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Assessment of Fungal Production of Laccases and Peroxidase Required as Potential Method for Biodegradation of Simulated Industrial Wastewater Containing Textile Green Azo Dye

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Abstract: The textile dye residues represent a growing threat to the surrounding environment of this vital industry. Therefore, the appropriate technology for the removal of dye residues from industrial effluents is an important task. One of these technologies is based on the bioremediation that depends on using certain microorganisms to remove these toxic wastes. It is well known that the microbial agents perform all biological transformations through immense enzymatic tools existing within the intact microbial cells. The use of the specific enzymes required for breakdown of certain industrial wastes can reduce the time of residues removal and consequently the cost of the process. In this study the production of two fungal enzymes namely laccases and peroxidases were assessed. To study these enzymes fifteen fungal strains were first screened for their growth on mineral salt medium supplemented with 0.5g/L of green azo dyes as sole source of carbon. The strains could grow on the above mentioned medium that indicates the production of the enzymes necessary for biodegradation of the dye and the support of biodegradation products for the growth of the fungal strains. The efficiency of the fungal strains production of the two important enzymes contributing to the azo dye biodegradation was tested on potatoes dextrose broth medium, throughout 15 days of incubation. In general the production of peroxidases was higher on potato dextrose medium than on mineral salt medium both supplemented with the green azo dye. The amounts of enzyme production were different from one strain to another. The fungal strains produced more laccases and peroxidases on potato dextrose medium than those produced on mineral salt medium. The most promising fungal strains for the production of the two enzymes are strains No. 1 and 2. In this study the direct response of fungal cells to the presence of azo dye in the medium was studied through tracking the fungal cell microscopy image analysis. The electron micrographs showed obvious differences in the cell wall and most of the cell components. The cell wall thickness after contact with the dye solution was almost doubled; moreover new components inside the cells were formed which might be due to the induction of specific enzymes such as the ligninolytic ones.

Key words: Legninolytic enzyme · Fungal strains · Bioremediation · Azo textile dye residues

INTRODUCTION

Dyes and chemicals widely used in textile, paper, plastic, food and cosmetic industries are easily recognized pollutants. Its presence, even in very low concentration, is highly visible and affects aquatic life and cause many diseases. The chemical pollutants discharged in textile industry in general and dye in particular if released into the surrounding environment without treatment will eventually contaminate the Nile River, which represent Egypt's lifeline as the source of all water required for agriculture sector, generation energy, industrial uses and human consumption [1, 2]. Due to the chemical stability of the dye components, conventional wastewater treatment technologies are often ineffective for handling wastewater containing synthetic textile dyes [3]. Considerable research has been done on color removal from industrial effluents to decrease their impact on the environment. These technologies include adsorption onto inorganic or organic matrices, decolorization by photo-catalysis or

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photo-oxidation processes, microbiological decomposition, chemical oxidation, ozonation and coagulation [4]. The advantages and disadvantages of some important methods are discussed in literature [5]. Adsorption has been shown to be the most promising option for the removal of non-biodegradable organics from aqueous streams. Activated carbons are the most common adsorbent for due to their effectiveness and versatility. But it is main disadvantage is the high price and difficult regeneration, which increases the cost of wastewater treatment. Thus, there is a demand for other adsorbents from inexpensive material and do not require any expensive additional pretreatment. In this context the low cost adsorbents can have give advantage for treatment of colored effluents [6, 7]. The degradation of wastewater containing synthetic textile dyes by fungal enzymes proved its success in many studies [8, 9]. Also many studies report that aerobic processes based on activated sludge can be used to degrade dyes further after anaerobic treatment [10, 11, 12, 13]. The idea isthat the aerobic microorganisms have oxidative enzymes that can break down the aromatic amines released during anaerobic color removal. Pure cultures have also been used for degradation of azo dyes. Promising results have been achieved with white-rot fungi, a type of basidiomycete also known as wood-decaying fungi [14]. These fungi produce extracellular enzymes to decompose lignin and thus find access to cellulose, which they use as a carbon source. Lignin is a complex organic molecule composed of many aromatic rings linked to one another. Azo dyes somewhat resemble the structure of lignin and researchers have suggested that lignin-degrading enzymes might also be able to degrade azo dyes. This idea proved correct and thus both white-rot fungi and their oxidative enzymes have been used for dye degradation [15, 16]. However, using pure cultures for treatment of wastewater remains a challenge. In fact, unwanted microorganisms found in water will start to grow and eventually overtake the white-rot fungi thereby destabilizing the system [17]. Thus this work aimed to assess the important role of many fungal enzymes in wastewater containing synthetic textile dyes biodegradation.

