and primers were purchased from Helini Biomolecules (Chennai, India).

Isolation of Microorganism

Twenty-five basidiomycetous carpophores (stalk of a fruiting body in fungi) were collected from the deep forest of Kolli Hills in Namakkal district, Tamil Nadu, India in early rainy season in the year 2008. Carpophores were picked and stored in thermocol boxes. The samples were then sprayed with 70% ethanol and soaked in Petri dishes for 5 minutes for surface sterilization. A sample of the interior flesh of the fruiting body was excised and placed on the PDA to isolate the fungi[20]. In addition, 0.01% (w/v) chloramphenicol or chlorotetracycline was added to the media in order to inhibit the growth of bacteria and 1% (w/v) of benomyl was added in order to select for wood decay fungi[21].

Screening Test

The ability of the fungal strains to secrete extracellular laccase was visualized according to the method of Kiiskinen *et al*[22]. The assay plate contained 15 ml of 4% PDA amended with 0.01% of guaiacol. The plates were incubated at 30°C for 1–3 days. The presence of brick red color around the mycelium was considered as guaiacol oxidizing laccase secreting organism.

Laccase Production in Liquid Media

The selected three positive fungal strains detected in the plate test, were subjected to qualitative determination of laccase production in submerged culture[20]. The strains were grown in 50 ml of 4% potato dextrose broth (PDB) in a 100 ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary shaker (120 rpm).

Dye Decolorization Potential

Dye decolorization potential of the isolated hypersecretory strain was carried out according to the method of Kiiskinen *et al*[22]. In brief 0.04% (w/v) Poly R-478 or RBBR was added individually to 15 ml of 4% PDA in Petri plates. The pH was adjusted to 5.5 before autoclaving at 121°C for 15 min. The dyes were added to the media after autoclaved as sterile filtered water solutions. The positive reaction on the plate was visualized within 5-6 d of incubation at 30°C.

Enzyme Assay

Laccase activity was determined using guaiacol as the substrate according to the method of Sandhu and Arora[23]. The assay mixture contained 4.80 ml of sodium phosphate buffer at pH 6.0 (100 mM), 0.1 ml of guaiacol (10 mM) and 0.1 ml of enzyme extract. One activity unit (U) was defined as the amount of enzyme oxidizing 1 μmol guaiacol per minute. The kinetic reaction was spectrophotometrically recorded at 470 nm (ε = 21,600/M/cm) incubated at 60°C for 30 min, in an UV-Visible Spectrophotometer 118 (Systronics, India). The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place. The enzyme activity was expressed as U/l.

In order to rule out the role of peroxidases in the oxidation and prove the oxidation was only by laccase, the enzyme was pre-incubated with catalase (1000 units/ml) (EC: 1.11.1.6) for 30 min at 30°C prior to assay to remove any endogenous hydrogen peroxide[24]. Lignin peroxidase (LiP) and manganese dependent peroxidase activities (MnP) were measured as follows:

Lignin peroxidase (LiP), (EC 1.11.1.14) activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde (ε = 9,300/M/cm) at 37°C as indicated by an increase in absorbance at 310

nm[25]. The reaction mixture (2.5 ml) contained 0.5 ml enzyme extract, 0.5 ml H_2O_2 (2 mM), 0.5 ml veratryl alcohol solution (10 mM) and 1.0 ml sodium acetate buffer pH 3.0 (10 mM). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per minute. The enzyme activity was expressed as U/l.

Manganese peroxidase (MnP), (EC 1.11.1.13) activity was measured with phenol red as the substrate at 610 nm[26]. Reaction mixture contained 0.5 ml enzyme extract, 0.1 ml phenol red solution (0.1%), 0.1 ml sodium lactate pH 4.5 (250 mM), 0.2 ml bovine serum albumin solution (0.5%), 0.05 ml manganese sulphate (2 mM) and 0.05 ml H_2O_2 (2 mM) in sodium succinate buffer pH 4.5 (20 mM). Activity was expressed as increase in absorbance at 610 nm per minute per milliliter. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per minute. The enzyme activity was expressed as U/l.

Fungal Identification Method

The classical method of identifying fungi is by using light microscopy. The spore structure of the fungi was identified by lactophenol cotton blue mounting method[27].

