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IDENTIFICATION AND MOLECULAR CHARACTERISATION OF LACCASE AND XYLANASE PRODUCING FUNGUS ISOLATED FROM PAPER MILL EFFLUENT

SATHIYAVATHI M [A*] AND PARVATHAM,R [B].

[a*.] PG and Research Department of Biochemistry, K.S.Rangasamy College of Arts and Science, Tiruchengode- 637215, Tamilnadu, India

[b.] Department of Biochemistry, Avinashilingam Deemed University for Women,Coimbatore-641043,Tamilnadu, India

SATHIYAVATHI M

PG and Research Department of Biochemistry, K.S.Rangasamy College of Arts and Science, Tiruchengode- 637215, Tamilnadu, India

ABSTRACT

The objective of this present study was to isolate a potential fungus and characterize the organism with respect to laccase and xylanase production. The fungal strains were isolated by conventional serial dilution technique from paper mill effluent. Morphological observations showed the presence of 12 isolates and all the 12 fungal strains were screened for their ability to produce both laccase and xylanase by plate screening method using the indicators guaiacol and congo red respectively. Among the 12 isolates, *Trichoderma* sp. was predicted to be the only strain to produce both laccase and xylanase. The morphological identity of the *Trichoderma* sp was subsequently corroborated by molecular techniques. The molecular characterisation was performed with partial 18S rRNA sequencing using ITS primer set ITS1/ITS4 targeted to ITS region of rDNA complex and Phylogenetic analysis. All the results obviously explored the presence of novel strain, *Trichoderma* sp MS 2010, which is an efficient producer of laccase and xylanase. Both enzymes were found to have important industrial applications, thereby *Trichoderma* sp MS 2010(Genbank accession no: HM 124450) could be exploited to create a pollution free industrial environment.

KEYWORDS

Guaiacol, Congo red, laccase, xylanase, PCR amplification, 18S rRNA, internal transcribed sequence, ITS primers, Phylogenetic analysis, *Trichoderma* sp.MS 2010.

1. INTRODUCTION

Laccases are benzenediol: oxygen oxidoreductases (EC 1.10.3.2) and are primarily found in higher plants and fungi (Mayer *et al.,* 2002). In fungi, ascomycetes, deutromycetes and basidomycetes contain laccase (Desai *et al.,* 2011). They belong to the class blue oxidases and catalyze the removal of a hydrogen atom from the hydroxyl group of ortho- and para substituted mono and polyphenolic substrates and from aromatic amines by one-electron abstraction to form free radicals capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation (Thurston,1994). Utilization of extensive range of potential substrates has evoked an attention in several industrial applications of laccases such as pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation (Gianfreda et al. 1999). Novel laccases with different substrate specificities and enhanced stabilities are more desirable for industrial applications, besides developing an effective, high yielding and economic production medium to enhance its utility (Desai *et al.,* 2011).

Endo-1, 4-β-D-xylanases (E.C.3.2.1.8) are hemicellulases accountable for random cleavage of the xylan backbone, thus, are industrially significant. (Zhou *et al.,* 2008). Among the microbial sources, filamentous fungi are particularly interesting since they secrete these enzymes into the medium and have high xylanase activity in contrast to yeasts and bacteria (Krisana, *et al.,* 2005). This feature makes fungal xylanases attractive to be used in various industrial processes. For instance, in pulp and paper industry, the xylanases are exploited in the pre bleaching process to decrease the utilization of the toxic chlorine chemicals (Savitha *et al.,* 2009). Besides that, xylanase treatment serves to increase the brightness of the pulp, which is very essential in developing chlorine free bleaching processes. (Suprabha *et al.,* 2008). Sequential treatment of pulp by xylanase/laccase system was found to be promising to remove 60% and up to 70% of the residual lignin from softwood Kraft pulp wheat straw chemical pulp respectively (Herpoel *et al.,* 2002).

