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Phialophora Melinii (NFCCI 3617): A Newly Isolated Psychrotolerant Fungus That Produces Enhanced Laccase Under the Influence of Organic Solvents

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Abstract

A psychrotolerant fungus, isolated from decomposing pine needle debris, is investigated for laccase production under the influence of 5 organic solvents. The fungus was identified as *Phialophora melinii* and was able to grow between 4 to 35 °C (opt. 25 °C) and 2-14 pH (opt. 5-7). In quantitative estimations that were carried out at optimum growth temperature and pH, the fungal laccase was estimated to be 21.0 ± 4.0 U/L. Native PAGE study revealed 35 kDa molecular mass of the fungal laccase. Supplementation of organic solvents namely, methanol, ethanol, acetone, n-propanol and iso-propanol in varying concentrations (0.5%-2.0%, separately), significantly affected the production of fungal laccase. Out of 5 solvents used, n-propanol was found to be the most efficient enhancer of laccase production. n-Propanol (0.5%) resulted in maximum enhancement (7 folds) in laccase production at 18th day of incubation. Methanol, iso-propanol and ethanol were able to enhance laccase production up to 5-6 folds in comparison to control with respect to the varying concentration and incubation length. Age of the fungal culture (incubation days) was observed as an important factor for laccase production. Use of low molecular compounds in enhancing the fungal laccase production may be considered as an eco-friendly approach.

Key words: *Phialophora;* Biodegradation; Laccase; Organic Solvents; Indian Himalayan Region

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INTRODUCTION

Laccases are the member of oxido-reductase group of enzymes with a wide variety of applications. They can oxidize several aromatic compounds by using one electron mechanism that further leads to the formation of water from molecular oxygen. Laccases are widely studied in fungi and plants, although they are also reported from bacteria and insects. Fungi from basidiomycota, ascomycota and deuteromycota phyla are the source of fungal laccases, however, basidiomycota are the best studied ones. Ascomycota and deuteromycota are also emerging as source of laccases from various ecological niches. Laccases are known for their role in the physiology of the fungi, their applications in industries such as textile, food, pharmaceuticals and synthetic chemistry, and as an eco-friendly approach with respect to the concept of "green chemistry" (Rodgers et al., 2010; Jeon et al., 2012; Rivera-Hoyos et al., 2013).

Laccases from the fungal sources have been widely studied with respect to biodegradation, lignin degradation in particular (Novotny et al., 2004; Strong & Claus, 2011). Temperature is one of the major governing factors in all the ecological processes. In low temperature environments, such as mountain ecosystem in Indian Himalayan Region (IHR), degradation is a slow process. Depending on the forest types, such as pine forest, degradation is further slow due to the recalcitrant nature of lignin polymer found in pine needles that persists for long

term and affects the biological diversity of soil (Rizvi & Rizvi, 1992). The psychrotolerant microbial communities, inhabiting these environments take the responsibility of various biological processes, including biodegradation (Mishra et al., 2012; Singh et al., 2012; Dhakar & Pandey, 2013; Yarzábal, 2014). The psychrotolerant fungi produce cold tolerant enzymes that may be relatively in smaller amounts but, at the same time, with the activity for longer periods (Dhakar et al., 2014a).

In view of the applications of laccases a variety of supplements have been used as enhancers of laccase production (Kunamneni et al., 2007; Bertrand et al., 2012). Antibiotics, vitamins, amino acids and several aromatic compounds have been reported to affect the laccase production (Dhawan and Kuhad, 2002; Dhakar et al., 2014a). Among aromatic compounds, guaiacol, 2,5- xylidine and 2,6-dimethoxyphenol have been reported as inducers while compounds such as TNT (2, 4, 6-trinitrotoluene) and 1, 4-hydroquinone have been found to act variably (enhancer or inhibitor) on laccase production from *Cerena unicolor*, *Ganoderma lucidum* and *Trametes versicolor* (Bettin et al., 2014). This indicates towards the importance of screening of supplements for their role in laccase production from specific sources. The aim of the present study is the characterization of a cold and pH tolerant strain of *Phialophora melinii* isolated from degrading pine needles with respect to its laccase production potential under influence of low molecular weight compounds.