Isolation of Genomic DNA

Twenty grams of mycelium was used for DNA isolation according to Hamelin *et al*[28], with some modifications. The fungal mycelium grown on PDB was harvested and any media attached to it was removed by washing with sterile water. The washed sample was ground to a fine powder in liquid nitrogen. 15 ml of extraction buffer (1.4 M NaCl, 0.1 M Tris-HCl (pH 8), 0.02 M EDTA, 200 µl β-2-mercaptoethanol, 2% cetyl-trimethyl-ammonium bromide) was added to 100 mg of the grounded mycelia. The mixture was incubated at 65°C for 1 h and extracted by adding 10 ml of phenol: chloroform: isoamyl alcohol (25:24:1). This was gently shaken and centrifuged at 15,000 rpm. Supernatant was transferred to a new tube and the aqueous phase was precipitated with cold isopropanol by incubating for 1 h at −20°C, and then centrifuged for 15 min at 15,000 rpm. The pellets were washed with 70% ethanol, air-dried, resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C.

PCR Targeting ITS Region and Sequencing the Amplicon

From the total genomic DNA, a DNA segment containing the 3’ end of the nuclear 18 S rDNA, ITS 1, 5.8 S rDNA, ITS 2 and the 5’ end of 28 S rDNA were amplified using primers ITS1 (5′TCCGT AGGTGAACCTGCGG3′) and ITS 4 (5′TCCT CCGCTTATTGATATGC3′)[29]. PCR amplification was performed in a total volume of 25 µl amplification reactions contained 10X *Taq* buffer, 2 mM $MgCl_2$, 0.4 mM dNTP mix, and 2 U proofreading *Taq* DNA polymerase. One micro litre of both forward and reverse primers (10 pmoles/µl) were added with 2 µl of genomic DNA. The volume of the mixture was made up to 50 µl by adding 21 µl of nuclease free water. The mixture was mixed gently and spun down briefly for about 30 min. PCR was performed in a thermocycler (Corbett Research, Austria). Initial denaturation of 95°C for 2 min was followed by 30 cycles of 94°C for 1 min, 58°C for 1 min (primer annealing), 72°C for 1 min (primer extension). A final extension of 72°C for 5 min was incorporated, followed by cooling to 4°C until recovery of the samples. Sequencing of the PCR products was carried out by Helini Biomolecules (Chennai, India) on an ABI Prism 3100-Avant Genetic Analyzer (USA) and the obtained sequence was aligned using the BioEdit sequence alignment editor version 7.0.4.1.

Analysis of PCR Product

Amplified PCR products were electrophoresed in a 2% agarose gel using 1X Tris–borate–EDTA buffer (100 mM Tris–HCl/l, pH 8.3, 83 mM boric acid/l, 1 mM EDTA/l) at 100 V and visualized by ethidium bromide staining. Sizes of the amplified products were determined using different size of standard DNA ladder. The reproducibility of DNA profiles was tested by repeating twice the PCR amplification with each of the selected primers. Only reproducible bands were considered for analyses.

Analysis of Sequence Data

A comparative study has been carried out with other rDNA sequences with the rDNA from the selected fungus (ST02) was accomplished using the BLAST[30] (http://www.ncbi.nlm.nih.gov). Thirty-four most similar meaningful sequences related to the isolated fungal sequence were downloaded and analyzed. Related fungal sequences were selected by basic local alignment search tool (BLAST) match and then by FASTA query of the National Centre for Biotechnology Information (NCBI) fungi data subset. The selected sequences were aligned by using clustalW2[31] version CLUSTAL 2.0.12. All the sequences were used to construct the phylogenetic

tree by using Geneious pro trial 4.8.5 software with the following parameters i.e. 65% similarity (5.0/-4.0) cost matrix, Gap open penalty = 12, Gap extension penalty = 3. The construction of phylogenetic tree was based on the Tamura *et al*[32] genetic distance model. The alignment was inspected closely to ensure that were no PCR chimaera events.