Morphological and biochemical distinctiveness of fungi are universally used for their identification, but differentiation of closely related cultures require extensive molecular techniques (Shahriarnour *et al.,* 2011). PCR amplification with universal primers targeted to conserved regions within the rRNA complex and subsequent DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Chen *et al.,* 2001). The PCR primer sets routinely used for amplification of ITS regions and rDNA are known to be ITS1 and ITS4 (White *et al.,* 2001).

Hence, fungi are highly diverse in nature; they have been recognized as a target for screening to find out the appropriate source of enzymes with constructive and novel characteristics (Bakri *et al.,* 2010). Plate-test screening based on polymeric dye compounds, guaiacol and tannic acid is an effective method to identify novel laccase producers (Kiiskinen *et al.,* 2004). In view of the industrial significance of laccase and xylanase, in this present study,

fungi were isolated from paper mill effluent and screened for laccase production using the indicator guaiacol and xylanase production using Congo red. This investigation led to the confirmation of fungal isolate which is the producer of both laccase and xylanase with the aid of molecular study and to introduce a new source of extracellular laccase and xylanase.

2. MATERIALS AND METHODS

2.1 Collection of Sample

The source for this study was effluent from Seshasayee Paper Boards ltd, situated at Erode. The effluent was collected from the storage reservoir approximately 1 km away from the discharge point of paper mill. The effluent was sampled in dry, sterile, polypropylene bottles, which were kept in ice during transportation and stored in the refrigerator (4^{o}C) till the isolation of fungi.

2.2 Isolation and morphological identification of Fungi

The isolation of fungi was carried out on potato dextrose agar (PDA) added with chloramphenicol (0.05%).One ml of the sample was made up to 10 ml with sterile distilled water and mixed. This suspension was serially diluted from 10^{-1} to 10^{-7}.Later, 1.0 ml each dilution was spread on the surface of PDA using an L-shaped glass rod and incubated at 28ºC for 5-7 days. The fungal cultures were transferred on the same medium without antibiotics until pure colonies were obtained. Fungal identification was carried out based on the colony morphology and structural characteristics as observed under the light microscopy. (Pang *et al.,* 2006).

2.3 Screening of fungal isolates for laccase production

Selection for laccase producing organisms was done on plates containing following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, and 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agars (pH-6) supplemented with 0.02% guaiacol. The species of fungi were inoculated into these plates and the plates were incubated at 30°C for 7 days (Coll *et al.,* 1993). After incubation, the plates were observed for the formation of reddish brown zones around the colony. The strain that showed colored zone was selected for further studies, since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium.

2.4 Screening of fungal isolates for xylanase production

The fungal isolates were screened for their abilities to produce extra cellular xylanase during their growth on Czapek's agar medium containing xylan as the sole carbon source. The composition of the medium was (g.L-1): Oat spelt xylan, 5.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2 and agar, 20.0 (Nakamura *et al.,* 1993). The inoculated plates were incubated for 7 days at 30ºC. The clearing zones formed around the fungal growth were more visible when the plates were flooded with 0.1% (w/v) Congo Red. After 30 mins of incubation, plates were washed with 1 M NaCl to remove the excess stain. Fungal strains, which produced distinct clearing zones around their colonies, were selected (Muthezhilan *et al.,* 2007).

2.5 Molecular identification of laccase and xylanase producing fungi using ITS primers

The fungus, which was observed to produce positive result in the screening test for both laccase and xylanase was subjected to genotypic identification. Genotypic identification was carried out by PCR amplification and partial sequencing of the 18S rRNA for the confirmation of morphological identity. The rDNA sequence of 18S ribosomal RNA gene was amplified by PCR and the PCR product

was bidirectionally sequenced using forward (ITS1-5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4-5'-TCC TCC GCT TAT TGA TAT GC-3') primers (Bakri *et al.,* 2010). Since, fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions (Anderson *et al.,* 2003). Primers were synthesized and PCR amplifications with subsequent sequencing were carried out by Synergy Scientific Services, Chennai, India. A comparative study of other rDNA sequences with rDNA sequence of *Trichoderma* isolate in this study was done using BLAST algorithm at the website http://www.ncbi.nlm.nih.gov. The nucleotide sequence of *Trichoderma* sp. has been assembled and submitted at the NCBI Genbank (Ahuja *et al.,* 2007).

