Biodiversity of laccase producing fungi in Egypt

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Laccase enzyme (EC 1.10.3.2) is a benzenediol, a multi-copper enzyme, and one of the three main ligninases that differs from the others in its ability to catalyze the oxidation of lignin components. It is widely distributed in fungi and because of its importance in bioremediation; the search for fungal laccases with different properties and potential applications is still on-going. In view of its importance in large scale application, the present endeavor is to search for highly efficient laccase producing fungi from different environmental habitats in Egypt. Cultural conditions such as temperatures, pH, carbon sources and nitrogen sources were optimized for the production of high extracellular laccase activity. By screening sources under investigation namely: soil, wood, seaweeds, sponge, ascidia, drifted decaying wood, plants and miscellaneous materials it was possible to encounter as many as 60 species belonging to 33 genera. Zygomycota represented by six species (10.16% of the total species number), teleomorphic Ascomycota (9 species, 15.25%), anamorphic Ascomycota (44 species, 74.57%) and Basidiomycota (1 species, 1.69%). Soil showed the highest Simpson's species diversity index of 0.83 while contaminated wax samples and *Adiantum* capillus-veneris showed the lowest value (0). All isolated taxa were tested for laccase production using a qualitative plate assay method by using guaiacol as color indicator. Sixteen isolates showed positive reaction indicating a lignin-degrading potentiality and out of them eight measured the highest zone diameter with high oxidation scale. The most promising taxa were endophytic namely: *Chaetomium globosum*, *Phoma exigua*, *Thanatephorus cucumeris* and *Sordaria fimicola*. pH 7, incubation temperature 30^{o}C, 1% maltose and 0.3% peptone supported the highest biomass and laccase production for *Chaetomium globosum*.

Key words – ascidia – *Chaetomium globosum* – entophyte – seaweeds – sponge – wood

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Introduction

Laccase, as a large blue copper-containing protein, is widely distributed in a wide range of living organisms e.g. higher plants and fungi (Leontievsky et al. 1997, Mendoza 2011), insects (Hattori et al. 2005) and bacteria (Claus 2004). Recently, a novel polyphenol oxidase with laccase-like activity was mined from a metagenome expression library from bovine rumen microflora (Beloqui et al. 2006). In 1883 Yoshida extracted and described laccase for the first time from the exudates of *Rhus vernicifera* (Japanese lacquer tree). In 1895 Bertrand demonstrated the presence of laccase in fungi for the first time. Over 60 fungal strains belonging to the phyla

Ascomycota, Zygomycota and Basidiomycota, particularly many white-rot taxa show laccase activity (Baldrian 2006).

Due to their higher redox potential, fungal laccases are used in many biotechnological important applications especially in the degradation of lignin and elimination of potentially toxic phenols arising during lignin degradation. One of the most important enzymes in the biopolpation process in pulp and paper industry are laccases which have the ability to depolymerize lignin and delignify wood pulps, kraft pulp fibers and chlorine-free (Bourbonnais et al. 1997, Lund & Ragauskas 2001, Chandra & Ragauskas 2002, Camarero et al. 2004, Rodríguez & Toca 2006, Vikineswary et al. 2006).

Fungal laccases also play an important role in physiological processes related to pathogenesis (Edens et al. 1999), morphogenesis i.e. fruiting body development (Kues & Liu 2000, Ohga & Royse 2001), pigmentation (Eggert et al. 1995) and cell detoxification (Bollag et al. 1988). Laccases from fungi have been used in food and beverage industry enhancement or modification (Ghindilis 2000, Minussi et al. 2002, Rodríguez & Toca 2006, Selinheimo et al. 2006, Minussi et al. 2007). Laccases are involved in green biodegradation of xenobiotic contaminants in soil (Rodríguez & Toca 2006) and polycyclic aromatic hydrocarbons (Anastasi et al. 2009). Laccases-mediator systems are powerful tools in dye processes and textile industries (Kunamneni et al. 2008). Laccases are also used as catalysts for the manufacture of anti-cancer drugs, in the cosmetic industry and have also been applied to nanobiotechnology (Xu 2005, Rodríguez & Toca 2006, Ponzoni et al. 2007, Kunamneni et al. 2008, Mikolasch & Schauer 2009).

In Egypt fungi producing laccases attracted the attention of various investigators by the late 1990s. Laccase activities produced by different ecological groups of Egyptian fungi have been the subject of many studies carried by Abdel-Raheem (1997), Yehia (2007), EL-Zayat (2008), Youssef et al. (2008) and Atalla et al. (2010). Other investigators studied the various applications of laccases in industry and medicine such as El Fallal (2001), Gomaa (2005), El-Fallal & El-Diasty (2006), Abd El-Thalouth et al. (2008), Abedin (2008), Gomaa et al. (2008), El-Fakharany et al. (2010), Abd El-Rahim & Moawad (2010), Abou-Okeil et al. (2010) and Gomaa et al. (2011). Molecular studies on characterization and cloning of laccases genes were carried by other Egyptian researchers viz. El-Shora et al. (2008) and Moussa (2009, 2011).

It is estimated that there are approximately 1.5 million fungi species in the world, of which around 4.6% are known (Hawksworth 2001). The Egyptian fungi are presently represented by 2281 taxa (1,035 species and 395 genera) out of the 101,202 world records (Abdel-Azeem 2010). The potential fungal resources of Egypt are globally important and vast areas are still unexplored. The extensive collection of fungi in unexplored areas remains a research priority (Abdel-Azeem 2010).

Marine-derived fungi represent a rich source of bioactive metabolites, especially enzymes, which have not yet been fully exploited in Egypt and have never been the target of any solid study before. Based on the aforementioned, the objective of the present work was to study the biodiversity of laccase producing fungi in Egypt through isolation, identification and qualitative assessment of laccase from different ecological habitats with special reference to marine habitat. In addition this study aims at optimizing cultural conditions supporting high biomass and laccase yield from a local isolate to enable the selection of such fungi with the greatest biotechnological potential.

Methods

Sampling – A range of terrestrial samples (soil, plants, and wood), marine (seaweeds, sponge, ascidia, and submerged decaying drifted wood), contaminated wax and water colour samples were screened in order to give a preliminary view of laccase producing mycobiota of Egypt.

One hundred rhizospheric soil samples were collected from the dominant plant species (31 plant species) between July 2010 and October 2010 from 20 different sites throughout different elevation wadis (Table 1) in Saint Katherine Protectorate. These were placed in sterilized polyethylene bags, closed

Table 1 Location of soil sampling sites and dominant vegetation

Wadi Level	Site No.	Site name	GPS reading			Dominant Plant Species per site
			N°	E°	Elevation (m)	
Low Elevation	1	Ain Hodra1	28° 54'.44	34° 25'.59	645	*Anabasis articulata* Moq.
	2	Rum	28° 54'.321	34° 25'.004	718	*Anabasis articulata* Moq. *Retama raetam* Webb & Berth. *Zilla spinosa* (Turra) Prantl.
	3	Sa'al 1	28° 45'.584	34°12'.287	966	*Acacia ehrenbergiana* Hayne *Aerva javanica* (Burm.f.) Juss. ex Schult. *Peganum harmala* L.
	4	Tebit el Marrah	28° 44.70'	34° 11'.50	949	*Acacia tortilis* (Forssk.) Hayne *Zilla spinosa* (Turra) Prantl. *Aerva javanica* (Burm.f.) Juss. ex Schult. *Retama raetam* Webb & Berth.
Mid Elevation	5	Nawamis	28° 28'.075	34° 05'.750	1345	*Artemisia judaica* L. *Achillea fragrantissima* (Forssk.) Sch. Bip. *Retama raetam* Webb & Berth.
	6	Hibran	28° 37'.641	33° 52'.945	1118	*Retama raetam* Webb & Berth. *Asclepias sinaica* (Boiss.) Musc. *Hyoscyamus boveanus* (Dunal) Asch. & Schweinf. *Tanacetum sinaicum* (Fresen.) Bremer & Humphries.
	7	Baidaa	28° 29'.78	34° 07'.703	1243	*Ochradenus baccatus* Delile *Aerva javanica* (Burm.f.) Juss. ex Schult. *Zygophyllum coccineum* L.
	8	Wadi Itlah	28° 35'.45	33° 55'.131	1370	*Artemisia herba-alba* Asso. *Origanum syriacum* L. *Alkanna orientalis* (L.) Boiss.
	9	Wadi Itlah 2	28° 35'.45	33° 55'.113	1389	*Fagonia mollis* Del. *Ochradenus baccatus* Delile *Verbascum sinaiticum* Benth.
	10	Gharba	28°39'.195"	33° 54'.250	1144	*Anabasis articulata* Moq. *Achillea fragrantissima* (Forssk.) Sch. Bip. *Peganum harmala* L.
	11	El Hadibi	28°34'.926	33° 55'.426	1459	*Matthiola arabica* Boiss. *Alkanna orientalis* (L.) Boiss. *Artemisia herba-alba* Asso.
	12	Shagg Tinia	28° 35'.547	33° 54'.933	1379	*Teucrium polium* L. *Achillea fragrantissima* (Forssk.) Sch. Bip. *Ficus pseudosycomorus* Decne.
	13	Sulaf	28° 38'.031	33° 52'.695	1145	*Retama raetam* Webb & Berth. *Launaea spinosa* (Forssk.) Sch.Bip. *Gymnocarpus decandrus* Forsk.

