Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 15	No. 4	pp. 1241-1248	2016

Original Research Paper

Ligninolytic Enzymes Production and Decolorization Potential of Native Fungi Isolated from Pulp and Paper Mill Sludge

Deepika Rajwar†, Sarita Joshi and J.P.N. Rai
Department of Environmental Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar 263145, India
†Corresponding author: Deepika Rajwar

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 02-12-2015
Accepted: 04-02-2016

Key Words:
Lignin degrading fungi
Ligninolytic enzymes
Black liquor
Decolorization

ABSTRACT

In this study, potential of 30 fungal strains isolated from pulp and paper mill sludge were assessed as biolignolytic microorganisms and their ability to produce ligninolytic enzymes viz., laccase (Lcc), lignin peroxidase (LiP), manganese peroxidase (MnP), xylanase and cellulase using different chromogenic substrates. A total of eight fungi exhibited conspicuous halos with their respective substrate. The fungal isolates were found to be significantly different in qualitative enzyme production. Maximum enzyme activity for Lcc, MnP and LiP were 26.24, 8.5 and 12.99 U/mL exhibited by fungal isolates *Trametes* sp. (LDFD5), *Nigrospora* sp. (LDF00204) and *Alternaria* sp (LDFD4) on 10th and 13th day, respectively, by solid state fermentation (SSF). Conspicuous reduction in colour unit upto 75.29 % and 76.37 % were observed by fungal strain LDF00204 at 30°C temperature and pH 5 respectively. Results depicted that fungal isolate *Nigrospora* sp. LDF00204 (Accession No. KP732542) exhibited a relatively noble characteristic of having ligninolytic enzymes, is able to degrade lignin and its co-compounds efficiently, and thus it could be utilized for decolorization of pulp and paper mill effluent and also for bioremediation of alike pollutants.

INTRODUCTION

The pulp and paper industries present an energy demanding and highly polluting sectors in the world which generate huge amount of wastewaters. It has a total production capacity of more than 3 million tons per annum (CPCB 2001). Chemical pulping and bleaching (kraft process) produce enormous amounts of inorganic chemicals, toxic components and wood residues known as black liquor which has a major impact on the environment. Black liquor generated by the industry has high biochemical oxygen demand (BOD), chemical oxygen demand (COD), pH and odour (Raghukumar & Rivonkar 2001). Moreover, the use of chemical compounds lead to the formation of xenobiotics e.g. dioxin, biphenyls, polybrominated diphenyl ether and chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (WHO 2007), influencing aquatic flora, fauna nutrient balance of soil and aquatic food chain as well (Makris & Banerjee 2002, Akhan 2008, Owens et al. 1994).

Treatment of the generated effluent is of great concern from environmental point of view. Biological methods are preferred over physico-chemical methods owing to its simplicity, low cost and no adverse impact on environment. Screening of fungi is a common practice to find out the potential fungal strains with the ligninolytic enzyme activity of industrial application. White rot fungi has been recognized as a model organism for biodegradation of lignocellulosic compounds. These fungi have strong oxidative enzymes viz., LiP, MnP and Lcc which have great potential to degrade lignin and its co-compounds (Linger et al. 2014). LiP and MnP helps in oxidation of aromatic compounds and terminal phenolic, nonphenolic substrates respectively, while Lcc are involved in breaking of phenolics and nonphenolics part and both have been reported in number of fungi (Dhouib et al. 2005). SSF technique using different lignocellulosic wastes, rich in nutrients and contain inducers of ligninolytic enzymes has been studied by many workers (Couto & Sanroman 2005, Singhania et al. 2009).

Decolorization of pulp and paper mill effluent using different microorganisms, where the effect of various factors like temperature, pH, carbon and nitrogen sources were investigated by several workers (Wu et al. 2005, Da Re et al. 2011). *Nigrospora* sp. has been reported as an endophytic fungus (Pawle et al. 2014) of plants and few species are characterized as pathogenic. Previous studies have emphasized its role for antimicrobial properties, secondary metabolites production and dye decolorization (Ilyas et al. 2013). Despite all these, *Nigrospora* sp. as source of ligninolytic enzyme and decolorization of pulp and paper mill effluent has not yet been studied thoroughly. Hence, the present study was undertaken to isolate ligninolytic enzymes producing fungi from pulp and paper mill sludge and also to assess their potential for decolorization of paper mill effluent.

MATERIALS AND METHODS

Chemicals and material: 2,2-azino-di-(3-ethylbenzo thialozin-6-sulfonic acid) (ABTS), birchwood xylan, guaiacol and azure B were purchased from Sigma (St. Louis, MO, USA) and 3,5-Dinitrosalicylic acid (DNS), was purchased from Hi Media, India. All other reagents were of analytical grade, made by known manufacturers. Wheat straw was purchased from local farm in Pantnagar, Uttarakhand.

