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Extraction of Manganese Peroxidase From Mangrove Wood Degrading Fungi

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ABSTRACT

Total seven fungal isolates were isolated from a degraded mangrove wood collected from the local coastal area. These isolates were screened for manganese peroxidase enzyme. Isolate MWF2 showed the production of MnP enzyme. The phylogenetic neighbors of the MnP producing isolate were identified using the partial gene sequence of the amplified ITS region.

KEYWORDS Lignin, degradation, Mangroves, enzyme, ITS, MnP

INTRODUCTION

Lignin is a structurally complex, important component of the plant tissues that provides rigidity and resistance from the microbial attack. However, there are a few microorganisms which are known to possess enzymes such as laccase, manganese peroxidase and lignin peroxidase that can efficiently degrade and mineralize lignin (Ahmad *et al*., 2010).

Isolating and studying various microorganisms which can degrade lignin is becoming important in recent years as these enzymes find applications in paper-pulp and various other industries (Perez *et al*., 2002).

Manganese peroxidase (MnP) is the most common lignin-modifying peroxidase produced by almost all wood-colonizing fungi and a few bacteria (Hofrichter, 2002). A perusal of literature indicates that there is dearth of information available on studying MnP isolated from wood degrading fungi in India. Therefore, the present study was undertaken.

MATERIALS AND METHODS

Isolation of fungi

The fungi were isolated from degraded mangrove wood collected from Devgad area of Sindhudurg district using a simple technique i.e. washing the sample 2-3 times in sterile sea water. It was then cut into small pieces. Four small pieces of the wood were placed in a petriplate and then the plates were poured with suitable Potato Dextrose Agar (PDA). This PDA was prepared in 75:25 distilled water with Benzyl penicillin salt to inhibit the growth of bacteria and incubated at 30±1°C for a week. Cultures subtending in the medium were sub-cultured repeatedly to obtain pure cultures. There were seven fungal isolates obtained from this source.

Qualitative assay for Manganese peroxidase enzyme

All the seven fungal isolates were screened for MnP by using agar plate method on a Guaiacol supplemented agar, which contained glucose 1 g, peptone 0.2 g, yeast extract 0.1 g, agar 1.8 g and 0.4 mM guaiacol in 100 ml distilled water.

Manganese peroxidase Assay

The fungal isolate that showed positive results for MnP production was subjected to fermentation, microfiltration and concentration by Tangential Flow Filtration (TFF). The crude enzyme was concentrated to 10 folds. This concentrated enzyme was used for quantitative assay by following the protocol described by Atalla *et al*., (2010) using guaiacol as a substrate. One unit of MnP activity was defined as activity of an enzyme that catalyzes the conversion of 1µM of guaiacol per minute.

Temperature stability of the enzyme

To analyze the temperature stability, the concentrated MnP enzyme sample was incubated at different test temperatures for 60 minutes. The test temperatures used in the present experiment were 30˚C, 40˚C, 50˚C., 60˚C and 70˚C respectively. At these tank points, the enzyme sample was taken and assayed for MnP activity. The enzyme activity was determined spectrophotometrically. .

Identification of enzyme producing fungal isolate

The enzyme producing fungal isolate was identified by using Internal Transcribed Spacer (ITS) gene sequences. DNA Extraction was carried out using Uniflex DNA isolation Kit (GeNei, 612117000051730) using the standard protocol. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -20˚C till further use.

The DNA isolated from the fungal sample was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer, 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 53°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used were procured from GeNei.

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100µl of PCR-A buffer was added to the 25µl of reaction. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product ITS1 - 5' TCCGTAGGTGAACCTGCGG 3' sequencing primer was used.

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI).

RESULTS

Qualitative assay for Manganese peroxidase enzyme

Out of the seven fungal strains, only one strain MWF2 showed positive results in qualitative screening. There was a halo of brown colour formed under and around the fungal strain (Positive for guaiacol oxidation), indicating the presence of Manganese peroxidase enzyme.