Results

Among the 25 samples collected, 3 strains were selected as positive organisms which formed halo of intense brown color around the fungal colonies. Based on the reddish brown zone, three strains were designated as ST01, ST02 and ST03 (Fig. 1). Among the three isolated positive organisms, hypersecretory strain was selected by quantitative production of laccase in the potato dextrose broth. Significant amount of laccase activities were measured for all the three strains. Sample was drawn from the culture for every 24 h to assay the production of laccase. Figure 2 shows the production of laccase by selected strains. Among the three strains, ST02 which showed highest production of laccase (410 ± 1.6 U/l) was selected for the further analysis. The selected hypersecretory positive strain ST02 was subjected to decolorization of indicator dye compounds. Figures 3a & b displays qualitative decolorization of Poly R-478 dye on incubation with ST02 strain. Initial pink color of the dye was decolorized to colorless on the 6 days of incubation with ST02. Figures 3c & d shows the RBBR dye was completely decolorized on 6 days of incubation. In the case of RBBR, initial blue color of the dye was decolorized to colorless. The selected strain ST02 decolorized both the dyes effectively.

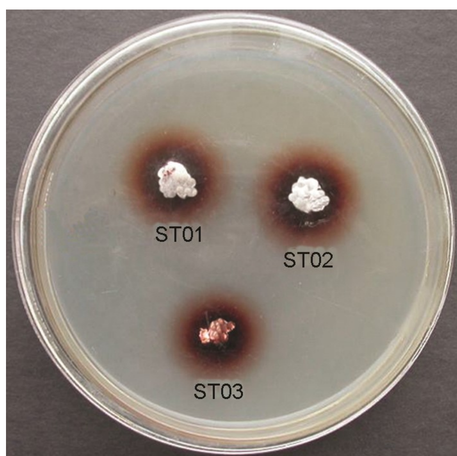

Fig. 1 — Oxidative polymerization of guaiacol to form reddish brown zones in the medium by positive strains.

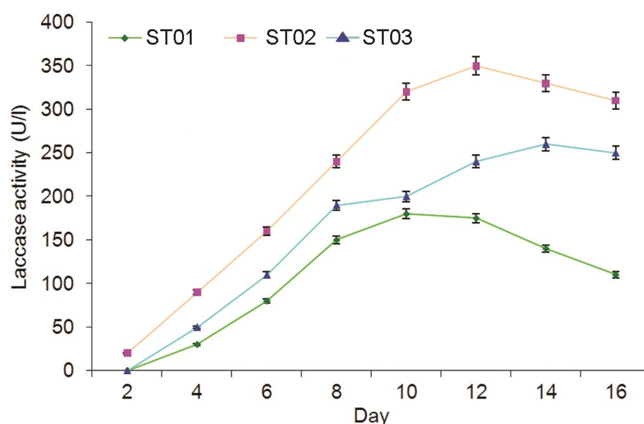

Fig. 2 — Laccase production by the selected three positive strains in potato dextrose broth.

The pure culture of ST02 on PDA was rich white aerial mycelium (Figs. 4a & b). The reverse of the mycelia was colorless, pigmentation was not produced even after two weeks of cultivation, hence the spores were used for identification. In the lactophenol cotton blue staining, spores were cylindrical to subcylindrical, thin-walled, hyaline, and not amyloid or dextrinoid, without germ pore. The hyphal system may be monomitic or dimitic, without binding hyphae. As the morphology was uninformative and ambiguous, rDNA sequence comparison was used for identification.

Genomic DNA was successfully isolated from the selected fungus ST02. The isolated DNA was confirmed by staining with ethidium bromide viewed under transilluminator (Fig. 5a). The genomic DNA was subjected to PCR-amplification of the ITS region

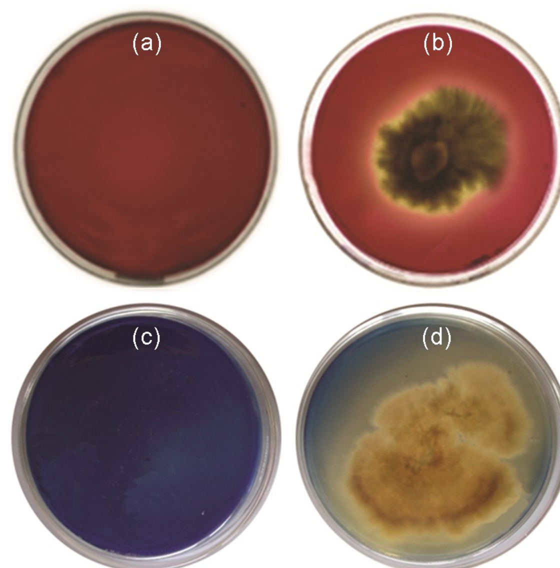

Fig. 3 — Poly-R478 oxidation by ST02 (a) Day 0, (b) Day 6; RBBR oxidation by 'ST02', (c) Day 0 d) Day 6.

of the rDNA using ITS1 and ITS4 primers. The approximate number of the base pair of the amplified PCR products was (~625 bp) determined (Fig. 5b).