Sample collection, isolation of microorganisms and culture conditions: Black liquor sample was collected from wood based pulp and paper mill and stored at 4°C for further experimental purpose. Soil sludge was collected from contaminated site of pulp and paper mill and was serially diluted for isolation of fungal strains. 1 g of soil was serially diluted upto 10^{-9} with sterilized distilled water. Different diluted suspensions, 10^{-2}, 10^{-4} and 10^{-6} were spread over potato dextrose agar (PDA) medium supplemented with black liquor (1%). Plates were incubated for 7 days at 28°C. The isolated cultures were grown and maintained on PDA plates or slants and sub-cultured every month. 10 mm agar plugs were taken from a 7 day old fungal colony and inoculated into 500 mL Erlenmeyer flasks with 100 mL of potato dextrose broth (PDB) medium (pH 5) for ligninolytic enzyme production. The flasks were incubated at 30°C on the shaker for 1-20 days under still conditions. Wheat straw was taken as lignocellulosic substrate for SSF to enhance enzyme production following the method of Pant & Adholeya (2006). All experiments were performed in triplicates.

Morphological characterization, DNA isolation and identification of fungal isolate: PDA plates were inoculated with seven days old cultures (8 mm) of fungal isolates. The plates were incubated at 28°C. The observation of mycelia and spore formation were noticed in full grown culture. Genomic DNA of fungal strain LDF4 was isolated by genome DNA kit (Qiagen Inc, USA). 18s rRNA gene amplification was done using universal primer ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990). Polymerase chain reactions (PCRs) were performed in an Eppendorf master thermocycler and PCR amplified product was sent to Chromous Biotech Pvt. Ltd. Bangalore, India for sequence analysis. The nucleotide sequence data were subjected to BLAST analysis using public databases GenBank (http://www.ncbi.nem.nih.gov/BLAST) to compare with already present sequenced data. Sequences were aligned using the CLUSTAL W program and phylogenetic tree was constructed using the neighbour-joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the software routines included in the MEGA 5 software. The sequence was submitted to NCBI GenBank for accession number.

Screening of fungal strains for ligninolytic enzyme production: The isolated fungal strains were screened for ligninolytic enzyme production by plate assay. Lignin modifying enzyme (LME) basal media (g/L- KH_2PO_4 1.0, Yeast Extract 0.01, $C_4H_{12}N_2O_6$ 0.5, $CuSO_4.5H_2O$ 0.001, $MgSO_4.7H_2O$ 0.5, $Fe_2(SO_4)_3$ 0.001, $CaCl_2.2H_2O$ 0.01, $MnSO_4.H_2O$ 0.001) was supplemented with 0.005% w/v α-naphthol for Lcc and 0.01% w/v Azure-B dye for peroxidase. The media containing (g/L)- ammonium tartrate (5g), KH_2PO_4 (1g), $MgSO_4$.$7H_2O$ (0.5g), yeast extract (0.1g), $CaCl_2.2H_2O$ (0.001g) was supplemented with 4% w/v birchwood xylan for xylanase and 2% w/v carboximethyl cellulose (CMC) for cellulase (Pointing 1999). All tests were performed in triplicate.

Quantitative enzyme assay: For quantitative enzyme production, potato dextrose broth (200 mL) was taken in 500 mL Erlenmeyer flasks and inoculated with fungi, individually. Additionally, wheat bran (5 g) was added as solid substrate for enhancing the enzyme production. Flasks were incubated at 30°C and SSF samples were extracted using 1:2.5 (w/v) of water at different time intervals. Samples were then filtered using a glass filter. Finally the filtrate was centrifuged at 10,000 rpm for 20 min to remove substrate particles (Makkar et al. 2001). Lcc activity was determined using 2, 2-azino-di-(3-ethylbenzothialozin-6-sulfonic acid) (ABTS) as the substrate. The reaction mixture contained 1 mM ABTS, 25 mM sodium acetate buffer (pH 3.8) and the crude enzyme. The rate of ABTS oxidation was determined spectrophotometrically at 420 nm (ε420 = 36,000M-1 cm-1) at room temperature (Bourbonnais & Paice 1990). LiP activity was determined by oxidation of veratryl alcohol to veratraldehyde and absorbance was measured at 310 nm (ε310 = 9300M-1 cm-1) according to Tien & Kirk (1984). The reaction mixture contained 2 mM veratryl alcohol, 50 mM sodium tartrate buffer (pH 3.0), and 50 µL culture filtrate. The reaction was initiated by addition of 0.2 mM of H_2O_2. MnP activity was assayed by measuring the oxidation of 2, 6-dimethoxyphenol (ε468 = 49,600M-1 cm-1). The reaction mixture consists of 100mM malonate buffer (pH 4.5), 2mM $MnSO_4$, 0.5 mM H_2O_2, 0.5 mM 2, 6-dimethoxyphenol and crude enzyme. MnP activity was measured at 270 nm by the formation of Mn^{+3}-malonate complexes (Wariishi et al. 1992). One unit (U) of enzyme activity was defined as activity of an enzyme that catalyzes the conversion of 1 µ mole of substrate per minute. Enzyme activity was expressed in international units. All experiments were run in triplicates along with controls.