3. RESULTS AND DISCUSSION

3.1 Morphological identification of Fungal Isolates

The primary identification of fungal isolates was carried out using morphological characterization as observed under light microscopy and the results were depicted in Figure-1 & Table-1 The results of the present study revealed the presence of 12 isolates in the effluent. They were found to be *Aspergillus niger* .,*A. flavus*., *A. fumigatus*., *A. terreus* .,*A. Awamori*., *A. oryzae*, *Fusarium* sp., *Alternaria* sp., *Curvularia* sp., *Rhizopus sp*., *Trichoderma* sp., and *Penicillium* sp.

Figure 1
Morphological observation of fungal isolates

Table -1
Morphological observation of fungal isolates

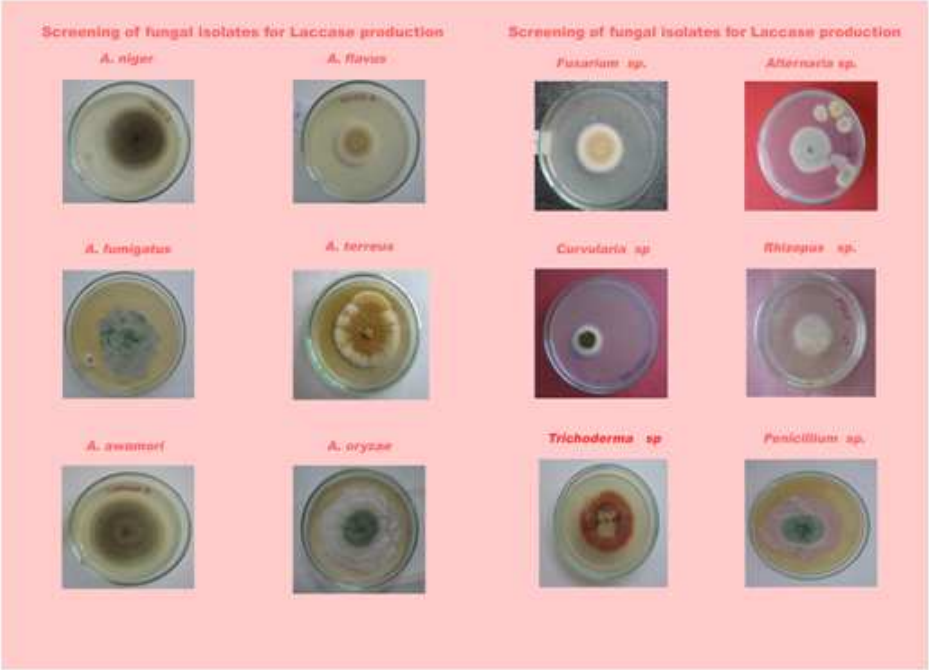
S.No	Colony color on PDA	Colony reverse color on PDA	Growth pattern	Identified fungal isolates
1	Black	White to yellow	Moderate growth with submerged mycelium	*A. niger*
2	Yellow-green	Goldish to red brown	Rapid growth	*A. flavus*
3	Blue-green to grey	White to tan	Moderate to slow growth	*A. fumigatus*
4	Cinnamon to brown	White to brown	Rapid growth with radial furrows, velvety, floccose	*A. terreuss*
5	Whitish yellow	Colourless motted clear yellow shade	Rapid growth; long bearing conidial with floccose margin	*A. awamori*
6	Greenish yellow to olive yellow then old gold and to shade of yellow brown	Colourless	Rapid growth, submerged mycelium, forming a tough basal felt	*A. oryzae*
7	Pale colored; whitish to yellow	Dark purple	Fast growing; cottony aerial mycelium	*Fusarium* sp.
8	Greyish white initial; later darkens and becomes greenish black or olive brown with a light border	Brown to black	Colony is flat, downy to woolly and fast growing	*Alternaria* sp.
9	White to pinkish gray initially and turns to olive brown or black as the colony matures	Black	Fast growing; woolly colonies	*Curvularia* sp.
10	White, at maturity black	Colourless	Fast growing; Cottony	*Rhizopus* sp.
11	White and scattered greenish patches become visible as the conidia are formed and may form concentric rings at times	Pale, tan, or yellowish	Fast growing; wooly becoming compact in time	*Trichoderma* sp.
12	Bright green with white margin	Dull yellow or greenish brown	Restricted growth; Larger, plane, Centrally umbonate	*Penicillium* sp.