MATERIALS AND METHODS

Microbial Media: Potatoes Dextrose Broth (PDB) and PDA media were purchased from Fabrique Par Laboratories Conda, SA. The Mineral Salt Medium (MSM) was prepared as described by (Chao et al., 2006) with modification. It consists of (g l^{-1}): $NH4NO_3$, 0.5; K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2g; NaCl, 0.5; $FeSO_4$, 0.02g; $CaCl_2$, 0.05; $CuSO_4$, 0.02g; pH 5. This medium was used as liquid or solid by adding 2% agar.

The Fungal Isolates: Fifteen fungal isolates were used during this study. The thirteen fungal isolates were isolated during our previous work. The other two fungal strains *Phanerochaete chrysosporium* and *Pleurotus ostreatus* also were included in this study for comparison. These strains were obtained from Microbiology Dept., Agricultural Research Center.

Fungi Maintenance and Inoculum Preparation: Each of the tested fungi isolates was maintained on a slant of PDA. The slant was incubated for one week at 28°C. The inoculum was prepared from each fungal strain by using the same medium in Petri dishes. A disk of each fungal growth was used as an inoculum. Each disk contains 10^5 spores.

Preparation of Biomass for Removing Process: Five grams of 72h mycelia of fungal strains were be transferred aseptically into 250 ml Erlenmeyer flasks containing the sucrose media (100 ml) along with tested dye. The flasks were shaken at 150 rpm and incubated at 30°C 72h. Dye decolorization was determined spectrophotometrically by monitoring the absorbance of samples at k max of the respective dyes using a UV–Visible spectrophotometer (Shimadzu UV 1800, Japan). Results were reported as means of decolorization of the three replicates.

The decolorization was expressed as % of the initial dye concentration and calculated as follows:

% Decolorization = 100 X (A0 _ At)/A0

where A0 is the absorbance value of the initial dye concentration and At is the absorbance value of the dye concentration in sample at time t.

Spore Suspension Preparation: One square cm of mycelium growth (4 to 5 days old culture slants of fungus) were transferred to plates PD medium and incubated for 4 to 5 days to develop enough spores on incubator at 28°C in growth medium. Spores were used as inocula after immobilization in dye removal experiments.

Screening for Dye Biodegradation: The fifteen fungal strains were screened for the green dye bio-degradation efficiency on solid MSM supplemented with 0.05g/L of the dye as a sole source of carbon. The Petri dishes containing solid MSM were inoculated by a disk of the

test fungi that has been maintained at PDA slant and then incubated for one week at 28°C. The grown fungi only were selected for further investigations.

Peroxidases and Laccases Production: Each of the selected fungal isolates was inoculated in 100-ml of both MSM supplemented with 0.05g/l of the Direct green dye and PDB. The flasks were incubated for 15-days at 28°C on rotary shaker at 150rpm. A volume of the culture was taken from both cultures every three days for peroxidases and laccases assay. One ml from the MSM culture was taken every day for determination of the remaining dye by HPLC.

Enzymes Assays: Both peroxidases and laccases activities were determined in both cultures, the enzyme crude extract after enzyme precipitation and the immobilized enzyme.

Peroxidases Assay: In this method, the rate of formation of purpurgallin from the reaction between pyrogallol as a hydrogen donor and hydrogen peroxide as an electron acceptor catalyzed by peroxidase was determined spectrophotometrically by measuring the rate of color development at 420 nm.

Unit Definition: That amount of enzyme which catalyzes the production of one milligram of purpurogallin in 20 seconds at 20°C. at a pH of 6.0.

Peroxidases activity was determined in the microbial culture filtrate, the crude enzyme precipitate and the immobilized enzyme.

Reagents:

- 100 mM Potassium phosphate buffer, pH 6.0.
- 0.50% (w/w) Hydrogen peroxide solution (H_2O_2) (freshly prepared).
- 5% (w/v) Pyrogallol solution (freshly prepared and Protected from light).
- Peroxidase Enzyme Solution: the microbial culture or the enzyme precipitate dissolved in solution (A).

