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# Screening of Fungal Isolates for Laccase Enzyme Production from Marine Sources.

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**ABSTRACT**

The objective of this study discusses the isolation and identification of a new marine fungal isolates highly active laccase producer. Twenty six fungal isolates obtained from decaying wood samples collected from Port-Saied, Ras-Ghareb and Ghamasa- Egypt, were screened for the presence of laccase enzyme activity. Results obtained from both qualitative and quantitative assay showed that the marine fungal isolate *Alternaria tenuissima* measured the highest zone diameter and colony diameter in agar plate screening test with guaiacol and showed a final specific activity of 54.103 U/mg protein with higher laccase activity 12.065 U/ml and the protein content was 0.223 U/ml when grown in Kirk's medium with half-strength sea water. It was recommended that the marine fungal isolate *Alternaria tenuissima* was the most promising one for laccase enzyme production.

**Keywords:** Marine-derived fungi, laccase enzyme, ligninases, bioremediation

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## INTRODUCTION

Fungi play an important role in degradation and mineralization of lignocellulosic substrates in the marine environment. These lignicolous fungi comprising Ascomycetes, Basidiomycetes and Deuteromycetes have distinct spore structures that set them apart from their terrestrial counterparts. Such fungi have been defined as obligate marine fungi "which grow and sporulate exclusively under marine conditions" (Kohlmeyer and Kohlmeyer 1979). Marine-derived fungi are able to produce biologically active secondary metabolites different from those produced by their terrestrial counterparts because they are adapted to the salinity found in marine environments (saleem, *et al.,* 2007 and Atalla, *et al.,* 2008). However, only recently this group of microorganisms has attracted attention as potential source of new generation of natural products and in biodegradation process the study of extracellular enzymes production by these microorganisms is very important in applied biotechnology (Bonugli-Santos, *et al.,* 2010). Atalla, *et at.,* (2010) found that high number of fungal strains isolated from different algae, sea grasses and decaying wood samples can produce laccase enzyme. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are one of the most important lignin-degrading enzymes and have great potential in industrial applications including the chemical, fuel, food, agricultural, paper, textile and cosmetic sectors (Sette *et al.* 2008). Laccases are multicopper oxidases that catalyse the oxidation of a variety of aromatic hydrogen donors with the concomitant reduction of oxygen to water. In general, these enzymes are monomeric or, rarely, homo- and hetero-dimeric or homo-tetrameric glycoproteins (Li, *et al.,* 2011). Most of the hazardous pollutants are phenolic in nature and persists in the environment. The ability of laccases to oxidize phenolic compounds and reduce molecular oxygen to water has led to intensive studies of these enzymes (Divya, *et al.* 2013).

Therefore, the major object of this study was to investigate the production of laccase enzyme by some local marine fungal isolates.

## MATERIALS AND METHODS

2-Methoxyphenol (Guaiacol) and 3,4-Dimethoxybenzyl alcohol (Veratryl alcohol) were purchased from Fluka Co. 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from MP. Bio (LCN) Co, USA.

Decayed wood samples collected from Port-Saied, Ras-Ghareb, El-Bahr El-Ahmar governorate and Ghamasa. The collected samples were brought to the laboratory in clean plastic bags and stored in ice box.

### Isolation of marine fungi

Samples were cut in small pieces, washed with sterile sea water, blotted between two folds of sterilized filter paper and transferred to petri dishes contained suitable medium(Höller, 1999; Rowley *et al*., 2003). Medium was used in isolation [Boyd & Kohlmeyer (B&K) agar]. Plates were incubated at 25 ºC for 4 days, exposed to daily examination to observe the developing growth. Fungal isolates were picked up under the dissecting microscope, transferred to biomalt agar (BIO) slants. Purification was carried out using single spore and hyphal tip techniques individually and transferred to suitable medium. Stock cultures were maintained on biomalt agar (BIO) slants and kept in the refrigerator at (5-6 ºC) for later use. Identification of the isolated fungi: Isolated fungi were identified in the National Research Centre, Chemistry of Natural and Microbial Products Dept. according to (Pitt and Hocking, 1985 and Kohlmeyer and Kohlmeyer, 1991).

### Media

Different types of media were used in this study.