Wadi Level	Site No.	Site name	GPS reading			Dominant Plant Species per site
			N°	E°	Elevation (m)	
High Elevation	14	Wadi Nasb	28° 29'.810	34° 07'.544	1235	*Retama raetam* Webb & Berth.
	15	Zewatein	28° 32'.300	33°55'.11	1841	*Achillea fragrantissima* (Forssk.) Sch. Bip. *Phlomis aurea* Decne. *Stachys aegyptiaca* Person
	16	Wadi Ahmer	28° 31'.726	33° 56'.452	1928	*Phlomis aurea* Decne. *Stachys aegyptiaca* Person *Thymus decussatus* Benth.
	17	Hreza	28° 32'.070	33° 56'.499	1939	*Tanacetum sinaicum* (Fresen.) Bremer & Humphries. *Pulicaria undulata* (L.) Mey. *Teucrium polium* L.
	18	Omgraf	28° 31'.56	33°55'.99"	1922	*Phlomis aurea* Decne. *Teucrium leucocladum* Boiss. *Tanacetum sinaicum* (Fresen.) Bremer & Humphries.
	19	Ghazna	28° 31'.290	33°56'.240"	1907	*Phlomis aurea* Decne. *Achillea fragrantissima* (Forssk.) Sch. Bip. *Chiliadenus montanus* (Vahl) Brullo.
	20	Wadi El-Arbaein	28° 32'.500	33°57'.56"	1800	*Artemisia judaica* L. , *Thymus decussatus* Benth. , *Asclepias sinaica* (Boiss.) Musc

soil samples were collected and mixed thoroughly to form one composite sample. by rubber band, and transferred to the laboratory until plated out. From each site, five samples were collected from each non terricolous substrate. *Adiantum capillus-veneris* L., *Phlomis aurea* Decne. and *Alkanna orientalis* (L.) Boiss. were collected from Wadi El-Talaah, Saint Katherine Protectorate, South Sinai for isolation of associated endophytic fungi. Wood samples of *Acacia tortilis* subsp. *raddiana* Savi and *Ceratonia siliqua* L. were collected from Wadi Feran and Wadi Itlah, South Sinai.

Six species of dried seaweeds collected from Suez Canal namely: *Ulva lactuca* L., *Caulerpa serrulata* (Forssåk.) Agardh and *C. prolifera* (Forssk.) Lamouroux from green algae, *Hypnea cornuta* (Kützing) Agardh and *Sarconema filiforme* (Sonder) Kylin from red algae and *Cystoseira myrica* (Gmelin) Agardh from brown algae.

Specimens of the sponge (*Hippospongia communis*) were collected from the western region of the Mediterranean Sea (El-Hamam, Sidi Abd-El-Rahman, Alam El-Room, Matrouh and Barrani). Ascidian samples belonging to *Phallusia nigra* Savigny, *Micsocosmus pupa* Savigny, *Styela plicata* Lesuer and *Polyclinum constellatum* Savigny were collected from Suez Canal.

Submerged decaying drift wood were collected from Mediterranean Sea coast at Port Said, El-Arish and Suez. Also contaminated samples of wax and water colours were collected from Port Said Governorate. The samples were transferred to the laboratory in an ice box for further study.

Isolation, maintaining and laccase screening media- The isolation and culture maintaining media for marine taxa were prepared with sea water (SW) and supplemented with Rose bengal (1/15,000) and chloramphenicol (50 ppm) for suppression of bacterial growth. Five media were adopted for isolation after Atlas (2004); these were: sea water rose Bengal chloramphenicol agar (SWRCA), sea water Czapek's yeast extract agar (SWCYA), sea water oatmeal agar (SWOA), sea water agar (SWA), and sea water potato dextrose agar (SWPDA).

The same media with distilled water were used for isolation from soil, plants and contaminated samples. For maintaining and identification of the isolated fungi, media such as vegetable agar (V8), oatmeal agar (OA), malt extract agar (MEA), potato dextrose agar (PDA) and potato carrot agar (PCA) were used.

Two screening media were used for qualitative assay of laccase: guaiacol supplemented agar (D'Souza et al. 2006a) and modified Czapek's agar supplemented with 0.02% guaiacol (Viswanath et al. 2008). The media were supplemented with sea water (50%) for marine organisms and distilled water for terrestrial taxa.

Isolation – A total of 1000 plates from 40 different environmental samples previously listed were used for isolation of mycobiota. Mycobiota of soil and contaminated samples were isolated using a dilution plate method (Garrett 1981). Pieces (5 mm^2, 4 pieces in each plate) of stem, root and wood from different plant species, *Adiantum capillus-veneris*, *Phlomis aurea*, *Alkanna orientalis*, *Acacia tortilis* and *Ceratonia siliqua* were surface sterilized (Fisher et al. 1993) and cut for isolation of endophytic fungi. The samples were washed in running water, immersed in 70% ethanol for 1-5 min. according to the thickness, dipped in 5% NaOCI for 3-5 min. and then in 70% ethanol for 30 seconds. The sterilized samples were plated on appropriate isolation media.

Seaweed samples were washed thoroughly in sea water and rinsed 3 times with sterile sea water (SSW). Samples were aseptically sliced into small pieces (5-10 mm^2) and placed in sodium hypochlorite solution (5 %) for 1 to 2 min according to the thickness of each algae, rinsed 3 times with SSW, immersed in ethanol (70%) for 2 min, rinsed several times with SSW and placed on different isolation media. Algicolous fungi were isolated by grinding 5 g (fresh weight) of each sample which was previously surface sterilized, as mentioned before, in a sterile mortar with 50 ml of SSW, homogenized with pestle and then 1 ml of liquid portion placed on agar plate of different isolation media.

To get rid of non-specific fungal propagules from seawater column on sponge surfaces, animal tissues were rinsed three times with sterile seawater. The surface of the sample was disinfected with ethanol (70%) for 2 minutes. The inner tissue was taken out with a scalpel and forceps and then cut into small cubes (~ 5 mm^3). A total of 15-20 cubes of each sample were placed on isolation media.

Ascidia tissues were rinsed three times with sterile seawater in order to get rid of any microbial contamination from seawater, rinsed in ethanol (70%) for 2 minutes, re-rinsed with SSW several times, dissected into pieces (5-10 mm^3) and then placed on different isolation media.

Fungi from submerged drift wood were isolated by direct isolation technique after washing the samples with sterile sea water, incubated in plastic boxes lined with wet tissue at room temperature and examined periodically over 3 months.

Identification of isolates – Taxonomic identification using morphology characteristics of fungal isolates down to the species level, on standard media, was mainly based on the following identification keys: Raper & Thom (1949), Pitt (1980) for *Penicillium* (on Czapek's yeast extract agar (CYA) & malt extract agar (MEA)); Raper & Fennell (1965) for *Aspergillus* (on Czapek's agar (CZ)); Ellis (1971, 1976) for dematiaceous hyphomycetes (potato carrot agar (PCA)); Booth (1971) for *Fusarium* (potato sucrose agar (PSA)); Arx (1981), Domsch et al. (2007), Watanabe (2002) for miscellaneous fungi (on MEA, PDA, CYA); Arx et al. (1986) and Cannon (1986) for *Chaetomium* (oat meal agar + lupin stem (OA+LUP)). The systematic arrangement follows the system of classification appearing in the 10th edition of Ainsworth and Bisby's Dictionary of the fungi (Kirk et al. 2008) and Species Fungorum (http://www.speciesfungorum.org/Names/Names.asp).