Decolorization of black liquor: The potential of screened fungal strain designated as LDF00204 was assessed based on decolorization of black liquor. An experiment for de-

colorization of black liquor was performed in 500-mL Erlenmeyer flask containing 250 mL sterilized black liquor, minimal salt media and pre grown fungal culture (LDF00204) in triplicates along with control. Flask was incubated at different temperatures (25°C, 30°C, 35°C) and pH (5, 7, 9) for 7 days at 140 rpm. A control set was run in parallel without addition of fungal strain. Samples were collected at every alternate day (1, 3, 5, 7 days), centrifuged at 10,000 rev/min for 30 min, the pH was adjusted to 7.6 and the sample absorbance was taken at 465 nm for calculating the colour units (Bajpai et al. 1993).

Statistical analysis: Data were statistically analysed using two-way ANOVA and the significant difference at $P<0.05$ among various treatments were given following SPSS software.

RESULTS

Isolation and identification of fungi: Thirty fungal isolates were obtained from pulp and paper mill sludge, rich in lignin and other toxic compounds at the point of effluent discharge by serial dilutions. Fungal isolates were characterized based on morphological features and spores formation. Fungal isolate LDF00204 (*Nigrospora* sp.) was characterized by fast growing colony and black spores. Mycelia were dull white which later on turned black. Other isolate LDFD4 (*Alternaria* sp.) was characterized by blackish-grey, septate, irregularly branched mycelia and pale brown septate conidiophores while the third isolate LDFD5 (*Trametes* sp.) was characterized by white mycelia which later on turned hard puffy body. Optimum temperature for growth was 28°C for these isolates.

For identification of fungal strain LDF00204 (*Nigrospora* sp.), genomic DNA was subjected to PCR amplification and 18srRNA gene sequence was compared with the available gene sequences of other organisms in GenBank at NCBI database at www.ncbi.nlm.nih.gov/BLAST. Constructed phylogenetic tree showed the nearest homology of *Nigrospora* sp. (LDF00204) with *Nigrospora* sp. KH00291. The ITS sequence was deposited in the GenBank database with the accession number KP732542.1.

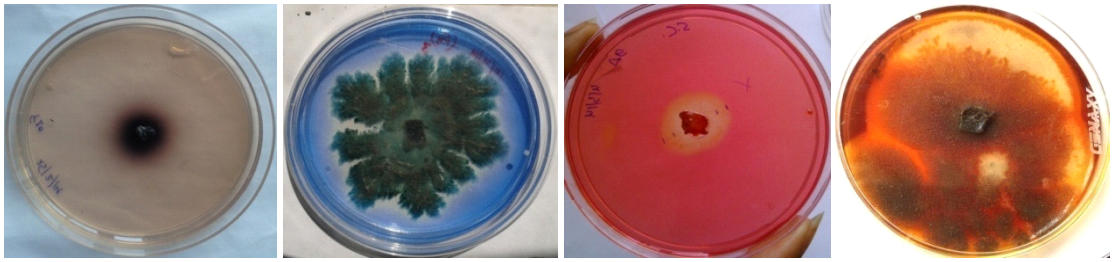
Screening of fungi for ligninolytic activity: Isolated fungi were subjected to plate assay for laccase, peroxidase, xylanase and cellulase enzymes production. Amongst 30 fungal strains, 8 produced the hydrolysis zone after reaction with different substrate (Fig. 1). Disappearance of blue colour of LME basal media confirmed the peroxidase activity while appearance of dark violet colour indicated the presence of laccase. Cellulase activity was confirmed by appearance of hydrolysis zone in CMC agar media and appearance of yellow zone indicated the presence of xylanase. Most of the strains showed laccase activity upto 75% followed by peroxidase (65%). Xylanase and cellulase activity were found in 24% and 35% of strains respectively (Table 1). *Trametes* sp. and *Nigrospora* sp. LDF00204 showed initial laccase activity on 1st and 2nd day (zone diameter 5.3 cm and 3.16 cm) while *Alternaria sp.* (zone dia 7.63 cm) and *Nigrospora* sp. LDF00204 (zone dia 6.66 cm) strain indicated maximum peroxidase activity on 5th and 7th day respectively. Cellulase and xylanase activity were observed in fungal strain LDFD2 (unidentified) (zone dia 5.7 cm) and *Alternaria* sp. (zone dia 7.6 cm) while absent in *Nigrospora* sp. LDF00204. Ligninolytic enzymes activity in all fungi were significantly ($P<0.05$) different, whereas, respective controls (without substrate) did not show any activity. On the basis of screening, fungal strains (*Nigrospora* sp. LDF00204, *Alternaria sp.* and *Trametes* sp.) were selected for quantitative enzyme production.

Estimation of enzyme production: Initially ligninolytic enzyme activities were observed in culture filtrate without adding any substrate. The selected fungal isolates were screened for quantitative production of Lcc, LiP and MnP. A noticeable difference in enzyme production was observed when lignocellulosic substrate (wheat straw) was added in culture media. Maximum production of laccase was observed by *Trametes* sp. (26.24 U/mL) followed by *Nigrospora*

Table 1: Ligninolytic enzyme production by fungal isolates with their respective substrates.