### Isolation medium

Boyd & Kohlmeyer (B&K) agar**:** contained**,** glucose 10 g, peptone 2 g, yeast extract 1 g, agar 18 g in 1l of 50% sea water (Kohlmeyer and kohlmeyer, 1979 and D'Souza *et al.*, 2006).

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**Screening media**

Two different types of media were used for qualitative and quantitative assay of laccase enzyme production.

**Qualitative media**

(D'Souza *et al.*, 2006): guaiacol-supplemented agar: contained**,** glucose 10 g, peptone 2 g, yeast extract 1 g, agar 18 g and 4mM guaiacol in1l of 50% sea water.

**Quantitative media (Rigas *et al*., 2005)**

Kirk's medium: composed of g/l 50%sterile sea water; $KH_2PO_4$ 0.20, $CaCl_2$ 0.01, $MgSO_4.7H_2O$ 0.05, ammonium tartrate 0.22, 2.2-dimethylsuccinic acid 2.90, glucose 5 , thiamine 0.1, Tween 80 0.10% v/v, veratryl alcohol 1.5 mM, and a mixture of trace elements (10ml) composed of (mg/l): $MnSO_4$ 33, $Fe_2(SO_4)_3$ 50, $ZnSO_4.7H_2O$ 43, $CuSO_4.7H_2O$ 80, $H_2MoO_4$ 50.

**Assay of laccase activity**

Laccase (EC 1.10.3.2) activity was measured based on the oxidation of the substrate 2,2'-azino–bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS). The rate of ABTS oxidation was determined spectrophotometrically at 420 nm.

The reaction mixture contained 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27 ºC), 300 µL ABTS (5 mM), 300 µL mycelial liquid fraction and 1400 µL distilled water. The mixture was then incubated for 2 min at 30 ºC and the reaction was initiated by addition of 300 µL hydrogen peroxide. The absorbance was measured immediately in one-minute intervals after addition of hydrogen peroxide. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 mole of ABTS ($\varepsilon_{420}$= 36,000 $M^{-1}$ $cm^{-1}$ ) per minute.

**Determination of total proteins**

The protein content of the culture filtrate was estimated according to the method of Lowry *et al.* (1951). And bovine serum albumin (BSA) used as a standard at known concentrations (20, 40, 80, 100, 150 and 200 µg).

## RESULTS AND DISCUSSION

**Isolation**

**Isolation of marine fungi from decayed wood samples**

Twenty six isolates belonging to nine fungal genera and 14 species have been isolated from different marine decayed wood samples collected from **Port-Saied,** Ras-Ghareb and Ghamasa (**Table 1).**

Ten fungal isolates were isolated from **Port-Saied samples**. The marine fungal genera associated with marine decayed wood samples collected from **Port-Saied** governorate were *Alternaria ,Aspergillus, Cladosporium, Fusarium, Helmenthosporium and Ulocladium* (Table 1). Out of ten fungal isolates belonging to five fungal genera isolated from different decayed wood samples collected from Ghamasa (*Alternaria, Aspergillus, Penicillium, Trichoderma* and *Verticillium*). While six fungal isolates belonging to two fungal genera, *Cladosporium* and *Aspergillus* were isolated from Ras-Ghareb, El-Bahr El-Ahmar governorate **Table (1).**

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**Table 1: Fungal isolates associated with marine decayed wood samples collected from Port-Saied governorate, Ghamasa, Ras-Ghareb, El-Bahr El-Ahmar.**

| Fungal isolates | Port-Saied | Ghamasa | Ras-Ghareb |
|---|---|---|---|
| *Alternaria alternata* | 3 | 0 | 0 |
| *Alternaria solani* | 0 | 1 | 0 |
| *Alternaria tenuissima* | 1 | 0 | 0 |
| *Aspergillus fumigatus* | 0 | 1 | 2 |
| *A. niger* | 1 | 2 | 2 |
| *A. terreus* | 0 | 2 | 1 |
| *Cladosporium herbarum* | 1 | 0 | 1 |
| *Fusarium verticilloides* | 1 | 0 | 0 |
| *Helmenthosporium sativum* | 1 | 0 | 0 |
| *Penicillium italicum* | 0 | 1 | 0 |
| *Penicillium corylophilum* | 0 | 1 | 0 |
| *Trichoderma viride* | 0 | 1 | 0 |
| *Verticillium albo-atrum* | 0 | 1 | 0 |
| *Ulocladium chartarum* | 2 | 0 | 0 |
| Total species | 10 | 10 | 6.00 |

