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Isolation, Screening and Morphological characterization of Laccase producing fungi

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Abstract

Research was carried out on the production of extracellular laccase enzyme by fungi isolated from different lignocellulolytic waste soils within the Bhilai-Durg region of Chhattisgarh, India. Soil samples were collected from lignocellulolytic waste such as flower, citrus peel and paddy straw soil and immediately transported to laboratory for analysis. Fungal colony was isolated by the serial dilution method on potato dextrose agar. A total of 49 fungal cultures were isolated from the soil samples. Isolated fungal culture were screened for production of laccase enzyme in guaiacol containing potato dextrose agar plate. Laccase producing fungi was morphological identified by Agharkar Research Institute in Pune. The fungal isolates were identified as Scytalidium lignicola, Humicola fuscoatra, Fusarium sp., Paecilomyces sp., Curvularia sp., Fusarium sp., Aspergillus niger, Chaetomium sp., Penicillium sp. And Histoplasma capsulatum

1. Introduction

Laccases belong to blue oxidase group. They used molecular oxygen for the oxidation of various phenolic and non-phenolic compounds by the mechanism of radical-catalyzed reaction. The general oxidation by laccase takes place by three types: the addition of oxygen to the substrate; the removal of hydrogen from the substrate; and the removal of electrons from the substrate. Fungal groups from the ascomycetes, deuteromycetes and basidiomycetes are the good laccase producers. White rot fungi also produces a valuable amount of laccase. Chaetomium thermophilum, Trametes versicolor and Pleurotus eryngii are great producers of laccase (Andlar et al. Mousa). Laccase enzyme has been found in plants such as lacquer, asparagus, mango, beet, peach, apples, prune, pears, mung bean, potatoes, pine, cabbage, sycamore and a variety of vegetables. It is broadly distributed in fungi, higher plants, bacteria and insects. Deuteromycetes, Ascomycetes, Basidiomycetes and many white rot fungi contain laccase. Over 60 fungal strains of phyla especially Basidiomycota, Ascomycota and Zygomycota show laccase activities (Loi et al. Ayla, Golla, and Pallipati). Laccase was found in the plant roots associated with bacterium including Bacillus subtilis, Azospirillum lipoferum, Escherichia coli, Bordetella compestris, Yersinia pestis, Pseudomonas syringae etc. (Sharma and Leung).

2. Materials and Methods

This study was focused on examining the fungi as a potential source for laccase enzyme production. The investigation included collection of lignocellulolytic waste soil samples; isolation of fungi; qualitative

and quantitative screening of the fungal isolates for laccase production and morphological identification of fungal isolates.

2.1. Collection of lignocellulolytic soil samples

Six soil samples were randomly collected from the 3 different places in the Bhilai-Durg region of Chhattisgarh, India. About a 5-10 cm deep layer of soil was collected in the sterile plastic bag and brought to the laboratory for further studies.

2.2. Isolation of fungi

The collected soil samples were dried at room temperature for seven days to remove moisture before being ground into powder with a Mortar and Pestle (Pulverizer). For isolation of fungi soil samples were serially diluted and made into a series of serial dilutions up to 10^{-6} dilutions. From the dilution 0.1 ml volumes were pipetted and spread on the isolation medium potato dextrose agar supplemented with streptomycin (50μg/ml) (for inhibition of bacterial contamination). The plates were incubated at 28°C for six days. After six days of incubation, a number of fungal colonies were observed on isolation plates. Colonies were sorted, picked up and transferred to an agar slant. The tubes were incubated at 28°C for six days and later preserved at 4°C.