Qualitative assay of laccase – For examination of laccase production, 5 mm diameter discs from 7 days old colonies were placed on the surface of guaiacol supplemented agar plates (D'Souza et al. 2006a) and direct inoculation into modified Czapek's agar plates and incubated at 25 ºC in dark for 7 days. The production of intense brown colour under and around the fungal colony was considered as a positive reaction resulting from guaiacol

oxidation (Coll et al. 1993, Okino et al. 2000). The diameters of coloured zone and growth rate were measured in mm. The white rot fungus *Pleurotus ostreatus*, a well known lignin-degrading fungus, was used as a positive control. Stock culture of *Pleurotus ostreatus* on millet grains was obtained from Mushroom Unit, Faculty of Agriculture, Mansoura University, Egypt and the spawn was kept at 4ºC until used for inoculation.

Optimization of growth and laccase production medium – *Chaetomium globosum* isolated from *Adiantum capillus-veneris*, as a high producer of extracellular laccase was maintained on OA slants at 30ºC for 7 days. Fungal spore suspension was prepared by adding 5 ml of sterile distilled water to freshly (7 days) grown cultures. The suspension was used to inoculate different media (100 ml) in Erlenmeyer flasks (250 ml).

In this study laccase production at different pH values (3, 4, 5, 6, 7, 8, 9) were analyzed and pH values were adjusted to the desired value by using 0.1 M HCl. The flasks were then incubated at different temperatures (25, 30, 35, 40 and 45ºC). For a time course study, the flasks were incubated on a gyratory shaker with shaking at 200 rpm at 30ºC for 10 days.

Various carbon sources at 1% final concentration were tested for laccase production instead of glucose using a synthetic medium (Viswanath et al. 2008) consisting of 0.3% peptone, 0.06% KH_2PO_4, 0.0001% $ZnSO_4$, 0.04% K_2HPO_4 0.00005% $FeSO_4$, 0.005% $MnSO_4$ and 0.05% $MgSO_4$ prepared in distilled water without inclusion of an inducer (0.02% guaiacol). The carbon sources included monosaccharide hexoses (fructose, galactose and rhamnose); monosaccharide pentose (xylose); disaccharides (sucrose, maltose, and lactose); sugar-alcohols (glycerol) and the polysaccharide (starch). Various nitrogen sources included inorganic nitrogen (potassium nitrate, sodium nitrate, ammonium nitrate); amino acid (glycine) and complex organic source (peptone and beef extract) at 0.3% final concentration were examined for laccase production.

At one day interval, flasks with growing culture of *Chaetomium globosum* were withdrawn and each sample was monitored for fungal growth, pH, and laccase activities. The culture in each flask was aseptically filtered through pre-weighed filter paper (Whatman No 1), mycelium dried at 70°C in an oven and the filtrate was centrifuged at 10000 rpm at 4°C for 15 minutes. The supernatant obtained was treated as the enzyme extract.

Enzyme activity was assayed at 30°C by using 10 mM guaiacol in 100 mM acetate buffer containing 10% (v/v) acetone. Absorbance of blank was measured at 470 nm, while the test samples with guaiacol were measured at 530 nm after 10 minutes of incubation. Enzyme activity is measured in u/ml which is defined as the amount of enzyme catalyzing the production of one micromole of coloured product per minute per ml.

Calculation

$$\text{Volume activity } (u/\text{ml}) = \frac{\Delta A_{470nm}/\text{min} \times 4 \times Vt \times \text{x dilution factor}}{€ \times Vs}$$

Where,
Vt = final volume of reaction mixture (ml) = 5.00
Vs = sample volume (ml) = 1
€ = extinction co-efficient of guaiacol = 6,740 /M/cm
4 = derived from unit definition & principle

Data analyses – The frequency of isolated taxa is expressed as number of cases of isolation of each species out of the total number of studied sites, while species diversity is calculated as Simpson's diversity index (Lande 1996).

Results

General features of isolated fungi – As shown in Table 2 it was possible to encounter as many as 59 species (108775 CFU) belonging to 33 genera from the various sources under investigation namely: soil, wood, seaweeds, sponge, ascidia, drifted wood, plants and miscellaneous materials.

Zygomycota was represented by six species (10.16% of the total species number), teleomorphic Ascomycota (9 species, 15.25%), anamorphic Ascomycota (44 species, 74.57%) and Basidiomycota (1 species, 1.69%). The prevailing genera were *Aspergillus* (15 species including anamorph stages of one *Emericella* and two *Eurotium* species; 25.42% of the total isolates), *Penicillium* (7 species including anamorph stage of *Talaromyces*, 11.86%), *Mucor* (3 species; 5.08 %), *Fusarium*, *Trichoderma* and *Ulocladium* (2 species each, 3.38%). The remaining taxa were represented by only one species each. The most abundant species were: *Aspergillus niger* (31.45% of the total isolates), *A. flavus* (16.24%), *Penicillium aurantiogriseum* (8.78%), and *Macrophomina phaseolina* (5.08 %).

Taxonomically, isolated species were assigned to 3 phyla with 4 classes, 11 orders, and 15 families. Taxa with uncertain position were distributed among classes, orders and families. Order Eurotiales accommodated the most species (23), while the orders Capnodiales, Cantharellales, Onygenales and Microascales accommodated only one species each. Family Trichocomaceae had the highest contribution to the isolated fungi (23 species out of 60) followed by Pleosporaceae (6 species), Mucoraceae (4 species) and the remaining families were represented each by only one or two species.

The species genus ratio (S/G) per family however shows that family Trichocomaceae was the most diverse taxonomical rank by recording a ratio of 3.8 followed by Mucoraceae and Pleosporaceae. In view of species richness, soil showed the highest richness index of fungi species (species richness = 42) among all studied habitats and followed by sponge (12 species). The remaining habitats showed moderate to poor species richness ranging from 1 to 9.

The distribution pattern of mycobiota based on the presence/absence in habitats under investigation is shown in Figure 1. It is clear from the figure that recorded taxa could be tentatively classified into three groups. Group 1, comprises taxa of occurrence restricted to a single habitat (38 species) e.g. *Sordaria fimicola*, *Aspergillus ustus*, *Aureobasidium pullulans* and *Stemphylium botryosum*. Group 2, consists of species occurring in two or more habitats but showing some sort of preference for higher occurrence in one of them (18 species) e.g. *Fusarium solani*, *Gymnascella dankaliensis*, *Aspergillus japonicus* and *A. fumigatus*. Group 3, contains species of common occurrence to almost all habitats (4 species) e.g. *Aspergillus niger*, *A. flavus*, *Penicillium aurantiogriseum* and *Trichoderma pseudokoningii*.

The diversity of fungi was measured for each habitat by calculating Simpson's diversity index (Lande 1996). Based on the results, soil showed the highest diversity index (Table 4) of 0.83 while contaminated wax samples and *Adiantum capillus-veneris* showed the lowest value (0).

Screening and qualitative assessment of laccase producing taxa

Out of 60 species screened in this study, only fifteen taxa had laccase activity compared with *Pleurotus ostreatus* as a positive control to differentiate between growth and oxidation characteristics. Thirteen species of Ascomycota, one Basidiomycota (*Thanatephorus cucumeris*) and one Zygomycota (*Mucor circinelloid*s) showed laccase production activity (Table 5).

Results showed that, the endophytic fungal isolates came first by showing high laccase production activity in comparison with other taxa isolated from different ecological habitats. *Chaetomium globosum* measured about 40 mm colour zone diameter and 55 mm growth colony diameter on the 7th day of cultivation, followed by *Phoma exigua* (32 mm and 50 mm, respectively), *Thanatephorus cucumeris* (23 and 30 mm) and *Sordaria fimicola* (23 and 59 mm).