1. MATERIALS AND METHODS

1.1 Site Description

Soil samples with degrading needles were collected from the pine forest (29°37'56"N 79°20'16"E, 2,480 m amsl, Soni-Binsar, Dist. Almora, Uttarakhand, India). The site experiences heavy rainfall during monsoon and occasional snowfall during winter season. The soil pH was 5.1 ± 0.5.

1.2 The Fungus

The fungus was isolated following the standard method (serial dilution). Morphological and physiological characterization was done on Potato Dextrose (PD) medium at 25ºC. Microscopic observations were recorded at 100 X (eye piece) using lacto-phenol cotton blue stain. Species level identification of the fungus was provided by National Fungal Culture Collection of India, Agharkar Research Institute, Pune. The fungus has been assigned the Accession no. NFCCI 3617. The temperature and pH tolerance was performed on PD between 4 to 35ºC and 1.0 to 14.0, respectively.

1.3 Laccase Production

The fungal culture was grown in modified Kirk & Farrell (1987) medium supplemented with ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) as described in Dhakar and Pandey (2013). The ABTS plate assays were performed at 4, 9, 14, 25 and 35ºC. Ligninolytic efficiency was calculated by zone diameter (mm)/ colony diameter (mm) * 100. Quantitative estimation of laccase activity was done by ABTS assay at 420 nm (Han et al., 2005) using spectrophotometric method. Reaction mixture contained crude enzyme, citrate phosphate buffer (pH 4.5) and ABTS (2 mM), it was incubated at room temperature for 2 min and absorbance was recorded at 420 nm. Enzyme activity was defined as 1 µM of ABTS oxidized per min.

1.4 Molecular Mass of laccase

Molecular mass of laccase was determined by performing native PAGE. Chilled acetone was added to crude enzyme and kept at -20ºC for overnight. Then it was centrifuged at 8,000 rpm for 20 min at 4ºC. Pellets were re-dissolved in the buffer (pH 4.0±0.5) and supernatant was discarded. The polyacrylamide gel constituted of separating (12.5%) and stacking gel (4.0%) according to Laemmli (1970) without SDS. The gel was incubated at room temperature for 30 min in the citrate-phosphate buffer of pH 4.0±0.5 following electrophoresis. It was then transferred to the ABTS solution (0.5%) in the same buffer and incubated at room temperature for 1 h. Green bands appeared by oxidation of ATBS through laccase.

1.5 Influence of Low Molecular Weight Organic Solvents on Laccase Production

Production of laccase was investigated under the influence of low molecular weight organic solvents. Laccase production medium was prepared by taking 50 ml in 250 ml Erlenmeyer flasks with pH 5.5±0.5.5 mm discs (1 per flask) of six days old culture were used for inoculation that was grown on PDA at 25ºC. Inoculated flasks were incubated at 25ºC. Five organic solvents (methanol, ethanol, acetone, n-propanol and iso-propanol) were supplemented between 0.5% to 2.0% at 4th day of inoculation, separately. These flasks were incubated at 25ºC in static conditions. Laccase activity was determined at every 6th day of inoculation up to 30th day. Culture broth was filtered with Whatman no. 1 filter paper and used as crude enzyme for further estimations (ABTS assays).

1.6 Statistical Analysis

All the experiments were done in triplicates. One way ANOVA with post hoc Tukey's test was used to analyse the results. The significance level was 5% ($p < .05$). Error bars show standard deviation in the graphs.