2.3. Screening for potent fungi

Qualitative screening was performed by the guaiacol plate assay method. Fungal isolates were point inoculated over the respective guaiacol (0.02%) containing potato dextrose agar plate and in incubated at 28°C for six days. After incubation laccase enzyme producing fungi were selected for quantitative screening. In the quantitative screening procedure, positive laccase producing fungal isolates were picked up from the previous qualitative screening step and inoculated into the potato dextrose broth medium for quantitative screening of potent laccase producing fungi. 50 ml of potato dextrose broth were autoclaved at 121°C for 15 minutes in a 250ml Erlenmeyer flask. After autoclaving flask were inoculated with positive laccase producing fungi. Inoculated flasks were incubated at 28°C for six days. After incubation crude enzyme was filtered by using Whatman No. 1 filter paper. For quantitative estimation of enzyme, a reaction mixture (5ml; 3ml sodium acetate buffer, 1ml of 2mM guaiacol and 1ml fungal culture filtrate) was made. The reaction mixture was incubated at 30°C for 15 minutes. After incubation of reaction mixture the absorbance was read by using UV-vis spectrophotometer (BioEra's-752) at 470 nm.

The laccase enzyme activity in U/ml was calculated by:

$$Volume \quad activity \quad (U/ml) = \frac{\triangle A470nm/min \times 4 \times Vt \times dilution\ factor}{€ \times Vs} \quad (1)$$

Where, 4= derived from unit definition & principle,

Vt = final volume of reaction mixture = 5.0 ml,

€ = extinction co-efficient of Guaiacol = 6,740/M/cm and

Vs = sample volume = 1 ml

2.4. Morphological identification of isolated fungi

The fungal isolates that produced the maximum laccase enzyme were identified through morphological characterization at the Agharkar Research Institute, Pune, India.

3. Results

3.1. Isolation of fungi

Isolation of fungal cultures was done using the Warcup soil plate method (WARCUP). A total of 49 fungi were isolated from soil samples i.e., Site A (12), Site B (20) and Site C (17). The isolated fungal colonies were later transferred to the potato dextrose agar slants.

3.2. Qualitative and quantitative screening

Qualitative screening was done by the assay plate method on a potato dextrose agar plate containing guaiacol. A reddish-brown color halo appeared in surrounding of the culture inoculated in the agar plate. This color indicated a positive laccase reaction by the isolated fungus. The result was recorded in cm using a measuring scale. Out of 49 culture isolates from the soil samples 10 cultures showed growth on potato dextrose agar plate and showed positive laccase reactions. Three culture isolates FWD (1.3 cm), FWJ (2.0 cm) and FWN (1.6 cm) from Site A; four culture isolates Peel B (2.0 cm), Peel E (1.0 cm), Peel J (0.8 cm) and Peel S (0.6 cm) from Site B and three culture isolates PSA (2.4 cm), PSO (1.3) and PSP (2.8 cm) from Site C showed positive reaction for laccase production. After qualitative screening, all the positive isolated cultures were assessed quantitatively using potato dextrose broth for laccase production at 470 nm in a UV-

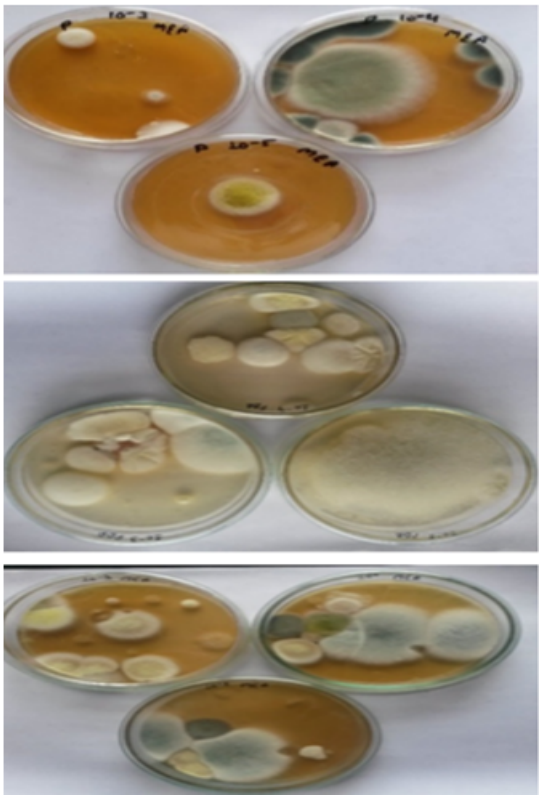

FIGURE 1. Fungal cultures isolated from lignocellulolytic soil samples from different sites of Bhilai-Durg region