Similar experiments for the identification of fungi through morphological and macroscopic observations were enumerated in several literatures. Singh *et al.,* (2006) isolated *Trichoderma* taxa from edible mushrooms and identified primarily with morphological observations. Among the 23 fungal strains which were isolated, four of the strains showed morphology typically for genus *Trichoderma* and four strains for genus *Aspergillus* (Gochev *et al.,* 2007). Macroscopic features were followed to study the morphological characteristics of the isolated basidiomycete, *Lentinus tuberregium* (Manjunathan *et al.,* 2011). Two ascomycetes and two basidiomycetes fungi with telomorphic and/or anamorphic stages (spores/conidia) produced in cultures were identified by morphology using the taxonomic keys (Verma *et al.,* 2010). Colony size of 15 isolates of *Trichoderma harzianum* on malt extract agar (MEA) was recorded after 14 days at 25°C. Chlamydospore production and size of conidiogenous cells and conidia were studied (Grondona *et al.,* 1997).

3.2 Screening of laccase producing fungal isolates

The laccase producing ability of fungi isolated from the paper mill effluent was examined by the screening method using solid media containing guaiacol as the indicator compound. A total of 12 species were screened and among these, *Trichoderma* sp. was found to cause the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Figure 2). Extracellular laccase activity was found in *Trichoderma* sp. which belongs to the group ascomycetes. Thus, this screening was used to select a promising ascomycetes strain *Trichoderma* sp.

Figure 2
Screening of laccase producing fungal isolates



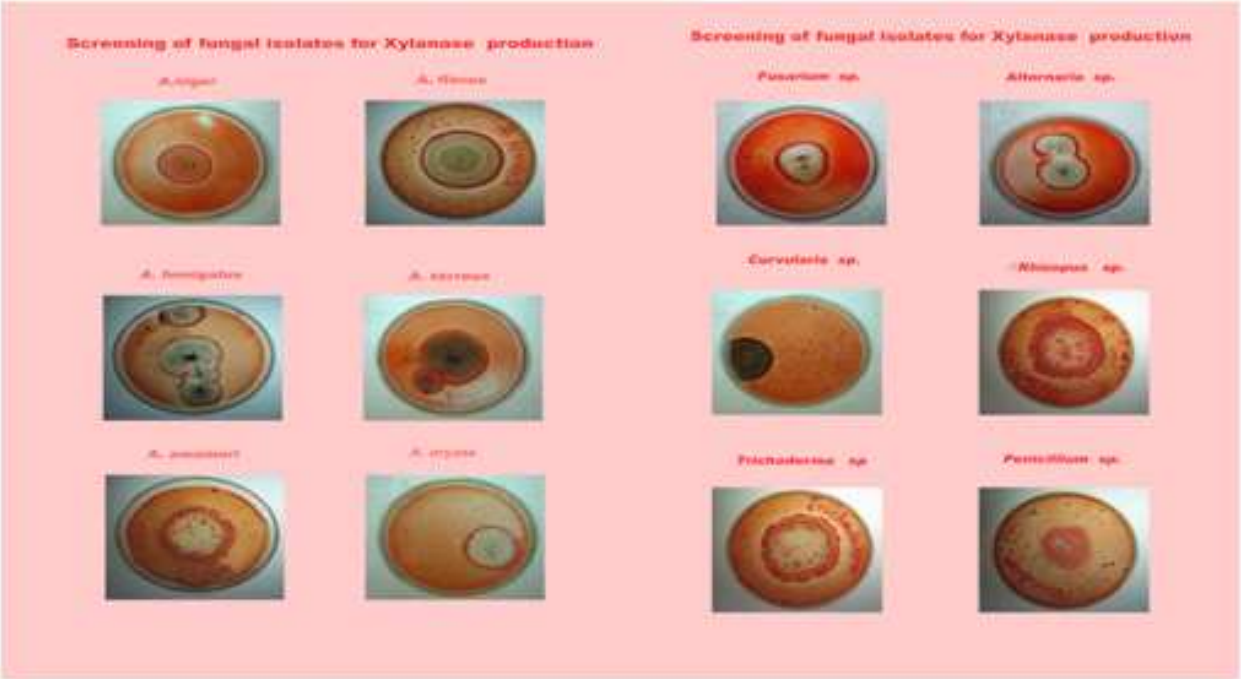
The results of this present investigation was supported by the screening process resulted in the isolation of 26 positive fungal strains, including three *Trichoderma* sp. (Kiiskinen *et al.,* 2004). Similar experiments were carried out to screen and select laccase producing fungi, *Stereum ostrea* and *Phanerochaete chrysosporium* (Viswanath *et al.,* 2008), *Pleurotus ostreatus* (Patel *et al.,* 2009), *Paecilomyces* sp (Liang *et al.,* 2007) and *Lentines edodes* (Shanmugam *et al.,* 2009). The ability of the fungal species to produce laccase was correlated with the effcetive decolorisation of azo dyes such as Reactive Black 5H was also exploited for the screening of laccase producers (Priyadarsini *et al.,* 2011).