Manganese peroxidase Quantitative Assay

As found in the qualitative assay, the MnP producer fungal

strain MWF2 was fermented and concentrated. MnP enzyme it produced was quantified as 0.82 U/ml.

Temperature stability of the enzyme

The MnP enzyme obtained from MWF2 fungal strain was found to be stable at 50˚C (Fig. 1).

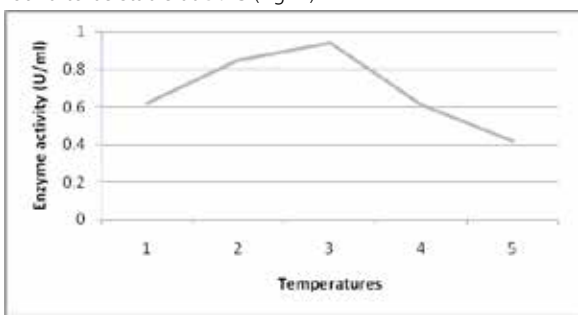

Figure 1: Effect of temperature on MnP enzyme activity (X axis: 1= 30˚C, 2= 40˚C, 3= 50˚C., 4= 60˚C., and 5= 70˚C.)

Identification of the fungal isolate using ITS gene sequence

The partial gene sequence of the amplified ITS gene of the extracted DNA from the isolate MWF2 produced a 425 bp product (Fig.2).

Figure 2: Partial gene sequence of 425 bp of the fungal isolate MWF2

This amplified sequence of the ITS gene when analysed using BLASTn tool indicated that the isolate was definitely a fungus as it showed 95% sequence similarity with other fungi of the genera *Aspergillus* and *Eurotiomycetes* which were present in the existing nucleotide database (Table 1).

Table 1: Phylogenetic neighbours of the isolate MWF2 based on partial ITS gene sequence

Description	**Ident**.
Aspergillus niger strain ATCC 16888 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95%
Eurotiomycetes sp. DC483 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95%
Eurotiomycetes sp. DC482 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95%
Aspergillus niger voucher MSR3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	95%

DISCUSSION

The results of the present study indicate that less explored areas like mangrove ecosystem could act as a hot spot to find novel microbes having the ability to produce bioactive compounds and secondary metabolites such as enzymes which can have industrial applications. Results are in agreement with those by Abraham (2016) who recently reported extraction of lignin peroxidase from a degrading wood collected from Ratnagiri, Maharashtra suggesting that this mangrove patch of Konkan needs to be further explored.

Manganese peroxidase (MnP) is a glycosylated haeme-containing enzyme and has been found to have important applications beides biodegradation of lignin, in the biodegradation of polycyclic aromatic hydrocarbons (PAH), humic acids , synthetic dyes, and polychlorinated biphenyls (PCB) (Oliveira *et al*., 2009). Although there many organisms which produce MnP, fungi are known to be the best MnP producers with maximum enzyme activity. So identification of novel fungal isolates having lignin oxidizing enzymes could be significantly important for industrial and environmental point of view. So findings of the present study are noteworthy.

In the present study, the extracted enzyme was found to have an activity of 0.82 IU. Though this is a promising activity but needs further purification as it is lower than the industrial requirements. So studies are ongoing to increase the activity using inducers such as calnexin.

In the present study, the extracted enzyme was found to be stable up to temperature of 70°C with an optimum temperature at 50°C. Report of such thermostable enzyme is quite important as previous reports indicate that MnP is generally stable only up to 40°C (Kanayama *et al*., 2002; Oliveira *et al*., 2009).

CONCLUSION

From the present investigation, we can conclude that mangrove ecosystem can provide novel microorganisms of industrial importance. We can also conclude that molecular marker like ITS gene sequence can be effectively used for the deducing the phylogenetic neighbors of the unknown microbes and create an evolutionary tree.

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