vis spectrophotometer. ANOVA (Analysis of Variance) and DMRT (Duncan's Multiple Range Test) analysis revealed statistically a maximum laccase production was recorded by PSA (2.56±0.03 U/L) from Site C and minimum laccase enzyme production was demonstrated by Peel S (0.54±0.02) from Site B.

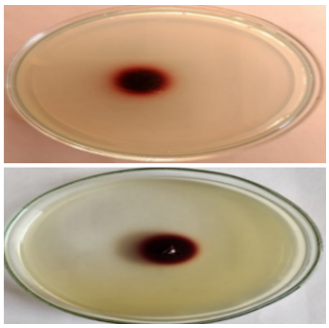

FIGURE 2. Qualitative screening of laccase producing fungi in guaiacol (0.02%) containing potato dextrose agar plate

3.3. Morphological identification of potent fungal isolates

For the identification of 10 laccase positive cultures they were sent to the Agharkar Research Institute Pune for morphological studies. The morphological study results revealed that two cultures Scytalidium lignicola (FWD) and Humicola fuscoatra (FWJ) belonged to the Chaetomiaceae family and one culture Fusarium sp. (FWN) to Nectriaceae family obtained from Site A. Likewise, two cultures Paecilomyces sp. (Peel B) and Aspergillus niger (Peel S) belonged to Trichocomaceae family, one culture Curvularia sp. (Peel E) to Pleosporaceae family and one culture Fusarium sp. (Peel J) to Nectriaceae family obtained from Site B. One culture Chaetomium sp. (PSA) belonged to Chaetomiaceae family, one culture Penicillium sp. (PSO) to Trichocomaceae family and one culture Histoplasma capsulatum (PSP) to Ajellomycetaeae family obtained from Site C.

Scytalidium lignicola (FWD): White in color, no exudates present, pigment absent, presence of terminal or intercalary arthroconidia and clamydospores, circular form, raised (cottony) elevation and entire margin.

Humicola fuscoatra (FWJ): White in color, no exudates present, no pigmentation occurs, moderately rapid-growth, circular form, raised (puffy) elevation and entire margin.

Fusarium sp. (FWN): White in color, exudates present, pigment absent, conidia present in two forms macroconidia and microconidia, circular form, raised (cottony) elevation and entire margin.

Paecilomyces sp. (Peel B): White in color, exudates present which is colorless, pigment absent, fast growing, hyline to darkdry chain of single-celled, circular form, raised (cottony) elevation and entire margin.

Curvularia sp. (Peel E): Black with off white covering in color, no exudates present, pigment absent, conidia are gridiron football or rugby to bean shaped three septa within each conidium, irregular form, irregular form, flat and raised elevation and entire margin.

Fusarium sp. (Peel J): White in color, exudates present, pink color pigment present, conidia present in two forms macroconidia and microconidia, circular form, raised (cottony) elevation and entire margin.

Aspergillus niger (Peel S): Black in color, no exudates present, no pigmentation occurs, phialosporous conidia, conidia borne on pale brown vesicle, irregular form, flat (powdery) elevation and

entire margin.

Chaetomium sp. (PSA): White in color, no exudates present, and no pigmentation occurs, dark walled (dematiaceous) fungi, lemon-shaped ascospores, irregular form, flat elevation and entire margin.

Penicillium sp. (PSO): White in color, exudates present which is brown in color, pigment absent, brush-like spore, circular form, raised (puffy) elevation and entire margin.

Histoplasma capsulatum (PSP): White in color after proper growth yellow in color with brown color exudate, pigment present, unicellular thick-walled macroconidia, circular form, raised (puffy) elevation and entire margin.