3.3 Screening of xylanase producing fungal isolates

The fungal isolates were screened for their abilities to produce extra cellular xylanase during their growth on Czapek's agar medium containing xylan as the sole carbon source. The inoculated fungal strains which produced distinct clearing zones in medium were selected. The results of this screening were depicted in **Fig 3.**

Figure 3

Screening of xylanase producing fungal isolates

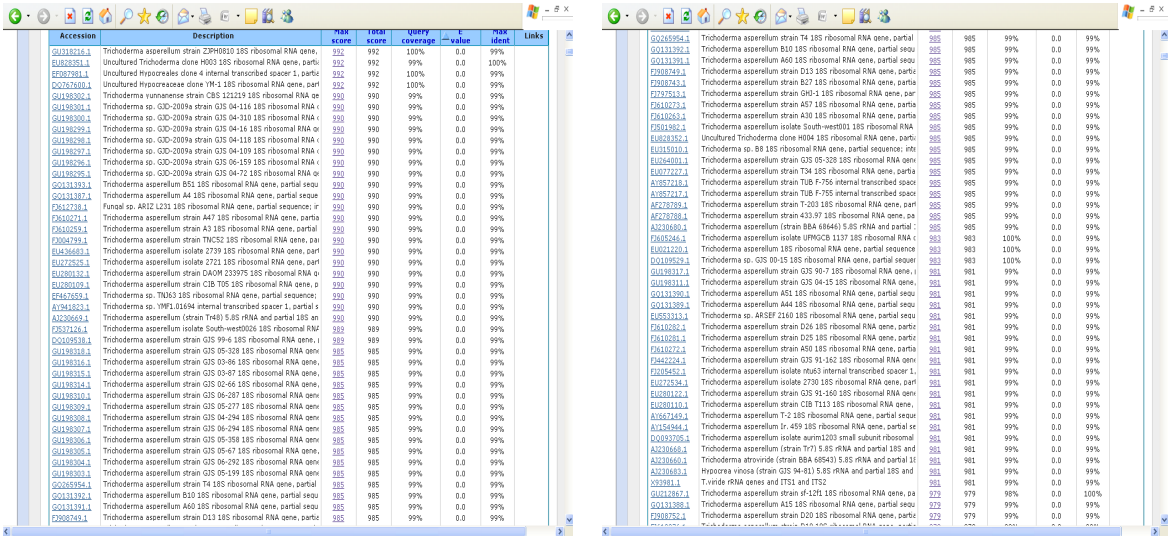


It was observed that all the 12 isolates exhibited xylanase producing ability. But the formation of clearing zones indicated that *Trichoderma* sp., *Penicillium* sp., and all *Aspergillus* spp.were the best producers of xylanase.This result was in concordance with similar screening work in which a total of 7 fungal strains were isolated from mixed soil and *Aspergillus niger* being predominant was used for xylanase production. Its ability to produce xylanase was further confirmed by the formation of reddish orange halo zone of hydrolysis in Congo red test by growing *A.niger* on oat spelt xylan agar plates (kavya *et al.,* 2009). Seventy fungal isolates were screened for their abilities to produce extracellular xylanase by means of formation of clearing zones around the fungal growth in Congo red test. A total of 34 fungal strains, comprised of *Aspergillus* spp, *Trichoderma* spp and *Penicillium* spp, which produced distinct clearing zones were selected (Suprabha *et al.,* 2008). Among the 69 strains isolated from Pitchavaram mangroves, *Penicillium oxalicum* was selected for xylanase enzyme production based on the formation of orange coloured digestion halos on treatment with Congo red (Muthezhilan et al.,2007). The ability of *Streptomyces Pseudogriseolus* to produce xylanase was screened qualitatively using Congo red (Aziz *et al.,* 2011).Sharma *et al.,* (2005) performed isolation of bacterial strains from environmental samples and screened their capability of xylanase production using Congo red and reported that the *Streptomyces* sp. CD3 was the maximum producer of xylanase among the isolated strains.

3.4 Molecular identification of laccase and xylanase producing fungus using ITS primers

Microscopic observation revealed the taxonomic identity of the fungus, which was observed to produce both laccase and xylanase. The fungus strain has shown the production of conidia, white and scattered greenish patches become visible as the conidia are formed and form concentric rings at times. The above mentioned characteristics designated the fungus to be *Trichoderma* sp.in taxonomy (Figure 1).The morphological identification of *Trichoderma* sp. was further substantiated with molecular study. PCR amplification of ITS region yielded a fragment of 540 bp length from the isolate of *Trichoderma* sp. The sequence of the isolate studied was compared with that of NCBI databases using BLAST network. The isolate was analyzed based on the similarity with the best-aligned sequence of the BLAST search (Singh *et al.,* 2006). Based on BLAST search of partial 18s rRNA gene sequence, partial 5.8s rRNA gene sequence and complete sequence of ITS region, the fungus was found to be closest homolog (100% identity) to uncultured *Trichoderma* sp. (Figure 4). The isolate was identified as *Trichoderma* sp.MS 2010 and submitted to GenBank under the accession number, HM 124450.