2. RESULTS

2.1 Morphology, Growth Characters and Ligninolytic Efficiency of the Fungus

The fungus produced white spiky colony on PD agar at 25ºC following 5 days of incubation. The microscopic examination revealed the development of septate

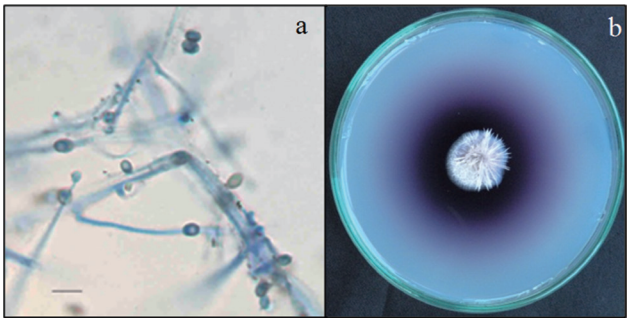

Figure 1
Microscopy (a) & ABTS plate assay (b) of the Psychrotolerant Fungus *Phialophora Melinii* (Bar= 5µm)

mycelium with single, oval shaped spores (2-3µm). The fungus was able to grow between 4 to 35ºC (optimum growth temperature 25ºC) and 2 to 14 pH (optimum 5-7). Based on colony morphology and microscopic features, the fungus was identified as *Phialophora melinii*. The fungus produced green zone around the colony on ABTS plate at 25ºC on day 6 of incubation (Figure 1a and b). The ligninolytic efficiency was determined maximum at 4ºC that decreased up to 25ºC and again increased at 35ºC (Figure 2).

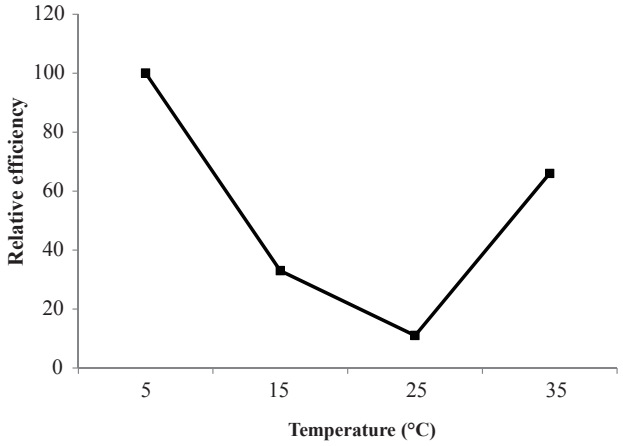

Figure 2
Qualitative Estimation of Ligninolytic Efficiency of *Phialophora Melinii* With Respect to the Biomass Production at Different Temperatures

2.2 Laccase Production and Its Molecular Mass

The fungus produced laccase throughout its growth temperature in Kirk & Farrell (1987) medium, showing preference for low temperature. The optimum production of laccase was recorded at 25ºC and pH 5, coinciding with the optimal growth requirements. The molecular mass of the laccase was determined to be 35 kDa (Figure 3). Besides, a light band of 100 kDa was also observed that may be attributed to the production of isozyme or some other related lignin degrading enzyme.

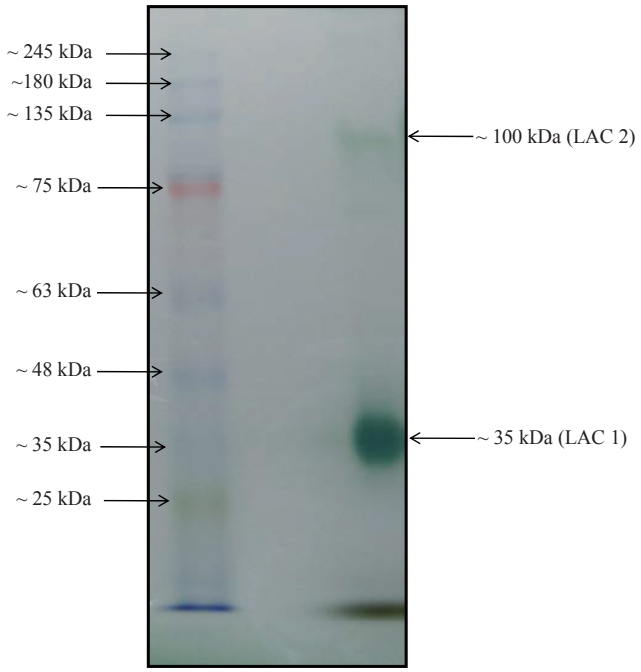

Figure 3
Molecular Weight Analysis of Laccase Produced by *Phialophora Melinii* on Native PAGE. Activity Staining Was Done Using ABTS. Lane 1: Protein Marker; Lane 2: Laccase Iso-Enzymes (LAC1&LAC2)