Fungal Strain	Laccase (α-naphthol)	Peroxidase (Azure B)	Cellulase	Xylanase
LDF4 (*Nigrospora* sp. LDF00204)	3.16±0.12 (1)	6.66±0.12 (7)	0±0	0±0
LDF21 (*Alternaria* sp.)	2.33±0.08 (2)	7.63±0.08 (5)	4.33±0.14 (3)	7.6±0.15 (5)
LDFC1	3.10±0.10 (3)	5.3±0.05 (7)	5.2±0.11 (3)	0±0
LDFD1	2.46±0.08 (2)	0±0	0±0	0±0
LDFD2	2.36±0.14 (4)	0±0	5.7±0.05 (3)	0±0
LDFD3	0±0	7.2±0.11 (8)	5.46±0.08 (3)	2.7±0.11 (5)
LDFD4	0±0	6.26±0.14 (9)	4.43±0.12 (3)	0±0
LDFD5 (*Trametes* sp.)	5.3±0.17 (1)	0±0	5.06±0.06 (3)	6.2±60.14 (5)

Values represent hydrolysis zone produced by fungal strains; Values in parenthesis denote day of initial or maximum enzyme activity; Fungal enzyme production is significant at $P<0.05$.



(a) Laccase (b) Peroxidase (c) Cellulase (d) Xylanase

Fig. 1: Positive plate assay test for ligninolytic enzymes production.

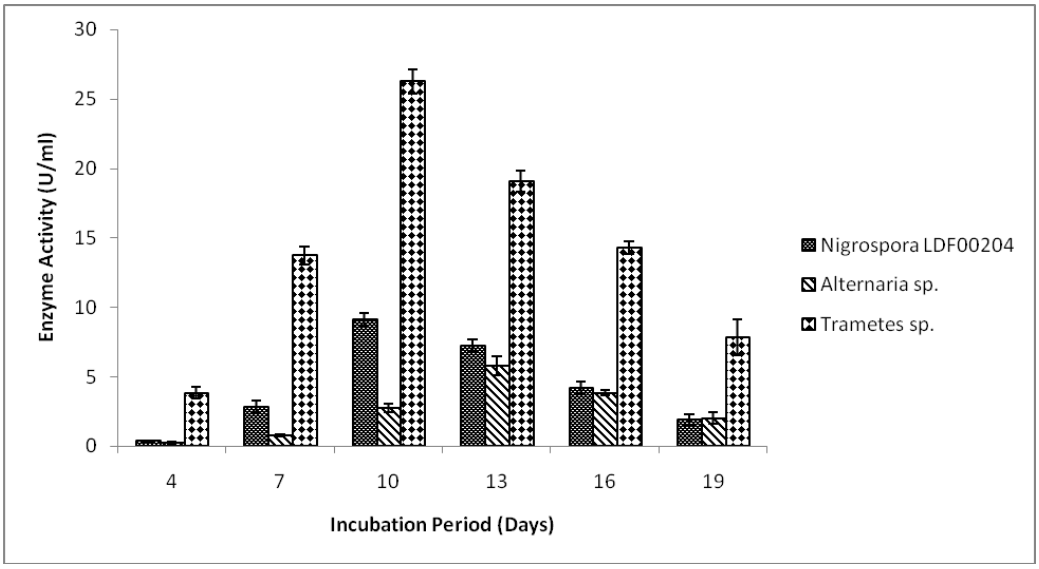


Fig. 2: Laccase production by fungal strains in SSF by *Nigrospora* sp. LDF400204, *Alternaria* sp. and *Trametes* sp. Error bar shows±S.E.

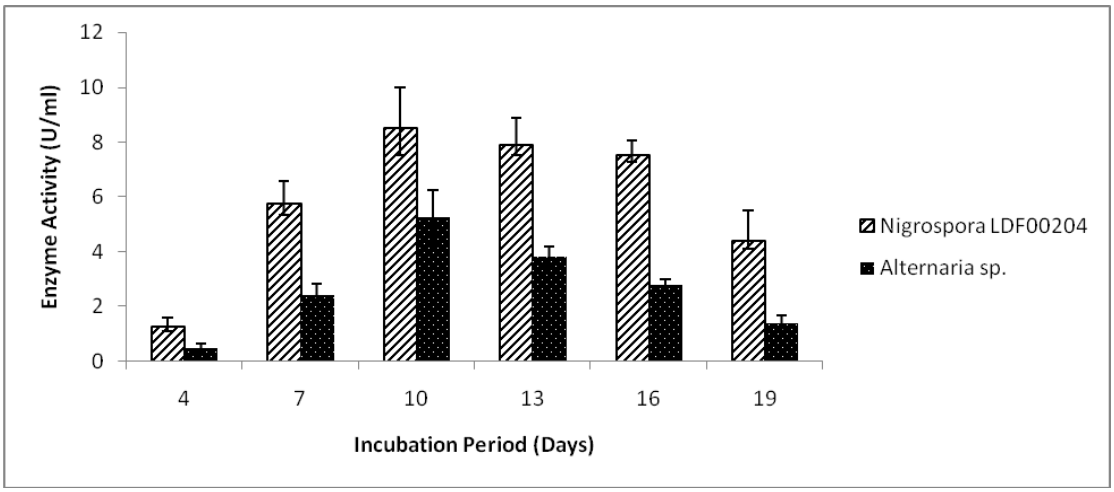


Fig. 3: MnP production by fungal strains in SSF by *Nigrospora* sp. LDF400204 and *Alternaria* sp. Error bar shows±S.E.