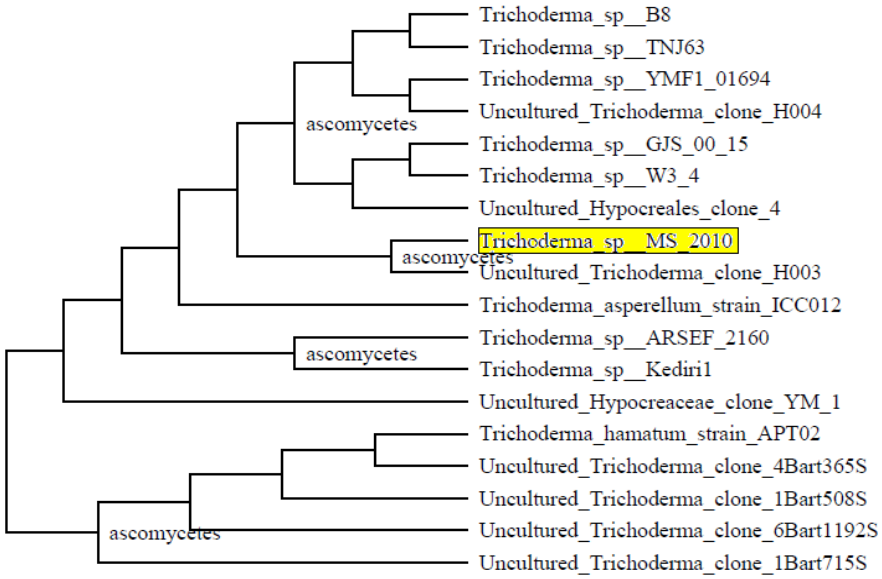
Figure 4
BLAST



3.5 PHYLOGENETIC INVESTIGATION

The isolated fungi was identified by molecular characterization technique in which genomic DNA was isolated and internal transcribed spacer (ITS) was amplified with suitable primers and phylogenetic analysis was done. Phylogenetic analysis was done with primers targeting ITS regions of fungus. The phylogenetic analysis revealed 100% similarity with uncultured *Trichoderma* sp (Figure 5) and hence the isolated fungus was identified as a new strain ,*Trichoderma* sp. MS 2010.

Figure - 5
Phylogenetic relationship of Trichoderma sp.MS 2010 with other Trichoderma sp.



The findings of this study go in parallel with the identification of the fungal strain BCC7928 by morphological characteristics as well as comparison of internal transcribed spacer (ITS) sequences of rRNA gene. Partial DNA fragment of the ITS was amplified by PCR using ITS-F and ITS-R primers. The PCR product, 600 bp in length was analyzed. On the basis of phenotypic characteristics and phylogenetic analysis of ITS data, the BCC7928 strain was identified as *Marasmius* sp (Ratanachomsri *et al.,* 2006). PCR was used to amplify 5.8S rDNA gene of two xylanase-producing isolates using ITS1 and ITS4 primers. The phylogenetic analysis confirmed that the two isolates were closely related to *Aspergillus terreus* and *Aspergillus tubingensis* with 100% of homology (Bakri *et al.,* 2010).

Priyadarsini, et al, (2011) isolated a white-rot fungus for laccase producing ability from decayed wood in a temperate forest and identified as *Trametes hirsuta* based on the morphological characteristics and a complete sequence analysis of it's ITS region using the fungal specific primer set ITS F and ITS R. A Laccase producing ascomycete, *Paraconiothyrium variabile* was isolated from soil and slide culturing on oatmeal agar was performed for microscopic characterization. Amplification of 18S rDNA was carried out with the primer set of NS1, nu-SSU-1536, nu-SSU-0817, and NS8. 