To identify the strain ST02, rDNA sequence comparison was used. The rDNA sequence of ST02 was compared with the rDNA sequence of other fungi present in the database. The obtained closely related sequence in the FASTA format and their length varies from 763 bp to 605 bp. The collected sequences were aligned by clustalW2 (www.ebi.ac.uk/Tools/clustalW2/index.html.). To confirm the identification, 5.8S rDNA region from ST02 was used. The sequence for the same have been deposited in the GenBank under the following accession number GU967699.1. The phenogram reflecting the phylogenetic relationship between ST02 was constructed using data from the BLAST analysis of the rDNA region (Figs. 6 & 7). The details of the organisms, respective accession number of sequences used to construct the trees are given in the Table 1.

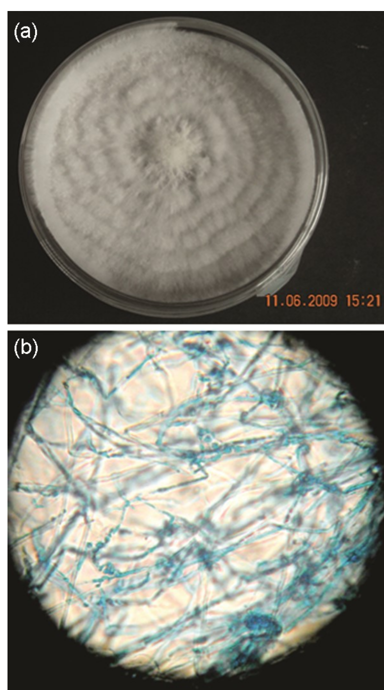

Fig. 4 — Pure culture of the isolated strain 'ST02' (a) and its mycelium structure at 40X (b).

Discussion

Among the 25 samples collected, three strains showed reddish brown halo around the growing mycelium in PDA amended with guaiacol, the substrate for laccase identification. The chromogen guaiacol is a very sensitive substrate that allows rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a color reaction[33]. The selected three strains produced the laccase, which catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Fig. 1). The obtained results have good agreement with the report of Kiiskinen *et al*[22] for the isolation of laccase producing fungus. Fungal strains that show positive reaction in the plate test for the secretion of laccase were further analyzed in liquid culture for the quantitative estimation. Among the three strains ST02 secretes laccase on second day of the culture whereas, other two strains secrete on third day. Maximum secretion of laccase was seen on 12th day of the culture (Fig. 2). Among the three strains, ST02 produced the maximum laccase activity (410 ± 1.6 U/l). The production of laccase peaks at the late stage of cultivation. The above observation

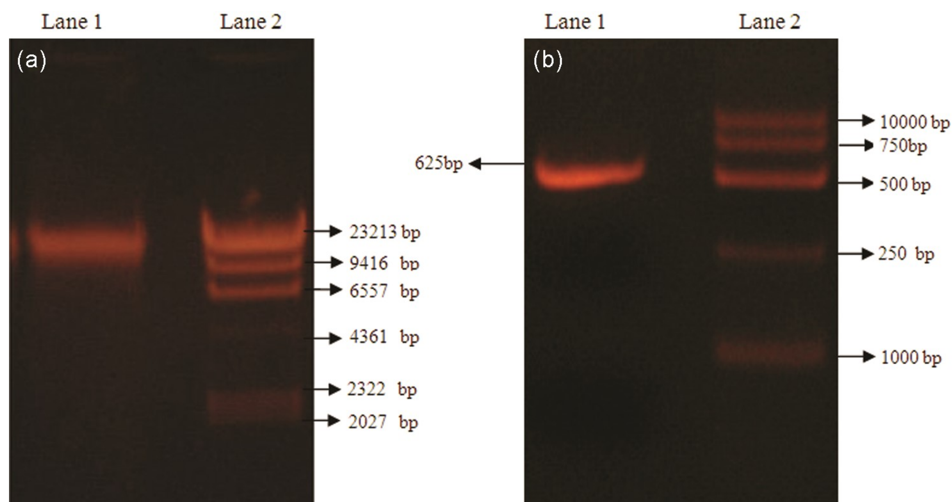

Fig. 5 —(a) Lane 1: Genomic DNA of ST02, Lane 2: DNA Ladder; (b) Lane 1: 5.8S rDNA (625 bp) Lane 2: DNA Ladder.