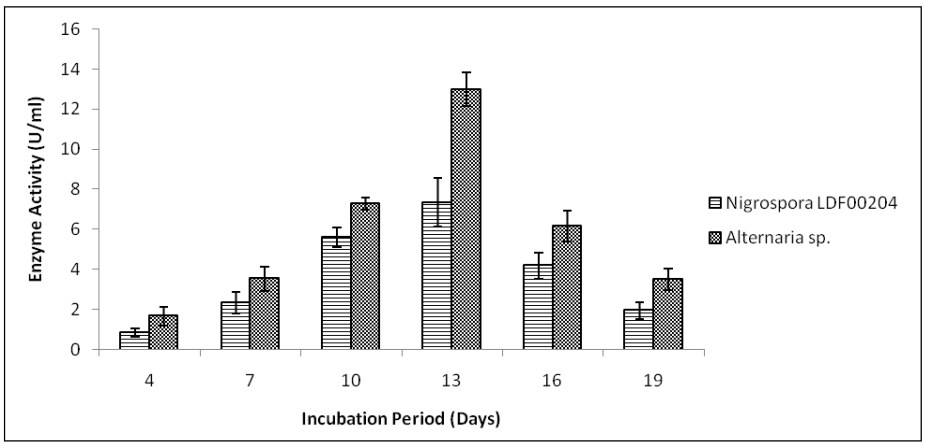


Fig. 4: LiP production by fungal strains in SSF by *Nigrospora* sp. LDF400204 and *Alternaria* sp. Error bar shows ±S.E.

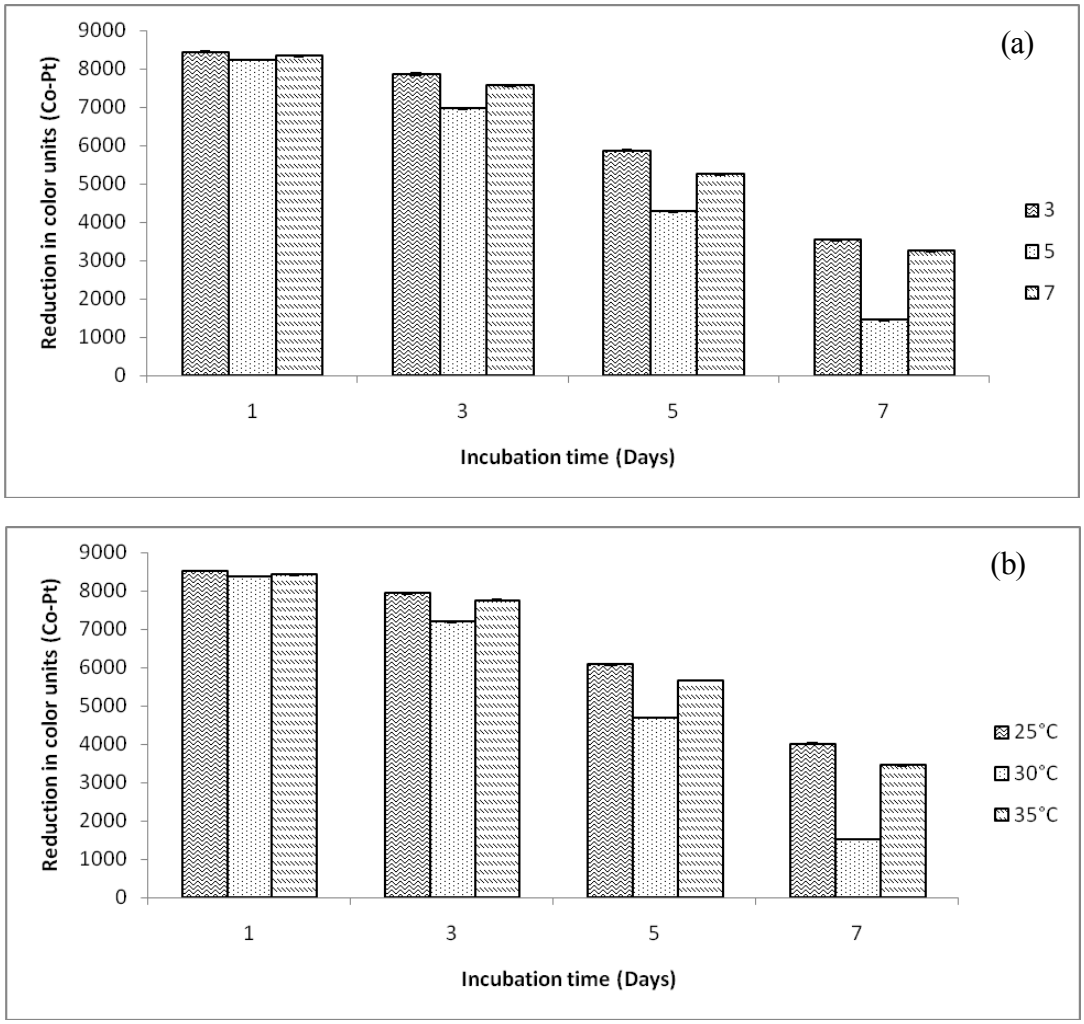


Fig. 5: (a) Effect of pH (b) Effect of temperature on decolorization of pulp and paper mill effluent by *Nigrospora* sp. LDF400204 during different time interval. Error bar shows±S.E.