5.8S in association with the two flanking internal transcribed spacers (ITS1 and ITS2), was amplified using primers ITS1 and ITS4 (Forootanfar *et al.,* 2011). Genotypic identification was carried out amplification and sequencing of 18S rDNA using forward (ITS4) and reverse (ITS5) primers. BLAST analysis of ribosomal RNA gene sequence described that the fungus was found to be closest homolog to *Fusarium oxysporum* (Barik *et al.,* 2010). ITS primers and intergenic spacer (IGS) primers for rDNA were used to amplify specific sequences of this region in order to make a distinction among Romanian *Trichoderma* isolates (Cornea *et al.,* 2008).The observations of this present study were concurred with these previous findings.

In a parallel study, Chakraborty *et al.,* (2010) observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in *Trichoderma* sp. The ITS PCR with ITS1 and ITS4 primers has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates. The results of dendrogram based on RAPD analysis evidently showed that all the nineteen isolates can be grouped into two main clusters. One cluster represents *T.viride* and other *T. harzianum*. Chen et al, (1999) established the identification of *Trichoderma harzianum* by RAPD-PCR analysis using the primers, 203, 211, 220, 230, 232, 238, 241.

3. CONCLUSION

Molecular methods being highly sensitive and selective, currently used to identify decay fungi. Environmental conditions may have intense impact on morphological and physiological characteristics, hence the accurate identification of fungal isolates turned out to be more difficult. Therefore, it was suggested that the molecular techniques are more significant for the characterization of the new fungal isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new fungal isolate is the purpose of the investigation.

This present investigation, with the aid of morphological observations and molecular techniques, revealed the existence of a novel *Trichoderma* sp. in paper mill effluent which could able to produce both laccase and xylanase, thereby allowing the synergistic actions of both enzymes. This salient feature is more desirable for efficient prebleaching of pulp. Hence, *Trichoderma* sp.MS 2010 identified in this study can be effectively exploited for the ecofriendly industrial processes.

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