Procedure:

Reagent	Test	Blank
Buffer solution (ml)	7.5	7.5
Hydrogen peroxide solution	50µl	50µl
Pyrogallol solution (ml)	0.5	---
Buffer solution (ml)	--	0.5 ml
Enzyme solution (ml)	2.5 ml	2.5ml

Mix by inversion and equilibrate to 20°C. Monitor the A 420nm until constant O.D. Then add:

Immediately mix by inversion and record the increase in A 420 nm for approximately 5 minutes. Obtain the A 420nm/20 seconds using the maximum linear rate.

Calculation:
One unit/ ml = Delta OD/20sec X 10.55/ 12 X 2.5

where:
12 = Extinction coefficient of 1 mg/ml of purpurogallin at 420 nm
2.5 = Volume (in milliliters) of enzyme solution.
10.55 is the total volume of the reaction mixture.

For determination of peroxidases activity in the crude enzyme precipitate, 0.1 ml of the enzyme solution was used; while 0.5 g of the alginate or chitosan materials were used for determination of the immobilized enzyme activity.

Laccases Assay: Laccase activity was measured with 2, 6-dimethoxyphenol (DMP, Sigma, St Louis, MO, USA) according to Palmieri et al., (1997) [27] at 477 nm by monitoring dopachrome formation and using the molar extinction coefficient ε 477 = 18400 M-1 cm^{-1}. Activities were expressed as µÌ min-1. One unit of laccase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µM of DMP/ min. at 25°C. Laccases activity was determined in the microbial culture filtrate, the crude enzyme precipitate and the immobilized enzyme.

Procedure: One ml of enzyme solution (the culture filtrate) was added into the cuvette followed by addition of 400µl of DMP solution (0.8% dissolved in acetate buffer, pH5) and 600µl acetate buffer solution. The auto zero was measured immediately and the O.D. was measured after 30sec and one min.

For determination of laccases in the crude enzyme precipitate, 0.1 ml of the enzyme solution was used; while 0.5 g of the alginate or chitosan materials were used for determination of the immobilized enzyme activity.

Calculation:
One units = (delta OD/min/ 18400) X 106 X2

where:
18400 is the molar extinction coefficient of DMP at 477nm
10^6 to convert the molar concentration to micromolar 2 is the dilution factor, where one mml of the enzyme solution is diluted by the reaction mixture solutions to two ml.

Enzymes Precipitation: 100 ml of the culture was filtered through a filter paper to remove the biomass. 400ml of cold acetone was added drop-wise from a reparatory funnel under continuous stirring in an ice path. The solution was left at 5°C overnight. The crude enzyme precipitate was separated by centrifugation under cooling at 6000 rpm for 10 min. The enzyme precipitate was dissolved in either 5-ml phosphate buffer (pH 6) for peroxidases or in acetate buffer (pH 5) for laccases.

RESULTS

Biodegradation of Direct Green Dye by Some Fungal Strains: The fifteen tested fungal strains was identified previously could grow on the MSM supplemented with 0.05g/l of the dye as a sole source of carbon. This indicates the ability of these strains to utilize the dye as a carbon source which means that these strains might be capable to degrade the tested dye. Therefore the capacity of these strains to produce peroxidases and laccases enzymes known to take part in the biodegradation of the dye was tested after growing the strains in both PDB and liquid MSM supplemented with 0.05 g/l of the dye as a sole source of carbon.

The Enzyme Production by Fungal Strains

Peroxidases Production: The peroxidases production by the fifteen fungal strains was studied in both MSM supplemented with the dye and PDB throughout the incubation period that lasted for 15 to compare the production efficacy of each fungal isolate under the two nutritional conditions pf the growth media. The levels of enzyme production was monitored by measuring, spectrophotometrically, the changes in purpurgallin concentration every one minute interval since the start of the reaction throughout five consecutive minutes to allow the enzyme reaction to reach the maximum and stabilize. Figures (1-5) show the peroxidase assay results in fungal cultures grown in MSM after 3, 6, 9, 12 and 15 days. Figures (6-10) show the assay of the same enzyme by the same fungal strains grown in PDB medium after the same sampling periods. The fungal strains were different in the peroxidases production and activities. The enzyme production as units/L is presented in Tables 1 and 2 for fungal strains grown in the two media.