sp. LDF00204 (9.12 U/mL) and *Alternaria* sp. (5.8 U/mL) (Fig. 2) on 10th and 13th day respectively and sharply decreased after respective days. *Nigrospora* sp. LDF00204 produced maximum quantity of MnP (8.5 U/mL) on 10th day followed by *Alternaria* sp. (5.25 U/mL) (Fig. 3), while, maximum LiP activity was observed in *Alternaria sp.* (12.99 U/mL), followed by *Nigrospora sp.* (7.34 U/mL) (Fig. 4) on 13th day but both (LiP and MnP) were absent in *Trametes* sp. Enzyme production among these fungi were statistically different ($P<0.05$). Respective controls showed no enzyme activities.

Decolorization study of black liquor: An experiment was conducted to assess the potential of *Nigrospora* sp. LDF00204 for decolorization of black liquor. Since high alkaline nature of black liquor hinders the growth of fungi, so it was diluted half of its concentration prior to use. Temperature and pH are two important factors which affect fungal growth, enzyme production and so the decolorization efficiency. To optimize these parameters, regimes of temperature (25, 30, 35°C) and pH (5, 7, 9) were studied. Initial colour units in control sample was recorded as 8550.66 Co-Pt. Maximum decolorization upto 1525.33 Co-Pt (82.16 %) at 30°C on and upto 1445.3 Co-Pt (83.09 %) at pH 5 was observed on 7th day of incubation (Fig. 5a and 5b), suggested optimum temperature and pH regime for growth of fungi and decolorization of black liquor, whereas, no change was observed in control set of experiments. Enzymes produced by *Nigrospora* sp. (LDF00204) were efficient to degrade lignocellulosic material in the effluent and can also be used in degradation of aromatic pollutants and recalcitrant compounds of alike nature.

DISCUSSION

In the present study, a total of eight fungal isolates exhibited positive results for ligninolytic enzyme production. These fungal strains LDF00204, LDFD4 and LDFD5 were characterized as *Nigrospora* sp, *Alternaria* sp. and *Trametes* sp. respectively. Based on the internal transcribed spacer (ITS) regions, fungal strain LDF4 was identified as *Nigrospora* sp. LDF00204 (accession no. KP732542.1).

Based on the screening of ligninolytic enzyme it was observed that the strains which were capable of producing laccase and/or peroxidase did not produce cellulase and/or xylanase or vice-versa. The difference in enzyme production depends on the fungal genetic variation and nature of substrate. However, white rot fungi efficiently produce all major ligninolytic enzymes, whereas, some produce only one or two of it (Singh & Chen 2008). The association of laccase with MnP or LiP as a combination of enzymes has been reported in fungi (Nerud et al. 1996). In the present study, combinations of laccase, cellulase and xylanase were observed in *Trametes* sp. but peroxidases activity was absent, which is well in line with findings of Pukahuta et al. (2004) and Tong et al. (2007). The enzyme production efficiency of *Nigrospora* sp. LDF00204, compared with that of *Phanerochaete chrysosporium*- most extensively studied species, showed that this fungus is equally competitive for ligninolytic enzyme production and lignin oxidation. Further, investigation on dye decolorization by *Nigrospora* sp. (Ilyas & Rehman 2013) reveals the presence of above said enzymes in it. However, few strains produced xylanase (*Trametes* sp, *Alternaria sp*, and LDFD3) but not laccase (fungal isolates LDFD3) or peroxidase (*Trametes sp*, LDFD2 and LDFD1), suggesting that phenoloxidases (peroxidase and laccase) participating in ligninocellulose degradation are not characteristic of every fungus. Moreover, culture conditions play an important role in production of peroxidases (Tien & Kirk 1988). Decreased hydrolysis zone, observed after few days of incubation, may be due to consumption of nutrients and poor growth of fungi with time. Such oscillatory nature of fungi with respect to enzyme production was reported by other investigators as well (Silva et al. 2009).