Mtui and Nakamura (2008) isolated a basidiomycete fungus *Flavodon flavus* from decayed sea grass leaves of *Thallasodendon ciliatum* collected about 50 m off the Coast Mjimwema, 20 km South of Dar-EL Salaam, Tanzania. Atalla, *et al.,* (2010). isolated thirty five fungal isolates from different decayed wood samples from Alexanderia of Egypt, while D'Souza *et al.* (2006) isolated 40 fungi from decayed wood pieces of mangrove swamps from Chorao Island in Goa, India. But a white-rot basidiomycete *Trametes trogii* was isolated from decayed acacia wood (from North West of Tunisia) (Zouari-Mechichi *et al*., 2006 These results agreed with those of (Sarma and Hyde, 2001) who stated that the lignocellulosic substrates in the marine environment, particularly mangrove wood, support a diverse mycota.

**Survey of marine fungal isolates for laccase enzyme activity:**

All fungal isolates were screened for the presence of laccase enzyme activity by using agar plate assay as a qualitative method for the determination of laccase enzyme production.

Pointing (1999) showed that qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required.

**Qualitative screening for laccase enzyme production assay (Guaiacol oxidation):**

Guaiacol oxidation is one of the most convenient qualitative assays for LMEs production among fungi. Twenty six of marine fungi were screened for guaiacol oxidation and radial growth rate on agar plates containing 4mM guaiacol as an aromatic model compound. The results showed that 17 fungal isolates negative for guaiacol oxidation, no halo of brown colour was formed under and around the fungal colonies, indicating the lack of ligninolytic enzymes.

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**Table 2: Qualitative assay for laccase enzyme production.**

| Fungal isolates | Mycelial growth and oxidation characteristics | | |
|---|---|---|---|
| | Colour zone diameter (mm) [a] | Oxidation scale [b] | Fungal colony diameter (mm) [c] |
| *Alternaria alternata* $_1$ | 28 | ++++ | 38 |
| *Alternaria solani* | 23 | +++ | 29 |
| *Alternaria alternata* $_2$ | 24 | +++ | 32 |
| *Alternaria tenuissima* | 30 | +++++ | 32 |
| *Helmenthosporium sativum* | 21 | ++ | 25 |
| *Ulocladium chartarum 1* | 25 | +++ | 30 |
| *Fusarium verticilliodies* | - | - | - |
| *Alternaria alternata* $_3$ | 14 | + | 26 |
| *Cladosporium herbarum* | 18 | ++ | 30 |
| *Ulocladium chartarum 2* | 12 | + | 25 |

(*Fusarium verticilliodies*) negative control

[a] Diameter of the oxidized zone in mm (measured on the 7th day of cultivation).
[b] Oxidation scale measured on the 7 th day of cultivation on B&K medium containing 4mM guaiacol: + diameter of the oxidized zone 0-10mm, ++ zone diameter 11-15 mm, +++ zone diameter 16-20 mm, ++++ zone diameter 21-30 mm, +++++ zone diameter up to 31mm.
[c] Diameter of the mycelia colony in mm measured on the 7th day of cultivation (the initial disc 10 mm diameter).