Fungal taxa isolated from marine sources showed degrees of oxidation scale ranging between weak to high. *Mucor*

Table 2 Total count (TC, colonies/ g), number of isolations (NCI, out of 520 samples) and percentage frequency of fungal taxa recovered on isolation media at 28°C

Species	TC	NC1	% F
Zygomycota			
Lichtheimia corymbifera (Cohn) Vuill.	97	2	0.38
Mucor circinelloid Tiegh.	258	13	2.50
M. hiemalis Wehmer	598	8	1.54
M. racemosus Fresen.	35	2	0.38
Rhizopus stolonifer (Ehrenb.) Vuill.	365	9	1.73
Syncephalastrum racemosum Cohn ex J. Schröt.	168	4	0.77
Ascomycota (teleomorphic)			
Achaetomium macrosporum Rai, Wadhwani & J.P. Tewari	457	6	1.15
Chaetomium globosum Kunze	5049	67	12.88
Emericella nidulans (Eidam) Vuill.	921	17	3.27
Eurotium amstelodami L. Mangin	190	4	0.77
E. chevalieri L. Mangin	163	5	0.96
Gymnascella dankaliensis (Castell.) Currah	496	20	3.85
Microascus cinereus Curzi	188	6	1.15
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	17	4	0.77
Talaromyces flavus (Klöcker) Stolk & Samson	33	5	0.96
Ascomycota (anamorphic)*			
Acremonium restrictum (J.F.H. Beyma) W. Gams	989	3	0.58
A. rutilum W. Gams	1230	7	1.35
Acrophialophora fusispora (S.B. Saksena) Samson	50	7	1.35
Agonomycete	277	14	2.69
Alternaria alternata (Fr.) Keissl.	279	23	4.42
Aspergillus candidus Link	27	6	1.15
A. flavus Link	17664	151	29.04
A.fumigatus Fresen	869	5	0.96
A.japonicus Saito	144	18	3.46
A.niger Tiegh.	34312	209	40.19
A. ochraceous G. Wilh.	734	7	1.35
A.sydowii (Bainier & Sartory) Thom & Church	197	2	0.38
A. tamarii Kita	1357	26	5.00
A. terreus Thom	1349	5	0.96
A. ustus (Bainier) Thom & Church	470	5	0.96
A. versicolor (Vuill.) Tirab.	897	6	1.15
A. wentii Wehmer	100	1	0.19
Aureobasidium pullulans (de Bary) G. Arnaud	3754	2	0.38
Botryotrichum piluliferum Sacc. & Marchal	29	9	1.73
Cladosporium cladosporioides (Fresen.) de Vries	504	64	12.31
Curvularia lunata (Wakker) Boedijn	7	3	0.58
Drechslera spicifera (Bainier) Arx	208	14	2.69
Fusarium oxysporum Schltdl.	175	16	3.08
F. solani (Mart.) Sacc.	5493	11	2.12
Humicola fuscoatra Traaen	433	6	1.15
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	61	9	1.73
Macrophomina phaseolina (Tassi) Goid.	5535	7	1.35
Myrothecium roridum Tode	47	5	0.96
Paecilomyces varioti Bainier	367	6	1.15
Penicillium aurantiogriseum Dierckx	9553	63	12.12
P. brevi-compactum Dierckx	927	34	6.54
P. canescens Sopp	848	8	1.54
P. chrysogenum Thom	5075	42	8.08
P. purpurogenum Stoll	2420	2	0.38
P. variabile Sopp	89	2	0.38
Phoma exigua var. *exigua* Desm.	32	11	2.12
Sarocladium strictum (W. Gams) Summerb.	332	36	6.92
Stachybotrys chartarum (Ehrenb.) S. Hughes	23	3	0.58
Stemphylium botryosum Sacc.	73	9	1.73

Species	TC	NC1	% F
Trichoderma pseudokoningii Rifai	988	44	8.46
T. viride Pers.	283	31	5.96
Ulocladium atrum Preuss	38	6	1.15
U. botrytis Preuss	86	5	0.96
Yeast	1327	17	3.27
Basidiomycota			
Thanatephorus cucumeris (A.B. Frank) Donk	88	7	1.35
Total	108775		

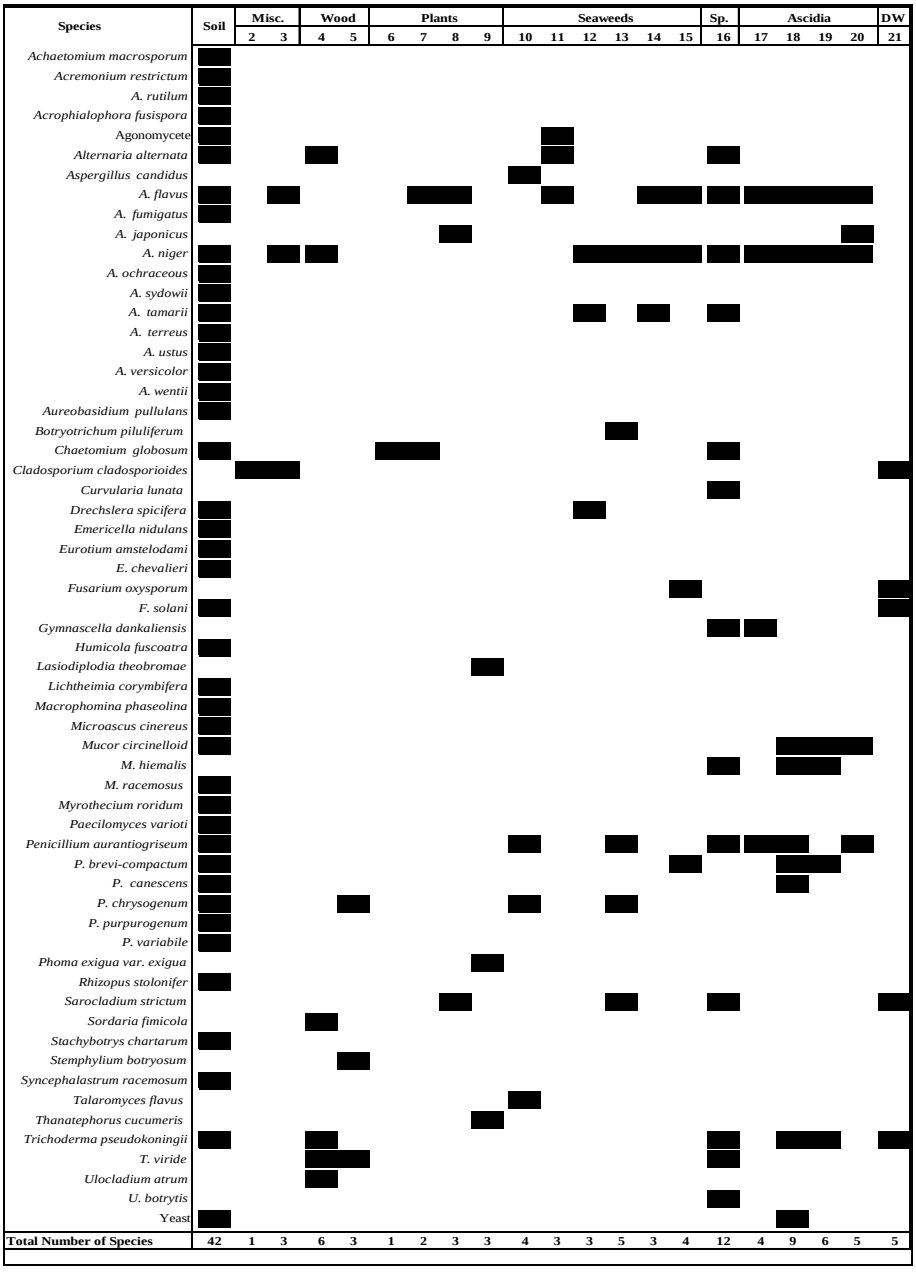

Fig. 1 – Distribution of isolated taxa in different environmental habitats and sites where: 2= contaminated wax samples, 3= contaminated water colours, 4= *Acacia tortilis* subsp. *raddiana* wood, 5= *Ceratonia siliqua* wood, 6= *Adiantum capillus-veneris*, 7= *Phlomis aurea*, 8= *Alkanna orientalis*, 9= roots of *Phlomis aurea* and *Alkanna orientalis*, 10=*Ulva lactuca*, 11= *Caulerpa serrulata*, 12= *C. prolifera*, 13= *Hypnea cornuta*, 14= *Sarconema filiforme*, 15= *Cystoseira myrica*, 16= *Hippospongia communis*, 17= *Phallusia nigra*, 18= *Micsocosmus pupa*, 19= *Styela plicata*, 20= *Polyclinum constellatum* and 21= drifted decaying wood.