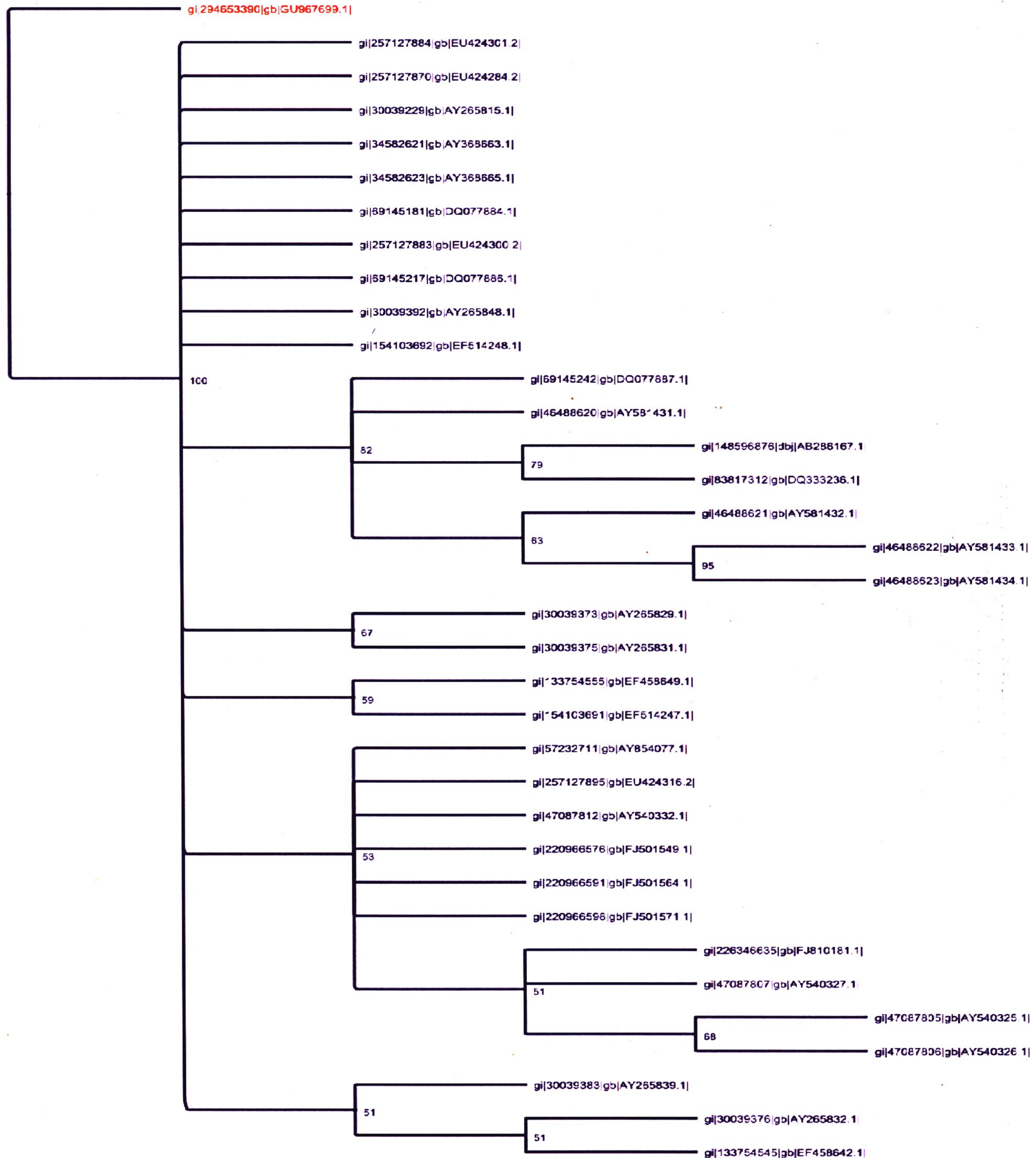

Fig. 6 — Phenogram constructed from 5.8S rDNA sequence homologous of ST02 (GU967699.1) by neighbor-joining (NJ) method.

shows that the secreted enzyme is not a primary metabolite and it was a secondary metabolite[34]. On 15th day onwards the secretion of laccase was decreased in all the three strains. Mazumder *et al*[35] observed that *Pleurotus ostreatus* have strong proteolytic activity in submerged fermentation (SmF) presumably caused subsequent laccase degradation, which lowered the ultimate laccase production in SmF compared to SSF. The above statement correlated with the report of Pant and Adholeya[36].