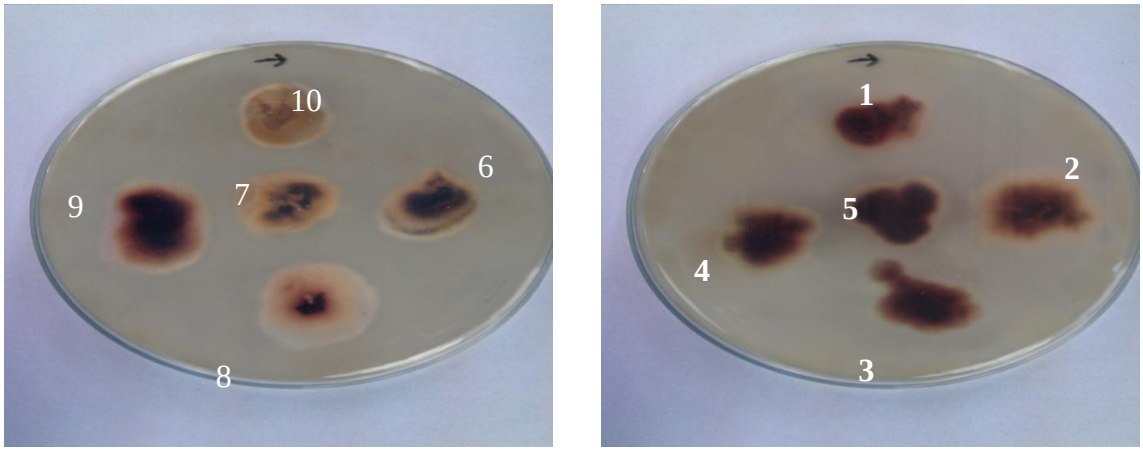


**Figure 1:: Photo of B&K agar plate showing positive guaiacol oxidation by 9 fungal isolates obtained from decayed wood samples after 7 days of inoculation. *1-Alternaria alternata* $_1$, *2-Alternaria solani* , *3-Alternaria alternate, 4-Helmenthosporium sativum, 5-Ulocladium chartarum 1, 6-Cladosporium herbarum, 7 - Ulocladium chartarum2, 8-Alternaria alternata* $_3$, *9-Alternaria tenuissima, 10--Fusarium verticilliodies* (negative control).**

Table (2) and Fig. (1) showed that only 9 fungal isolates positive for guaiacol oxidation identified as, *Alternaria alternata, Alternaria solani, Cladosporium herbarum, Helmenthosporium sativum, Alternaria tenuissima* and *Ulocladium chartarum* obtained from decayed wood samples where, a halo of intense brown color was formed under and around the fungal colonies. Results showed that *Alternaria tenuissima* (NRC 9) measured about 30 mm colour zone diameter and 32 mm growth colony diameter on the 7th day of cultivation. So, that the marine fungal isolate *Alternaria tenuissima* (NRC 9) is the most active fungus in the qualitative assay. Screening of local fungi for ligninolytic activities was performed using agar plate screening tests with guaiacol as a qualitative assay (Atalla, *et al.,* 2010).

These results agreed with D'Souza *et al.* (2006) who stated that, out of 40 fungi isolated from decayed mangrove wood, 3 isolates showed positive reaction for laccase activity when grown in the presence of guaiacol.

Mtui and Masalu (2008) demonstrated the guaiacol oxidation by mycelial cultures of a marine fungal isolate *Laetiporus sulphureus* isolated from mangrove forests of coastal Tanzania after 7 days of incubation. The ability of *L. sulphureus* enzymes to degrade the aromatic model compound indicated that they are the

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main decomposers of cellulose, hemicellulose and lignin contained in the mangrove trees, this implies that the enzymes can also be used in detoxification of aromatic pollutants such as agrochemicals and industrial effluents.

**Enzymes production in liquid medium**

Nine fungi showed positive reaction for laccase activity when grown in the presence of guaiacol were grown in liquid culture medium (Kirk´s medium) for enzyme activity. Where laccase and protein of the culture filtrate were measured.

The results summarized in **Table (3)** and **Fig. (2)** showed that, *Alternaria tenuissima* which was isolated from Port-Said was the most active fungal isolate. The amount of laccase produced by *Alternaria tenuissima* was 12.065U/ ml and the protein content was 0.223 U/ml with specific activity 54.103U/mg protein. These results agreement with the work by Atalla *et al.,* (2010) who demonstrated the ability *Trematosphaeria mangrovei* to produce laccsae enzyme.