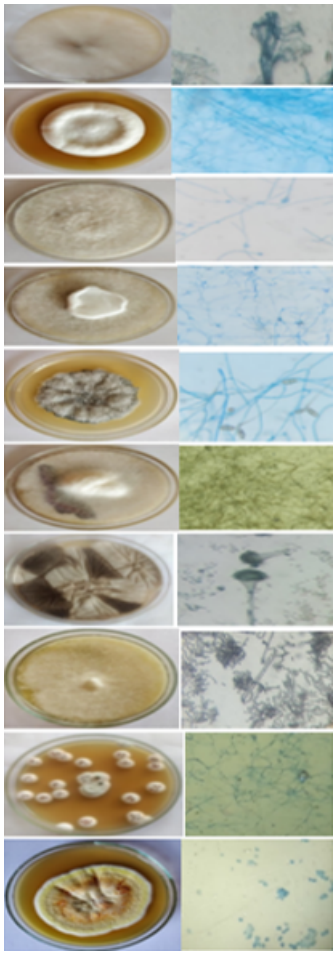

FIGURE 3. Microscopic images of isolated fungi, Scytalidium lignicola (FWD), Humicola fuscoatra (FWJ), Fusarium sp. (FWN), Paecilomyces sp. (Peel B), Curvularia sp. (Peel E), Fusarium sp. (Peel J), Aspergillus niger (Peel S), Chaetomium sp. (PSA), Penicilliumsp. (PSO) and Histoplasma capsulatum (PSP)

4. Discussion

In the present study three different types of lignocellulolytic waste soil samples were collected from different areas of Bhilai-Durg region of Chhattisgarh, India. A total of 49 fungal cultures were isolated from the soil samples. A total of 12 fungal cultures isolated from Site A, 20 cultures from Site B and 17 cultures from Site C. The result of morphological studies shows that three cultures belong to Chaetomiaceae family (Scytalidium lignicola, Humicola fuscoatra and Chaetomium sp.), one cultures belong to Pleosporaceae family (Curvularia sp.), three cultures belong to Trichocomaceae family (Penicillium sp., Paecilomyces sp. and Aspergillus niger), two cultures belong to Nectriaceae family (two cultures of Fusarium sp.), and one culture belong to Ajellomycetaeae family (Histoplasma capsulatum). The findings were similar to (Toker, Evlat, and Koçyi˙i) they isolated the six fungi from marine water (Cakalburunu Lagoon), they found laccase producer fungi (Alterneria sp. D11, Alterneria sp. D21, Cadophora sp. D43, Cadophora luteo-olivacea D51, Phoma sp. K2BR and Phoma sp. K21); (Nuhu et al.) isolated the fungi from soil and screening the fungus with tannic acid and fungus were morphologically identified. A total of 10 out of 49 fungal isolates were found to have laccase activity on guaiacol (0.02%) containing potato dextrose agar plate. Similar findings were obtained with environmental sample isolated fungi, they showed maximum laccase activity with guaiacol as a substrate (Bello et al. Vandelun Ado et al. Devasia and Nair Nnamchi, Ezeofor, and Amadi Thakkar and Bhatt). Author (Ali et al. Mtibaà et al.) identified the fungi and screened with the DMP and ABTS in solid medium plate. After qualitative screening laccase positive cultures further used for quantitative screening using sodium acetate buffer with fungal culture filtrate (enzyme source) and guaiacol as a substrate.

5. Conclusion

In the present study laccase producing fungi were isolated. A total of fourty nine lignocellulolytic fungi were obtained from soil samples from Bhilai-Durg region of Chhattisgarh, India. The presence of reddish brown halo in the guaiacol containing patato dextrose agar medium resulted in the production of the laccase enzyme by ten isolates, accord-

ing to qualitative analysis. Morphological studies revealed that isolated fungi belongs to Chaetomiaceae, Pleosporaceae, Trichocomaceae, Nectriaceae and Ajellomycetaeae family.

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