The quantitative production of major ligninolytic enzymes by SSF using lignocellulosic waste were studied in *Nigrospora* sp. LDF00204, *Trametes* sp. and *Alternaria* sp. Lignocellulosic wastes are good source of carbohydrates and inducers, promoting the growth of fungi and enzyme production. Out of the three fungal species, maximum production of Lcc was observed in *Trametes* sp., which confirms the findings of Lorenzo et al. (2002) who observed enhanced Lcc production in *Trametes versicolor* after addition of lignocellulosic residue in culture media. While other two fungal species produced less amount of Lcc which favours that Lcc not only depends on the fungal strains but also on many fermentation factors such as medium compositions, pH, aeration rate etc. (Alves da Cunha et al. 2003). MnP production was not observed in *Trametes* sp., suggests that phenoloxidases are not characteristic of every fungus while *Nigrospora* sp. LDF00204 and *Alternaria* sp. were capable of producing MnP and also laccase. It goes with the findings of Nerud et al. (1996) who reported that association of MnP and laccase is most common in white rot fungi. Maximum production of MnP was observed on the 10th day of incubation, which is well in line with the study of Vaithanomsat et al. (2010) who reported the MnP production in WRF after 8th day of incubation. Pahkala et al. (1996) reported 50 ppm of Mn in wheat straw, which acts as an inducer and enhance the production of MnP. Maximum production of LiP in *Alternaria* sp. during SSF fermentation was observed after 4th day of incubation. Similar findings of enzyme production were observed by Wu et al. (2002). Moreover, it was observed that fungal

growth and enzyme production in SSF depends on the temperature, pH, moisture, incubation time and inoculums size (Barlev & Kirk 1981).

A positive correlation between ligninolytic enzyme production and decolorizing efficiency of white rot fungi have been reported by many workers (Revankar & Lele 2006, Zhang et al. 2006). Present study on *Nigrospora* sp. LDF00204 depicts that temperature and pH play an important role in fungal biomass and enzyme production, leading to decolorization. Maximum reduction in colour of the effluent (82.16 %) was observed at 30°C, suggests maximum fungal biomass at this temperature which led to maximum enzyme activity. Similar condition for effluent treatment was observed by *P. chrysosporium* at 30°C (Sharari et al. 2011). pH 5 was found best suitable for fungal growth, enzyme activity and decolorization of black liquor (83.09 %), which supports the contention of Haddadin et al. (2002). The pH range of 4-6 for treatment of pulp and paper mill effluent has been reported by Lara et al. (2003) which further supports the study. Highest decolorization was achieved at 7th day, indicates that LiP and MnP were most active at this point. Immobilization of fungus on lignocellulosic substrate favours the enzyme activity in the treatment and it was maintained during the incubation time.

CONCLUSION

Present study manifests that the fungal strains possess ligninolytic enzyme potential and has ability to alleviate the toxic components of black liquor. SSF by wheat straw enhanced the production of enzymes. In the present study, the fungal strain *Nigrospora* sp. LDF00204 was harnessed for the production of ligninolytic enzymes and their application in treatment of black liquor which seems to be novel. Results also highlighted that growth conditions (pH, temperature and incubation period) also regulate bioremediation of recalcitrant compounds. This study can be further done for continuous removal of pollutants at reactor scale. In addition, the study suggests the potential of these fungal strains in many other biotechnological applications.

ACKNOWLEDGEMENTS

The author is thankful to Department of Science and Technology (Ministry of Science & Technology) for providing fellowship to conduct the study, and also to GBPUAT, Pantnagar for providing research facilities.

REFERENCES

Akhan, J.C. 2008. Physicochemical determination of pollutants in waste water and vegetable samples along the Jakara wastewater Channelin Kano Metropolis, Kano State, Niigeria. Europian Journal scientific Research, 23: 122-23.

Alves da Cunha, M.A., Barbosa, A.M., Giese, E.C. and Dekker, R.F.H. 2003. The effect of carbohydrate carbon sources on the production of constitutive and inducible laccases by *Botryosphaeria* sp. J. Basic Microbiol., 43: 385-392.

Bajpai, P., Mehna, A. and Bajpai, P. 1993. Decolorization of kraft bleach plant effluent with the white rot fungus *Trametes versicolor*. Process Biochem., 28(6): 377-384.

Barlev, S.S. and Kirk, T.K. 1981. Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun., 99(2): 373-378.

Bourbonnais, R. and Paice, M.G. 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett., 267: 99-102.

Couto, S.R. and Sanroman, M.A. 2005. Application of solid-state fermentation to ligninolytic enzyme production. J. Biochem. Eng., 22: 211-219.

CPCB, 2001. Comprehensive industry document for large pulp and paper industry. COINDS/36/2000-2001.

DaRe, V. and Papinutti, L. 2011. Black liquor decolorization by selected white-rot fungi. Appl. Biochem Biotechnol., 165(2): 406-415.

Dhouib, A., Hamza, M., Zouari, H., Mechichi, T., Hmidi, R., Labat, M., Martinez, M.J. and Sayadi, S. 2005. Screening for ligninolytic enzyme production by diverse fungi from Tunisia. World J. Microb. Tech., 21: 1514-1523.

Haddadin, M.S., Al-Natour, R., Al-Qsous, S. and Robinson, R.K. 2002. Biodegradation of lignin in olive pomace by freshly isolated species of basidiomycete. Bioresour. Technol., 82: 131-137.

Ilyas, S. and Rehman, A. 2013. Decolorization and detoxification of Synozol red HF-6BN azo dye by *Aspergillus niger* and *Nigrospora* sp. Iranian J. Environ. Health Sci. & Eng., 10(1): 12.