Table 4 Simpson diversity index of all studied habitats.

Habitat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Simpson diversity	0.84	0.60	0.82	0.74	0.76	0.00	0.61	0.77	0.62	0.00	0.47	0.65	0.62	0.68	0.52	0.57	0.80	0.66	0.71	0.85	0.79
Standard Deviation	0.00	0.01	0.01	0.01	0.01	0.00	0.02	0.01	0.02	0.00	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
Total Count	97418	1275	1540	464	773	211	338	343	188	122	114	148	181	250	323	223	177	279	247	3668	486

Where: 1= soil, 2= contaminated wax samples , 3= contaminated water colours , 4= *Acacia tortilis subsp. raddiana* wood, 5= *Ceratonia siliqua* wood, 6= *Adiantum capillus-veneris*, 7= *Phlomis aurea*, 8= *Alkanna orientalis*, 9= roots of *Phlomis aurea* and *Alkanna orientalis*, 10=*Ulva lactuca*, 11= *Caulerpa serrulata* , 12= *C. prolifera* , 13= *Hypnea cornuta*, 14= *Sarconema filiforme* , 15= *Cystoseira myrica*, 16= *Hippospongia communis*, 17= *Phallusia nigra*, 18= *Micsocosmus pupa*, 19= *Styela plicata*, 20= *Polyclinum constellatum* and 21= drifted decaying wood

circinelloid (isolated from ascidia) and *Aspergillus tamarii* (from seaweeds) showed weak activity (Table 5), while Agonomycete, isolated from green seaweed, measured about 5 mm colour zone diameter and 12 mm growth colony diameter (moderate laccase activity). Both marine isolates of *Talaromyces flavus* and *Aspergillus candidus* were similar in their oxidation scale and growth, having 28, 25 and 28, 38 mm, respectively.

The fungal endophyte isolates *Ulocladium atrum* and *Alternaria alternata* showed moderate to high oxidation (14, 19 mm and 19, 21 mm, respectively). Other terricolous taxa e.g. *Aureobasidium pullulans*, *Penicillium purpurogenum* and *Stachybotrys chartarum* have the lowest oxidation activity in comparison with the previous taxa.

Optimization of laccase production - Production and activity of laccase was measured by culturing of *Chaetomium globosum* in medium containing different carbon and nitrogen sources at different incubation degrees and pH. Maximum production of laccase (13.3 μ/ml) was reached on the 6th day of incubation in culture broths with maximum biomass between 5-7 days (145-172 mg dry weight).

The optimum pH and incubation temperature were at 7 and 30 ^{o}C, respectively. High level of laccase production was recorded using maltose (3.12 μ/ml) out of the 9 different carbon sources followed by glycerol in comparison with glucose. Other carbon sources had poor growth of the fungus and production of laccase. Laccase production is induced by using peptone in comparison with the five examined nitrogen sources, which showed reduced rate of laccase production. Results of potassium nitrate and sodium nitrate as nitrogen source came second after peptone (2.8 μ/ml), while beef extract was a very poor nitrogen source for production of laccase. Finally, we observed that there is a high increase in laccase production (13.3 μ /ml) when the culture is grown in optimum medium composed of 1% maltose, 0.3% peptone, 0.06% KH_2PO_4, 0.0001% $ZnSO_4$, 0.0%4 K_2HPO_4 0.00005% $FeSO_4$, 0.005% $MnSO_4$ and 0.05% $MgSO_4$ at pH 7 and 30^{o}C (Figs. 3-8).

Table 5 Qualitative assay for laccase enzyme production

Fungal Isolate	Source of Isolation [a]	Mycelial growth and oxidation characteristics		
		Color zone diameter (mm)[b]	Oxidation Scale[c]	Fungal colony diameter (mm)[d]
		Zygomycota		
Lichtheimia corymbifera	S	-	-	60 mm
Mucor circinelloid	As, S	4 mm	+	54 mm
M. hiemalis	As, Sp	-	-	60 mm
M. racemosus	S	-	-	39 mm
Rhizopus stolonifer	S	-	-	70 mm
Syncephalastrum racemosum	S	-	-	35 mm
		Ascomycota (teleomorphic)		
Achaetomium macrosporum	S	-	-	67 mm
Chaetomium globosum	P, S, Sp	40 mm	+++++	58 mm
Emericella nidulans	S	-	-	25 mm
Eurotium amstelodami	S	-	-	28 mm

Fungal Isolate	Source of Isolation [a]	Mycelial growth and oxidation characteristics		
		Color zone diameter (mm)[b]	Oxidation Scale[c]	Fungal colony diameter (mm)[d]
E. chevalieri	S	-	-	26 mm
Gymnascella dankaliensis	As, Sp	-	-	34 mm
Microascus cinereus	S	-	-	15 mm
Sordaria fimicola	W	23 mm	++++	59 mm
Talaromyces flavus	GS	28 mm	++++	28 mm
Ascomycota (anamorphic)*				
Acremonium restrictum	S	-	-	30 mm
A. rutilum	S	-	-	32 mm
Acrophialophora fusispora	S	-	-	27 mm
Agonomycete	GS, S	12 mm	++	5 mm
Alternaria alternata	GS, S, Sp, W	19 mm	+++	21 mm
Aspergillus candidus	GS	25 mm	++++	38 mm
A. flavus	As, BS, GS, P, RS, S, Sp, Wc	-	-	53 mm
A.fumigatus	S	-	-	23 mm
A.japonicus	As, P	-	-	45 mm
A.niger	As, BS, GS, RS, S, Sp, Wc, W	-	-	55 mm
A. ochraceous	S	-	-	22 mm
A.sydowii	S	-	-	17 mm
A. tamarii	Gs, Rs, S, Sp	7 mm	+	18 mm
A. terreus	S	-	-	15 mm
A. ustus	S	-	-	9 mm
A. versicolor	S	-	-	12 mm
A. wentii	S	-	-	9 mm
Aureobasidium pullulans	S	4 mm	+	6 mm
Botryotrichum piluliferum	RS	-	-	8 mm
Cladosporium cladosporioides	Dw, Wc, Wx		-	34 mm
Curvularia lunata	Sp	9 mm	+	19 mm
Drechslera spicifera	GS, S	-	-	11 mm
Fusarium oxysporum	BS, Dw	-	-	10 mm
F. solani	Dw, S	-	-	13 mm
Humicola fuscoatra	S	-	-	8 mm
Lasiodiplodia theobromae	P	-	-	11 mm
Macrophomina phaseolina	S	-	-	17 mm
Myrothecium roridum	S	-	-	15 mm
Paecilomyces variotii	S	-	-	22 mm
Penicillium aurantiogriseum	As, GS, RS, S	-	-	19 mm
P. brevi-compactum	As, BS, S	-	-	50 mm
P. canescens	S	-	-	60 mm
P. chrysogenum	As, GS, RS, S, W	-	-	25 mm
P. purpurogenum	S	8 mm	+	11 mm
P. variabile	S	-	-	22 mm
Phoma exigua var. exigua	R	32 mm	+++++	50 mm
Sarocladium strictum	Dw, P, Sp, RS	-	-	8 mm
Stachybotrys chartarum	S	17 mm	+++	19 mm
Stemphylium botryosum	W	-	-	58 mm
Trichoderma pseudokoningii	As, Dw, S, Sp, W	-	-	70 mm
Trichoderma viride	Sp, W	-	-	64 mm
Ulocladium atrum	W	13	++	19 mm
U. botrytis	Sp	-	-	22 mm
			Basidiomycota	
Thanatephorus cucumeris	R	23 mm	++++	30 mm
** Pleurotus ostreatus*		38	+++++	24

[a] Where: As= Ascidia, BS= Brown Seaweeds, Dw= Drifted Wood, GS= Green Seaweeds, P= Plants, R= Root, RS= Red Seaweeds, S= Soil, Sp= Sponge, W= Wax, W= Wood, Wc= Water colors

* *Pleurotus ostreatus* a well known lignin-degrading white rot fungus used as a positive control.

[b] Diameter of the oxidized zone in mm (measured on the 7th day of cultivation).

[c] Oxidation scale measured on the 7th day of cultivation on modified Czapek's agar medium containing 0.02% 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.

[d] Diameter of the mycelial colony in mm measured on the 7th day of cultivation (the initial disc 5 mm diameter).