2.3 Influence of Low Molecular Weight Organic Solvents on Laccase Production

Supplementation of low molecular weight organic solvents in the medium resulted in varying effects on laccase production. Further, the concentration of the solvents and the incubation time also influenced the production. Out of 5 solvents, n-propanol (0.5%) was recorded as the best enhancer. It enhanced laccase production up to 7 folds (147.1 ± 19.3 UL^{-1}) at 18th day of incubation, in comparison to control. Increased concentration of n-propanol (1.0 and 1.5 %) resulted in enhancing effect, varying with the incubation period being highest (6 and 4 folds), respectively, at 24th day of incubation. The lowest enhancement was recorded with the highest concentration (2.0%) of n-propanol. It was interesting to note that in all the concentrations under consideration, the highest effect of n-propanol was recorded between 18th and 24th day of incubation (Figure 4). The stimulating effect of n-propanol appears to be expressed at transcriptional level during a particular period.

Methanol (1%), the second potent inducer, was recorded to be effective throughout the incubation period (up to 35th day). The enhancement ranged from 1.5 (27.1±7.2 UL^{-1}) to 6.0 folds (128.3 ±14.8 UL^{-1}) at different days of incubation. Similar to n-propanol influence, further increase in concentration of methanol reduced the enhancing effect. The effect of methanol was recorded to be on higher side in the early incubation period (12th

and 18th day) (Figure 5). Although n-propanol produced higher laccase in comparison to methanol in later stage of incubation, enhancement effect of methanol was expressed during early incubation.

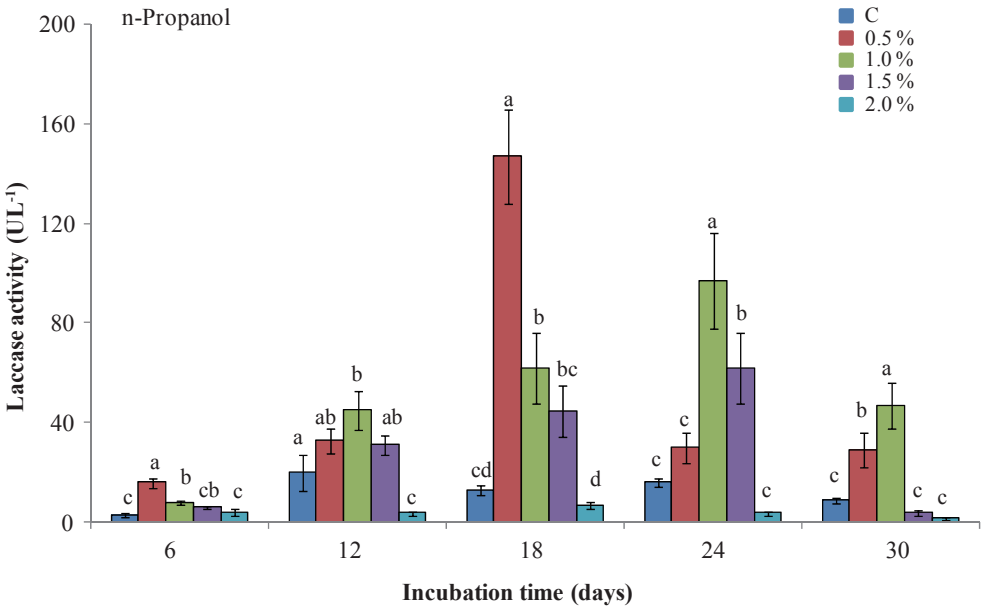

Figure 4
Influence of N-Propanol on Laccase Activity at Different Days of Incubation. Bars With Different Letters Present Significant Difference (*P*<.05) With Respective Incubation Time