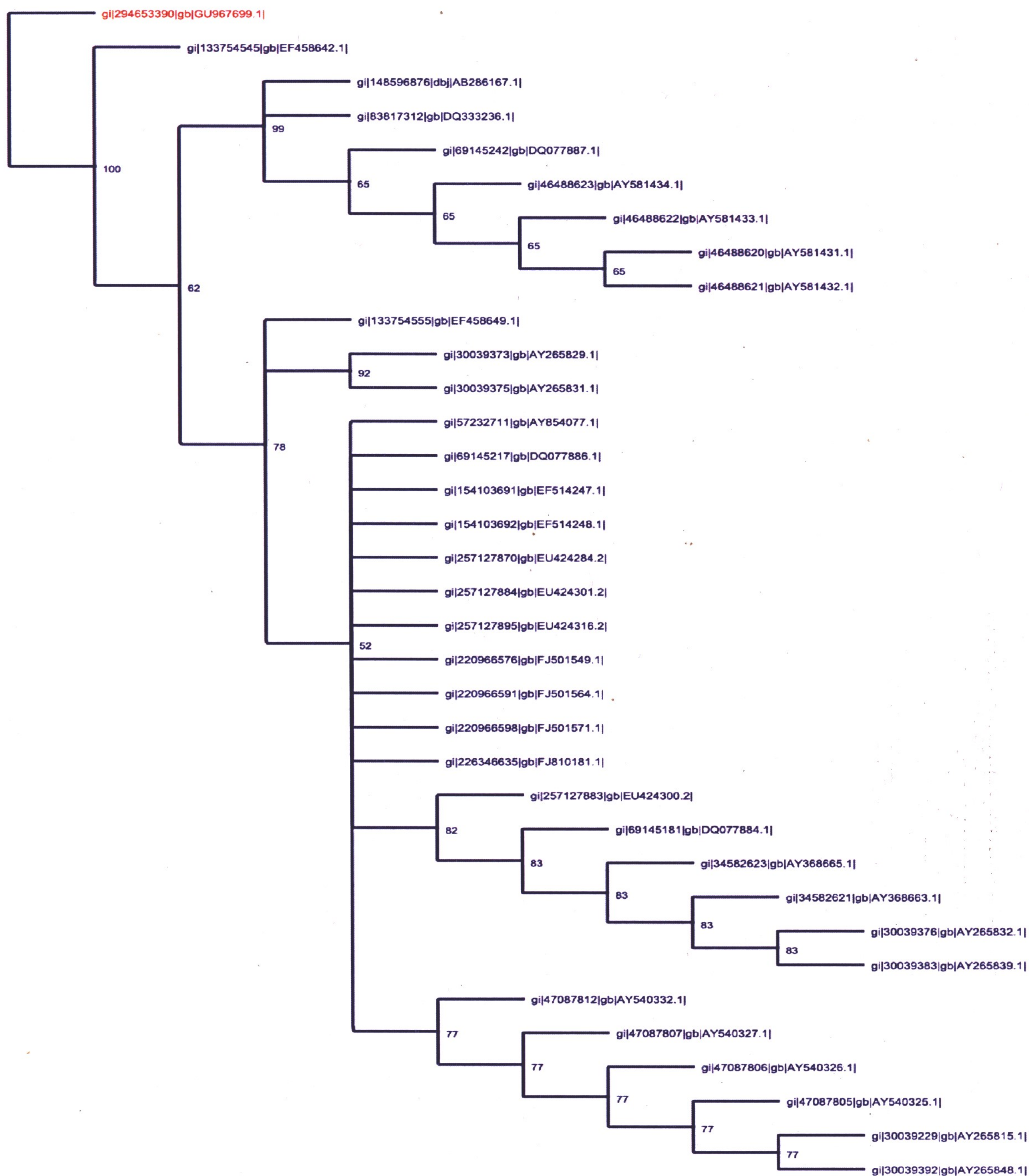

Fig. 7 — Phenogram constructed from 5.8S rDNA sequence of ST02 (GU967699.1) by UPGMA method.