**Table 3: Quantitative estimation of laccase enzyme produced by the selected marine fungal isolates.**

| Fungal isolates | Laccase (U/ml) | Protein content (mg/ml) | Specific activity (U/mg) |
|---|---|---|---|
| *Alternaria alternata* $_1$ | 11.315 | 0.254 | 44.547 |
| *Alternaria solani* | 8.88 | 0.221 | 40.181 |
| *Alternaria alternata* $_2$ | 9.925 | 0.278 | 35.701 |
| *Alternaria tenuissima* | 12.065 | 0.223 | 54.103 |
| *Helmenthosporium sativum* | 6.425 | 0.191 | 33.639 |
| *Ulocladium chartarum 1* | 8.485 | 0.161 | 52.702 |
| *Alternaria alternata* $_3$ | 6.97 | 0.117 | 59.573 |
| *Cladosporium herbarum* | 5.705 | 0.189 | 30.185 |
| *Ulocladium chartarum 2* | 5.845 | 0.179 | 32.654 |

* The values are mean of three replicates

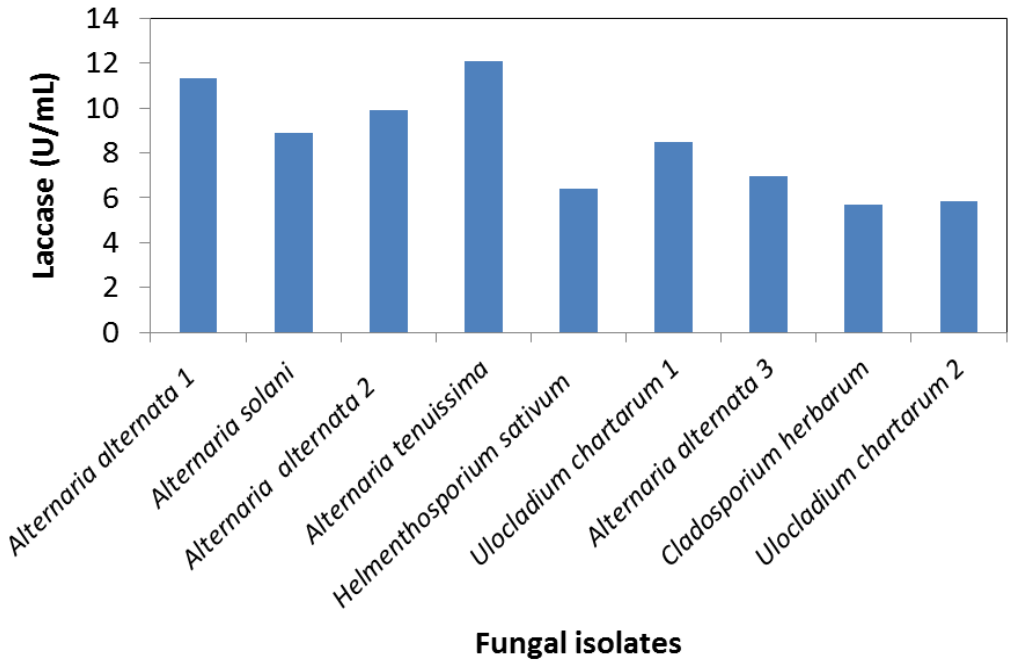


**Figure 2: Laccase enzyme production by the selected marine fungal isolates.**

These results are in consistent with Hou *et al.* (2004) findings, who demonstrated that laccase was the only ligninolytic enzyme activity detected in the supernatant when the fungus was grown in liquid culture with or without shaking.

*Alternaria alternata*$_1$, *Ulocladium chartarum1* and *Alternaria solani* produced levels of laccase activities as following, 11.315, 8.485 and 8.88 U/ml with specific activity of 44.547, 52.702 and 40.181U/mg protein.

Lower laccase activities of 5.845 and 32.654/ml were observed with the fungal isolates *Alternaria alternate 3* and *Ulocladium chartarum 2*.

The appreciable amount of laccase which produced by *Alternaria tenuissima* can be used for application in several bioprocesses, such as biopulping, biobleaching, bioremediation, food technological uses, and treatment of industrial waste water (Hublik and Schinner 2000; Robinson *et al*., 2001). So that fungi producing laccase are currently the focus of much attention (Pointing *et al*., 2000) and this places a high value on the importance of fungi in coastal ecology (Bucher *et al*., 2004).

## CONCLUSIONS

It can be concluded that, from a high number of fungal strains isolated from decaying wood samples collected from three different sites (Port-Saied, Ras-Ghareb and Ghamasa), the marine fungal isolate *Alternaria tenuissima* (NRC 9) which was isolated from Port-Said was the most active fungal isolate for laccase production. It can be used for application in several bioprocesses, such as biopulping, biobleaching, bioremediation, food technological uses, and treatment of industrial waste water.

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