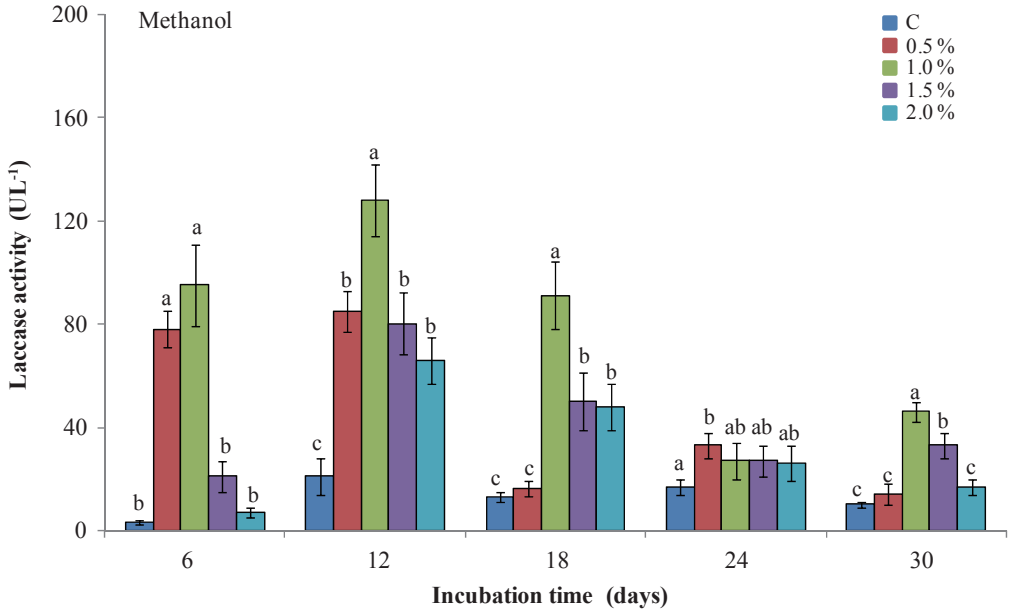

Figure 5
Influence of Methnaol on Laccase Activity at Different Days of Incubation. Bars With Different Letters Present Significant Difference (*P*<.05) With Respecaive Incubation Tioe

The enhancement in laccase production with rest three solvents, iso-propanol, ethanol and acetone, was relatively on lower side. It was recorded maximum 112.3±9.2 UL^{-1} (1.5 % at 18th day), 115.0 ± 23.2 UL^{-1} (1.5% at 18th day), and 57.4 ± 8.7 UL^{-1} (0.5% at 12th day), respectively, with isopropanol, ethanol and acetone. The enhancing effect of iso-propanol was on higher side in middle of the incubation period (12-18th days) with 0.5%-2.0% concentration. Lower concentration (1.0%) was effective in early period of incubation (6th day). A distinct pattern was recorded in case of ethanol where the low concentration (0.5%-1.0%) was more effective during

early incubation (6^{th} and 12^{th} day). Acetone, the weakest enhancer, resulted in 4 and 3 folds increase with 0.5 and 1.5% (57.1 ± 4.2 UL^{-1} and 33.2 ± 6.1 UL^{-1}) at 12^{th} and 18^{th} day of incubation, respectively. In the later period (24^{th} to 30^{th} day) acetone acted as an inhibitor and suppressed laccase production in comparison to control.

DISCUSSION

Soil is the key component of the forest ecosystem. It inhabits the microbial communities responsible for various functional activities including biodegradation. The soil fungal community, in particular, plays important role in the degradation and biogeochemical cycle of carbon especially (Dighton et al., 2005). In low temperature environments, such as that prevails under mountain ecosystem of IHR, the degradation is likely to be a slow process and governed by psychrotolerant microbes. Soil microbial diversity with respect to biological processes including plant growth promotion, biocontrol and biodegradation from IHR is increasingly getting attention from both basic and applied importance (Pandey et al., 2001b; Bisht et al., 2003; Trivedi et al., 2007; Trivedi & Pandey, 2008; Rinu & Pandey 2010, 2011; Sati et al., 2013; Dhakar et al., 2014b). The peculiar feature of these studies is the involvement of ascomycetous fungi (Dhakar et al., 2014a) in a major role along the basidiomycetes (Dhakar & Pandey, 2013) that are otherwise better known for lignin degrading activities. The fungus *Phialophora melinii*, isolated from degrading pine needles, has been demonstrated for its potential in biodegradation. Higher ligninolytic efficiency of the fungus at suboptimal temperature was an indicative of the triggering of secondary metabolites under stress conditions. Such characters are relevant as means of survival strategies possessed by these organisms under low temperature environments. The psychrotolerant fungi, ascomycetes in particular, that have shown their dominance in low temperature environments of IHR (Pandey et al., 2001a; Pandey & Palni, 2007; Dhakar et al., 2014b) suggest the need for focused research along these lines.