The selected hypersecretory positive strain (ST02) was further investigated to analyze the potential of decolorization of dyes like Poly R-478 and RBBR individually. Dye decolorization and halo formation as a result of oxidation of indicator colored compounds were due to lignolytic enzymes production[22,37]. The isolated strain ST02 decolorizes both the indicator dyes successfully (Fig. 3 a, b, c & d).

According to Pant and Adholeya[20] a strain that turns the poly R-478 into yellow or colorless was considered as a ligninolytic enzyme producing organism. The studies of Palmieri *et al*[38] and Erkurt *et al*[39] proved that the decolorization of anthraquinonic dye RBBR was solely due to the laccase activity. The decolorization of RBBR dye was used as a tool for biodegradation studies it offers an additional

Table 1 — List of sequences producing significant alignments with *Pleurotus ostreatus* isolate RP7 (ST02) 5.8S rDNA

Accession No	Name of the strain	Length	Q.C*	M.I **
GU967699.1	*Pleurotus ostreatus* isolate RP7	625	100	100
AB286167.1	*Pleurotus eryngii*	660	86	91
AY265815.1	*Pleurotus columbinus* strain CBS 281.32	639	86	91
AY265829.1	*Pleurotus ostreatus f. florida* strain ATCC 38539	638	86	90
AY265829.1	*Pleurotus ostreatus f. florida* strain ATCC 38539	638	86	90
AY265831.1	*Pleurotus ostreatus, f. florida* strain ASI 2016	638	86	90
AY265832.1	*Pleurotus floridanus* strain CCRC 36038	640	86	91
AY265839.1	*Pleurotus ostreatus* strain CCRC 36249	640	96	91
AY265848.1	*Pleurotus spodoleucus* strain ASI 2012	640	86	91
AY368663.1	*Pleurotus floridanus* strain CCRC 36210	640	86	91
AY368665.1	*Pleurotus ostreatus* strain ASI 2029	640	86	91
AY540325.1	*Pleurotus ostreatus* strain S042	673	86	91
AY540326.1	*Pleurotus sapidus* strain S046	669	86	91
AY540327.1	*Pleurotus sapidus* strain S047	703	86	91
AY540332.1	*Pleurotus ostreatus* strain S474	678	86	91
AY581431.1	*Pleurotus nebrodensis* isolate W6	620	84	91
AY581432.1	*Pleurotus nebrodensis* isolate W7	605	84	91
AY581433.1	*Pleurotus nebrodensis* isolate W8	624	86	91
AY581434.1	*Pleurotus nebrodensis* isolate WW	678	86	91
AY854077.1	*Pleurotus ostreatus* isolate AFTOL-ID 564	630	86	91
DQ077884.1	*Pleurotus ostreatus*	640	86	91
DQ077886.1	*Pleurotus spodoleucus*	639	86	91
DQ077887.1	*Pleurotus nebrodensis*	639	86	91
DQ333236.1	*Pleurotus eryngii* clone 3	692	86	91
EF458642.1	*Pleurotus ostreatus* strain MUCL 28511	763	90	90
EF458649.1	*Pleurotus sajor-caju* strain MUCL 31674	710	86	91
EF514247.1	*Pleurotus ostreatus* strain CGMCC 5.37	629	86	91
EF514248.1	*Pleurotus ostreatus* voucher HMAS 66080	662	86	91
EU424284.2	*Pleurotus cornucopiae* strain ACCC50234	617	82	92
EU424300.2	*Pleurotus ostreatus* strain CBS 593.82	679	86	91
EU424301.2	*Pleurotus cornucopiae* strain ACCC50375	630	83	92
EU424316.2	*Pleurotus sapidus* strain ACCC50155	679	86	91
FJ501549.1	*Pleurotus ostreatus/Coprinus comatus* fusant isolate SMCC4.11.15	661	86	91
FJ501564.1	*Pleurotus ostreatus* isolate SMCC4.03.14	661	86	91
FJ501571.1	*Pleurotus ostreatus/Coprinus comatus* fusant isolate SMCC4.11.7	660	86	91
FJ810181.1	*Pleurotus sapidus* strain dd08093	703	86	91

Q.C* = Query Coverage M.I** = Max Identity

advantage compared to usual substrates. Moreover, dyes are stable, soluble, possess high molar extinction coefficients and low toxicity and can be applied in simple, rapid and quantitative spectrophotometric assays. RBBR has widely been used since this compound represents an important group of organopollutants[40-41]. The correlation between the guaiacol and polymeric dyes were good. The color reactions with synthetic dyes and guaiacol are more easily detectable, detect more laccase positives, and these compounds can thus reliably be used for laccase activity screening[22]. According to Masalu[42] the plate-test with indicator compounds is an efficient and simple method for screening the bioprospecting fungi with lignolytic enzymes for industrial applications.