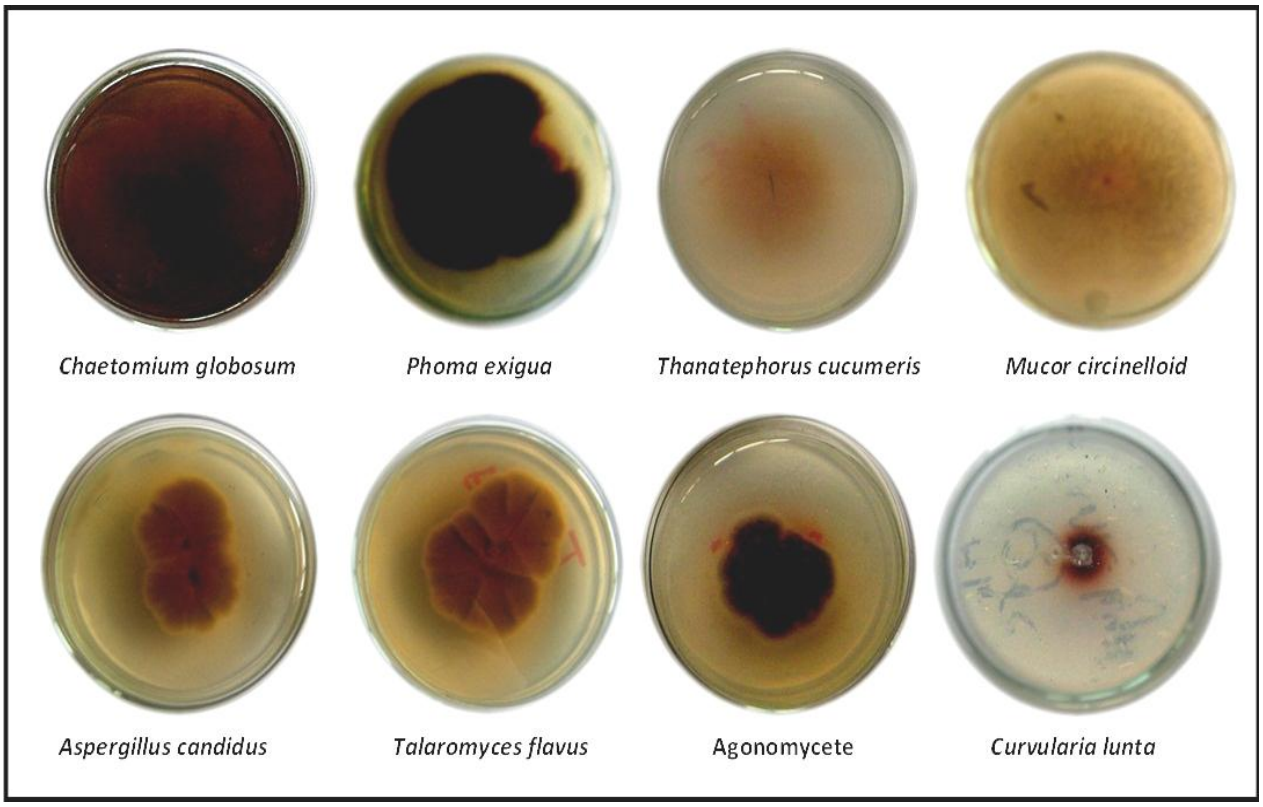

Fig. 2 – Oxidative polymerization of guaiacol to form reddish brown zones in the medium.

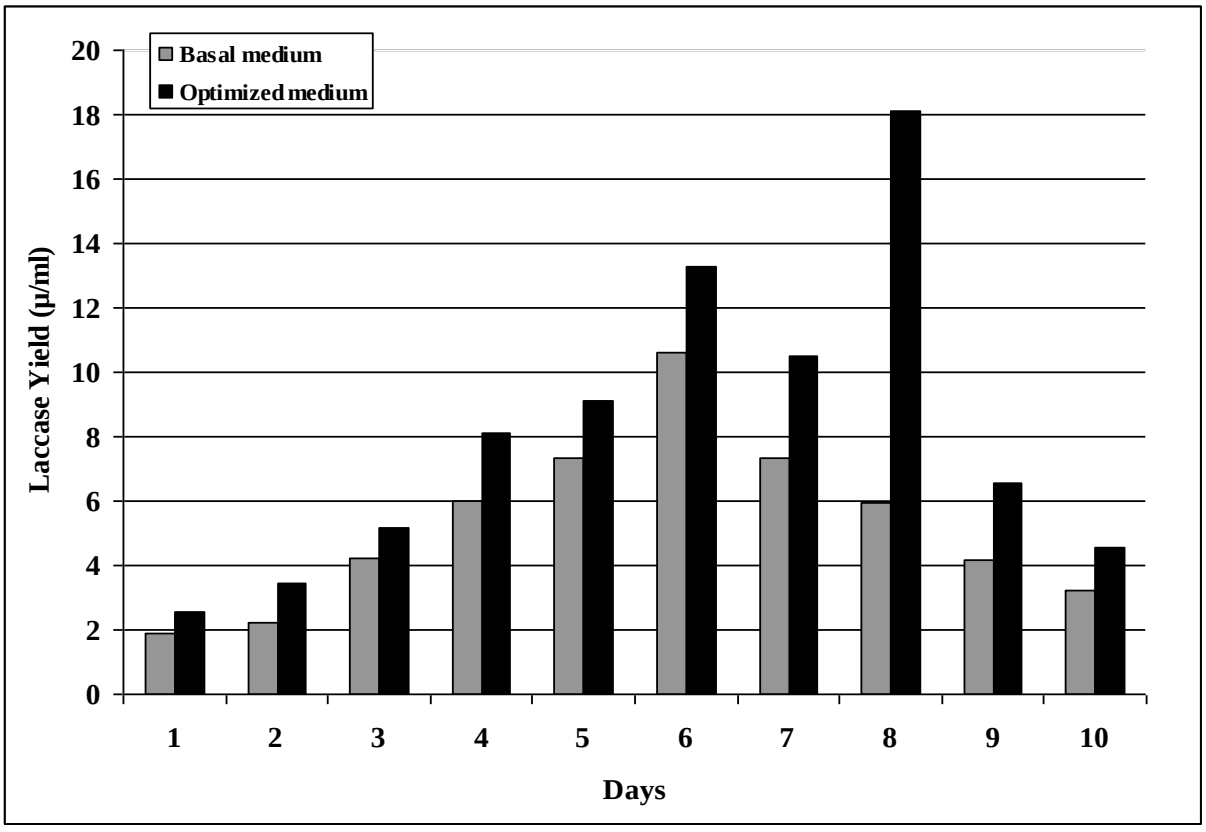

Fig. 3 – Laccase yield of *Chaetomium globosum* on basal and optimized media.