Lara, M.A., Rodrguez-Malaver, A.J., Rojas, O.J., Holmquist, O., Gonzalez, A.M., Bullon, J., Penaloza, N. and Araujo, E. 2003. Black liquor lignin biodegradation by *Trametes elegans*. Int. J. Biodeterior. Biodegrad., 52: 167-173.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. and Beckham, G.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proceedings of National Academy of Sciences, 1-6.

Lorenzo, M., Moldes, D. and Rodriguez Couto, S., and Sanroman, A. 2002. Improvement in laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. Bioresour. Technol., 82: 109-113.

Makris, S.P. and Banerjee, S. 2002. Fate of resin acids in pulp mills secondary treatment systems. Water Research, 36: 2878-82.

Nerud, F. and Misurcova, Z. 1996. Distribution of ligninolytic enzymes in selected white rot fungi. Folia Microbiol., 41: 264-266.

Owens, J.W., Swanson, S.M. and Birkholz, D. 1994. Environmental monitoring of bleached kraft pulp mill chlorophenolic compounds in a Northern Canadian River System. Chemosphere, 29(1): 89-109.

Pahkala, K., Mela, T., Hakkola, H., Järvi, A. and Virkajärvi, P. 1996. Production and use of agrofibre in Finland. Final report of the study, Part I. Production of agrofibre crops: Agronomy and varieties. Agricultural Research Centre of Finland, Jokioinen, Finland, pp. 84.

Pant, D. and Adholeya, A. 2006. Isolation and screening of potential fungi for decolorization of distillery wastewaters. In: A. Mendez-Vilas (Ed.), Modern Multidisciplinary Applied Microbiology Exploiting Microbes and their Interactions, Weinheim: Wiley. pp. 95-102.

Pawle, G. and Singh, S.K. 2014. Antimicrobial, antioxidant activity and phytochemical analysis of an endophytic species of *Nigrospora* isolated from living fossil *Ginkgo biloba*. Curr. Res. Environ. & Appl. Mycol., 4(1): 1-9.

Pointing, S.B. 1999. Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Divers., 2: 17-33.

Pukahuta, C., Suwanarit, P., Shinagawa, E., Hoshida, H. and Nishizawa, Y. 2004. Combination of laccase, xylanase, and cellulase in lignocellulose degradation by white rot fungi, *Lentinus polychrous* Lev. and L. squarrosulus Mont. Kasetsart Journal (Natural Science), 38: 65-73.

Raghukumar, C. and Rivonkar, G. 2001. Decolorization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat. Appl. Microbiol. Biotechnol., 55: 510-514.

Revankar, M.S. and Lele, S.S. 2006. Enhanced production of laccase using a new isolate of white rot fungus WR-1. Process Biochem., 41: 581-588.

Sharari, M., Jahan Latibari, A., Guillet, A., Aurousseau, M., Mouhamadou, B., Rafeiee, G., Mirshokraei, A. and Parsapaghouh, D. 2011. Application of the white rot fungus *Phanerochaete chrysosporium* in biotreatment of bagasse effluent. Biodegradation, 22: 421-430.

Silva, I.S., Grossman, M. and Durrant, L.R. 2009. Degradation of polycyclic aromatic hydrocarbons (2-7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. Int. Biodeterior. Biodegrad., 63: 224-229.

Singh, D. and Chen, S. 2008. The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes. Appl. Microbiol. Biotechnol., 81: 399-417.

Singhania, R.R., Patel, A.K., Soccol, C.R. and Pandey, A. 2009. Recent advances in solid-state fermentation. Biochem. J. Biochem. Eng., 44: 13-18.

Tien, M. and Kirk, T.K. 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proceedings of the National Academy of Sciences USA, 81(8): 2280-2284.

Tien, M. and Kirk, T.K. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol., 161: 238-248.

Tong, P., Hong, Y., Xiao, Y., Zhang, M., Tu, X. and Cui, T. 2007. High production of laccase by a new basidiomycete, *Trametes* sp. Biotechnol. Lett., 29: 295-301.

Vaithanomsat, P., Apiwatanapiwat, W., Petchoy, O. and Chedchant, J. 2010. Production of ligninolytic enzymes by white-rot fungus *Datronia* sp. KAPI0039 and their application for reactive dye removal. Int. J. Chem. Eng., 162(504): 1-6.

Wariishi, H., Valli, K. and Gold, M. 1992. Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: Kinetic mechanism and role of chelators. J. Biol. Chem., 267: 23688-23695.

WHO 2007. Dioxins and their effects on human health. Resource document. World health organization. http://www.who.int/mediacentre/factsheets/fs225/en/.

Wu, J., Xiao, Y.Z. and Wang, Y.P. 2002. Treatment of pulp mill wastewaters by white rot fungi. J. Biol., 19: 17-19.

Wu, J., Xiao, Y.Z. and Yu, H.Q. 2005. Degradation of lignin in pulp mill wastewaters by white rot fungi on biofilm. Bioresour. Technol., 96: 1357-1363.

Zhang, M., Wu, F., Wei, Z.Y., Xiao, Y.Z. and Gong, W.M. 2006. Characterization and decolorization ability of a laccase from *Panus radis*. Enz. Microb. Technol., 39: 92-97.