The molecular mass of laccase has been reported between 50 to 140 kDa (Rivera-Hoyos et al., 2013). In a recent study, laccases from *Streptomyces* spp. have been reported with molecular mass between 38 to 114 kDa (Lu et al., 2013). The molecular mass of the laccase in the present study is on further lower side. The presence of isoforms may further facilitate to the fungal activity. Detection of laccases in wider range of molecular mass along with their iso-forms is likely to be an important aspect for molecular investigations relevant to diversity of laccases. Though a huge literature is available on the regulation of laccase, there seems to be a need to understand the role of the iso-forms with respect to the laccase producers.

The production of laccase has been found to be affected by a number of nutritional and supplemental factors apart from the physical factors such as temperature and pH. Most of the studies have involved carbon and nitrogen sources as enhancers of laccase production. In view of the recent "green chemistry" approach, involvement of simple low molecular and eco-friendly compounds as enhancers of laccase will be a suitable option. The low molecular weight organic solvents can affect laccase expression by involving at response element level or by creating other physiological conditions such as oxidative stress (Bertrand et al., 2013). The enhancement efficiency of low molecular compounds may be attributed to their structural similarities to various lignin forms. They can contribute to the improved expression of the enzyme.

In the present study, n-propanol was recorded as the best enhancer followed by methanol. Isopropanol, ethanol and acetone were also recorded as enhancers but with lower efficiency. Although, ethanol has been reported as the potent enhancer of laccase production (Lee et al., 1999; Lomascolo et al., 2003; Alves et al., 2004), in general, there are many studies suggesting role of enhancers to be fungal specific. Acetone has been reported to enhance laccase production from *Trametes hirsuta* (Dhakar & Pandey, 2013) and isopropanol from *Rhizoctonia solani* (Crowe & Olsson, 2001). Besides response of the individual fungus, age of the culture (incubation length) and the concentration of supplement are other important factors in influencing the enzyme production. Ethanol with $CuSO_4$ suppressed laccase production from *Pleurotus sajor-caju* in the early stage of incubation (5-9 days) but enhanced the enzyme production at later period (Bettin et al., 2014). The response of same compound can be observed as inhibitory or stimulatory and in early or later phase of incubation for the production of laccase, depending on the organism and its metabolic stages. The present study demonstrates a versatile case with respect to the screening and selection of promising organic solvents.

CONCLUSION

The extremophiles are known as a potential source of several bioactive compounds and novel products. The cold adaptive microorganisms have the capability to perform wide variety of activities as they are the principal component of nutrient cycles in the respective environment. Biodegradation is one of the major ecological processes and regulated by the psychrotolerant microorganisms under the low temperature environments. The degradation can be accelerated by using eco-friendly approaches to input higher C in the cycle. Specific study sites, such as pine forest, are likely to provide biodegrading organisms that although may be with lower activity but for prolonged periods important to fulfill the

requirements of low temperature ecosystems. The isolation of organisms from these sites will be of ecological as well as biotechnological relevance. The production of laccase might be associated with the stress of the temperature that induces its response elements and stimulates the expression of the enzyme. To facilitate the carbon flux from the carbon containing biomass, it is necessary to increase the production of the responsible enzyme. Use of eco-friendly low molecular weight compounds in laccase production will support the bioprospecting of psychrotolerants in biotechnologcal applications.

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