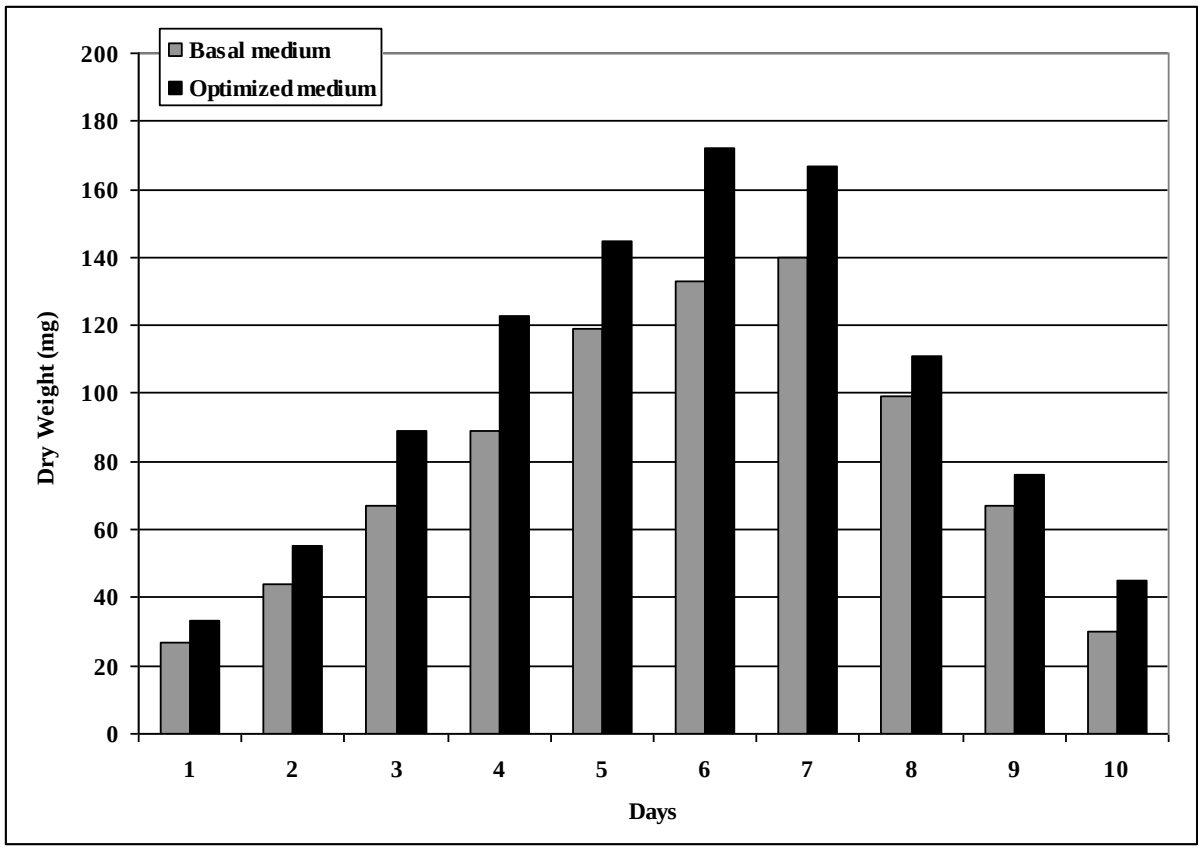

Fig. 4 – Dry weight (mg) of *Chaetomium globosum* on basal and optimized media.

Discussion

Saint Katherine wadis soils showed relatively low counts of fungal populations (97418 CFU in 20 sites) in comparison with the majority of Egyptian cultivated soil (Abdel-Azeem 2009). In this study an average count of 4870.9 CFU/ g was recorded. Most of the taxa have been isolated previously from Egyptian desert soils, but this is the first survey concerning laccase producing taxa in Saint Katherine World Heritage site. The structure of terricolous mycobiota in Saint Katherine area characterized by low counts associated with a narrow spectrum of species have been also reported by other investigators in different arid soils (Moubasher et al. 1985, Abdel-Hafez et al. 2000, Abdel-Sater 1999, Abdel-Azeem 1991, 2003, 2009, Abdel-Azeem & Ibrahim 2004, Abdel-Moneim & Abdel-Azeem 2009.

We found that the composition of marine-derived fungi isolated from seaweeds, drifted decaying wood, sponge and ascidia during this study in agreement with findings of Abdel-Moneim et al. 2010, Atalla et al. (2010).

It is noteworthy that the initial isolation and enumeration of the laccase producing taxa was not carried out by using a substrate specific selective medium, even though, about 25% of the isolates were found positive to laccase production. Fifteen species were recorded to produce laccase during this survey 13 of which were Ascomycota. This finding is in agreement with Hatakka (2001) who showed that Basidiomycota and saprobic fungi are the most widely known species that produce substantial amount of laccase in changeable quantity.

The optimal temperature range for fungal laccase activity ranges from 30º to 60ºC (Wood 1980, Youn et al. 1995, Nishizawa et al. 1995). Laccase from *Chaetomium globosum* examined in this study had an optimal temperature range of 30ºC, which is similar to that of values obtained for laccases from other taxa (Youn et al. 1995, D'Souza et al. 2006a, El-Zayat 2008, Viswanath et al. 2008). *Phanerocheate chrysosporium*, *Pleurotus ostreatus* and *Ganoderma lucidum* had an optimum temperature for growth and production of lignocellulose degrading enzymes of 25-35 ºC (Punnapayak et al. 2002).

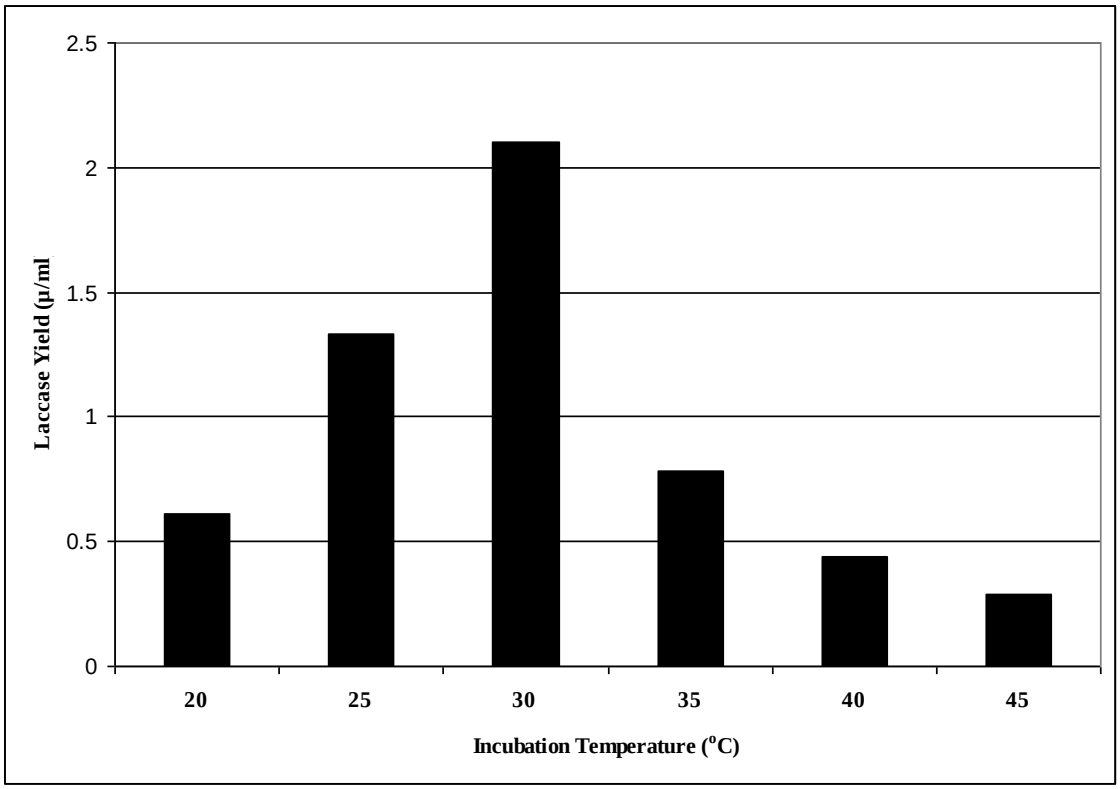

Fig. 5 – Effect of incubation temperature on laccase production by *Chaetomium globosum.*

The optimum pH for laccase activity reported in most fungi is between 3-5 (Galhaup et al. 2002, Jordaan & Leukes 2003, Wesenberg et al. 2003, Niku-Paavola et al. 2004, D'Souza et al. 2006b). Laccase from *Chaetomium globosum*, has maximum activity at pH 7. This result is in agreement with studies by El-Zayat (2008), Nyanhongo et al. (2002) and Kunamneni et al. (2007). While others have mentioned that when fungi are grown at pH 5.0, laccase is produced in excess, most studies have shown that pH between 4.5 and 6.0 is suitable for enzyme production (Thurston 1994).

Most laccase synthesis was obtained when maltose was used as the carbon source and peptone as the nitrogen source; lower yields were obtained with an inorganic nitrogen source. These results are consistent with those of Mikiashvili et al. (2006), Wang et al. (2006) and El-Zayat (2008). The high activity of laccase obtained from the examined fungus, without inducer, is higher than that obtained from *Lentinus* spp. (Pukahutal et al. 2004) and than that from *Chaetomium globosum* (El-Zayat 2008, Varnaitė et al. 2011).