The morphological characters of the isolated strain spores were uninformative and confused with other related genera (Fig. 4a & b). Hence, the molecular method was used for the identification of isolated strain. The ITS regions of fungal rDNA have been successfully used for species identification[20]. The isolated strain ST02 was identified using rDNA homology. Figures 5a & b shows the isolation and amplification of 5.8S rDNA of isolate ST02 from the genomic DNA, which was ~625 bp, this was closer to other similar sequences reported in NCBI database in

Table 1 (AY265829.1; AY854077.1; EF514247.1). The 5.8S rDNA sequence of ST02 (GU 967699.1) was blasted in the nucleotide sequence data base of PubMed and the first blast hit was *Pleurotus ostreatus* strain MUCL 28511 (EF458642.1) which showed 90% query coverage and maximum identity. Another organism *P. ostreatus* strain CCRC 36249 (AY265839.1) shared maximum query coverage of 96% with the ITS sequence of the isolated strain ST02. Fungi showing more than 90% similarity in the 5.8S ITS sequence can be considered as belonging to the same species[43], whereas for 18S rDNA sequence homology needs > 99% sequence identity can safely be considered as belonging to the same genus[44]. Since the 5.8S rDNA is a hyper variable region, the evolutionary distance resolved by ITS is usually restricted to demarcating within the species level and cannot be completely relied. The 5.8S rDNA sequence homology leads to the conclusion that the isolated strain ST02 (GU 967699.1) belongs to the genus *Pleurotus* and species *ostreatus*. Peterson *et al*[45] reported three *Penicillium* sp based on the ITS sequence and large subunit rDNA. Following a similar approach, a new fungal strain of *Bjerkandera audusta* was identified by Kowalska *et al*[46].

A few other facts also correlated with the identification of the strain ST02. Phylogenetic tree was constructed by two different methods (Neighbor joining and UPGMA method) for the obtained related ITS sequence of the isolated strain. The main virtue of NJ relative to these other methods is its computational efficiency. Unlike the UPGMA algorithm for phylogenetic tree reconstruction, NJ does not assume that all lineages evolve at the same rate (molecular clock hypothesis) and produces an unrooted tree. Phenogram was constructed using NJ method and the evolutionary distance calculated by the Tamura-Nei model shows that the strain *P. ostreatus* voucher HMAS 66080 (EF514248.1) was very close to the isolated strain (Fig. 6). Alam *et al*[47] reported that the size of ITS1 and ITS2 region varied among the strains and ITS2 was more variable than that of ITS1, whereas the 5.8S sequence was identical to the *Pleurotus* sp. Figure 7 shows the phenogram, constructed by UPGMA method with the same sequence that was used for NJ method and the distance was calculated by Tamura-Nei model, *P. ostreatus* strain MUCL 28511 (EF458642.1) was very close to the isolated strain ST02, moreover this was only strain which shares 90% of and query coverage 90% maximum identity to the isolated selected strain. Zervakis *et al*[48] used the 5.8S rDNA regions as nucleotide sequence divergence portion for molecular phylogeny of mushroom species *Pleurotus cystidiosus* and allied taxa. The above discussion gives strong correlation with 5.8S rDNA sequence with UPGMA phyogenetic analysis. The above organism was deposited and acknowledged by Commonwealth Agricultural Bureaux International (CABI) as "*Pleurotus ostreatus* IMI 395545".

Conclusion

This study showed that high laccase producers can be discovered from environmental samples by very simple plate test screening methods. Guaiacol is a sensitive substrate for screening the laccase producing organisms. Dye decolorization potential of the fungus was also determined for the future applications. Using 5.8S rDNA sequences, a number of fungi species have been identified. The present study has proved that 5.8S rDNA sequence analysis is a valuable tool for the detection of genus and species of unknown fungus. In addition, their evolutionary relationships could provide an important clue for further exploration of new strains. NJ and UPGMA method of phylogenetic analysis is more reliable method to find new fungal strains based on ITS sequence.

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