It can be concluded that the distribution pattern of mycobiota, based on the presence/absence in habitats under investigation, showed that recorded taxa (60 species) can be classified into three groups. The first group comprises taxa of occurrence restricted to a single substrate (38 species); the second contains species occurring in two or more habitats but showing some sort of preference for higher occurrence in one of them (18 species); and the third of species common to almost all habitats (4 species). The endophyte isolate *Chaetomium globosum* was selected based on fast and high oxidation rate of guaiacol on agar plates. Culture conditions (pH of 7, 30º C, 1% maltose as a carbon source and 0.3% peptone as a nitrogen source) increased laccase synthesis in submerged cultures of this species. In general the study underlines the need to explore not only more organisms but also lignocellulosic substrates with different composition to express and evaluate the real potential of fungi producing hydrolases and oxidases. In addition, the medium composition must be optimized for each enzyme producer taxon. The present study must be considered, therefore, as a preliminary one and additional investigations are warranted.

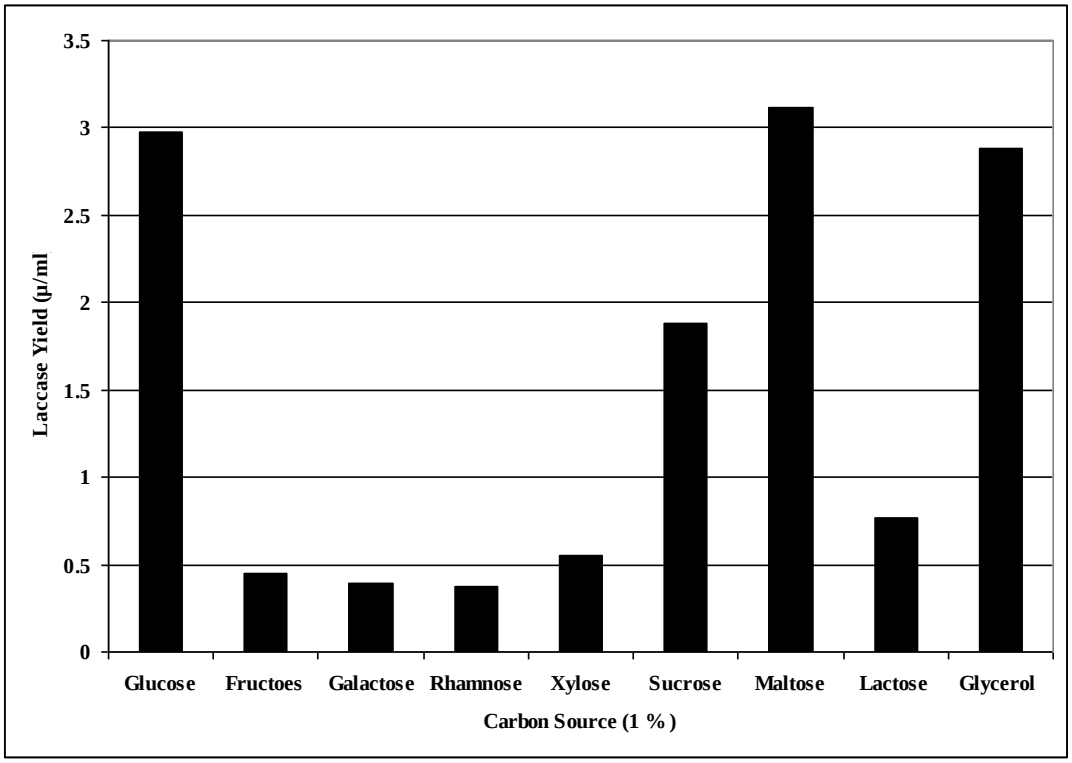

Fig. 6 – Various pH values in relation to laccase production by *Chaetomium globosum*.

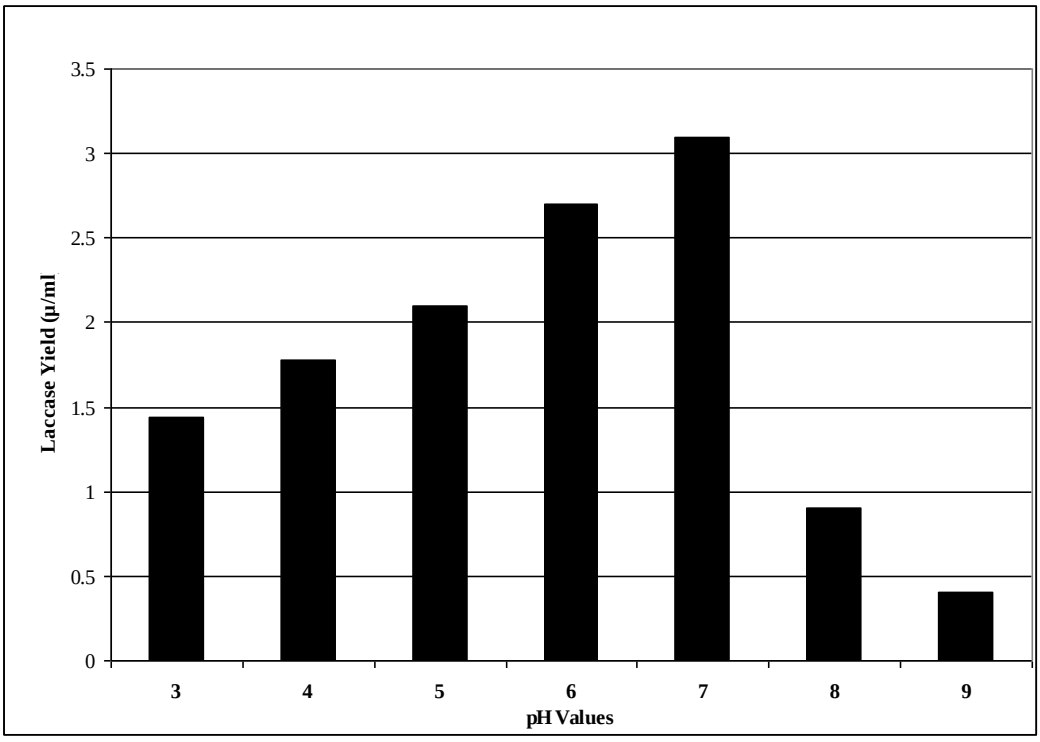

Fig. 7 – Effect of different carbon sources on laccase production by *Chaetomium globosum* at 30°C.

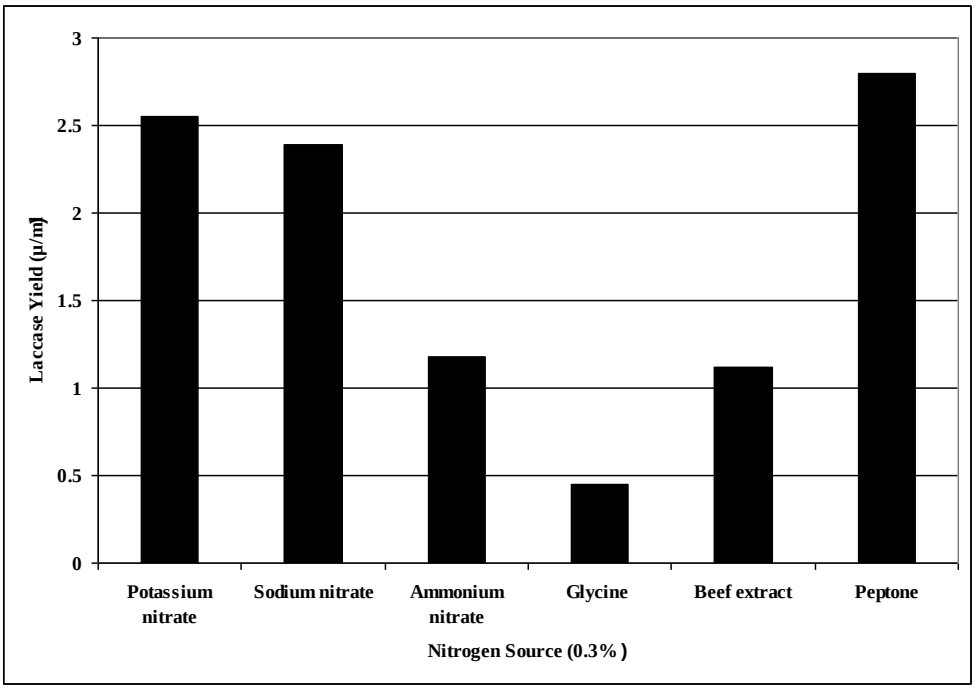

Fig. 8 – Effect of different nitrogen sources on laccase production by *Chaetomium globosum* at 30°C.

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