Peroxidase Production in MSM: The optimum time for maximum peroxidase production varied depending on the fungal strain and the medium type. In case of MSM none of the fungal strains showed maximum peroxidases production after three days. Four isolates (1, 5, *Aspergillus tubingensis* 11 and *Phanerochaete chrysosporium*) showed their maximum production after six days. The production was 15.8, 15, 6.1 and 18.8 Unit/ L for the four fungal strains respectively. Five other fungal isolates (6, 31, 39, 53, *Pleurotus ostreatus* and *Phanerochaete chrysosporium*) showed their maximum peroxidase production after nine days. Their enzyme production was 22.2, 31.8, 25.3, 25.9 and 34.4 Units/l, respectively. After 12 days, only two fungal strains (21 and 24) showed their maximum peroxidase production (20.4 and 13.2 U/L, respectively). The remaining four fungal strains (2, F2, 20 and 37) showed their maximum peroxidase production after 15-days. Their enzyme production was 12.6, 14.1, 47.8 and 24.3 U/L, respectively.

Peroxidase Production in PDB: The enzyme production by fungal strains in PDB medium showed that only one fungal strain (37) gave maximum peroxidase production after 3-days (32.2 U/L). This value is higher than that obtained from the same fungus in MSM, which was 24.3 U/L after 15 days of incubation.

Three fungal strains (5, 11 and 20) showed the maximum peroxidase production after 6-days of incubation (102.1, 91.9 and 17.1 U/L, respectively). These values are also higher than that obtained by the fungi 5 and 11 in MSM after the same incubation period. The production increased more than six folds for the fungus 5 and about 15 folds for the fungus 11. However, the peroxidase production by the fungus 20 was higher in the MSM (47.8 U/L) than in the PDB (17.1 U/L), although it was obtained after 15-days of incubation in MSM.

After 9-days of incubation, only one fungal strain (6) showed the maximum peroxidase production (19.5 U/L). This fungal strain showed the maximum peroxidase production after the same incubation period in MSM, although it was higher than that obtained in PDB (22.3 U/L).

After 12-days of incubation, five fungal strains (1, 2, F2, 39 and 53) showed the maximum peroxidase production. The enzyme produced by strains 1, 2, F2 and 39 in PDB was higher than that obtained by the same strains grown in MSM medium.

After 15-days of incubation, the remaining five fungal strains (21, 24, 31, *Pleurotus ostreatus* and *Phanerochaete chrysosporium*) showed the maximum peroxidase production (31.2, 38.1, 90.8, 44.9 and 44.8 U/L, respectively). All of these values are also higher that those obtained by the same fungal strains in MSM medium.

Table 1: Peroxidase production (Units*/L) in MSM by the fifteen fungal strains

	Incubation time (days)				
Fungal strains number	3	6	9	12	15
1	3.84	15.83	14.65	7.27	7.27
2	2.44	9.14	8.79	1.17	12.66
F2	3.61	4.45	13.01	2.46	14.18
5	5.35	15.00	9.03	1.52	6.56
6	5.72	3.40	22.27	1.29	8.79
11	4.60	6.10	1.29	4.34	3.17
20	5.35	9.73	15.59	36.10	47.83
21	6.89	1.52	20.05	20.40	11.84
24	3.70	0.82	12.07	13.25	12.07
31	6.80	18.17	31.88	13.72	13.48
37	6.14	0.82	10.90	10.78	24.27
39	9.99	12.43	25.32	12.54	20.75
53	11.30	7.74	25.91	19.46	17.11
Pleurotus ostreatus	13.22	7.27	34.46	17.35	21.22
Phanerochaete chrysosporium	2.91	18.87	8.67	9.26	10.43

*One unit was expressed as the enzyme amount that catalyzes formation of 1mg of purpurgallin per 20sec.

Table 2: Peroxidase production (Units/L) in PDB by the fifteen fungal strains

	Incubation time (days)				
Fungal strains number	3	6	9	12	15
1	2.1	7.0	12.3	83.7	18.9
2	3.5	9.4	12.7	36.6	7.6
F2	4.5	16.1	15.4	74.1	13.4
5	3.0	102.1	14.7	16.6	18.5
6	7.0	7.5	19.5	19.1	18.8
11	4.3	91.9	13.4	84.2	48.1
20	2.5	17.1	5.5	7.4	16.6
21	12.3	10.6	11.8	25.9	31.2
24	6.4	7.9	11.1	34.9	38.1
31	12.0	44.0	10.2	12.5	90.8
37	32.2	21.3	11.8	2.2	11.1
39	6.6	16.1	5.6	55.6	35.0
53	6.8	4.3	13.9	18.4	10.9
Pleurotus ostreatus	34.6	27.7	26.0	3.3	44.9
Phanerochaete chrysosporium	21.9	21.6	22.2	13.6	44.8

*One unit was expressed as the enzyme amount that catalyzes formation of 1mg of purpurgallin per 20sec

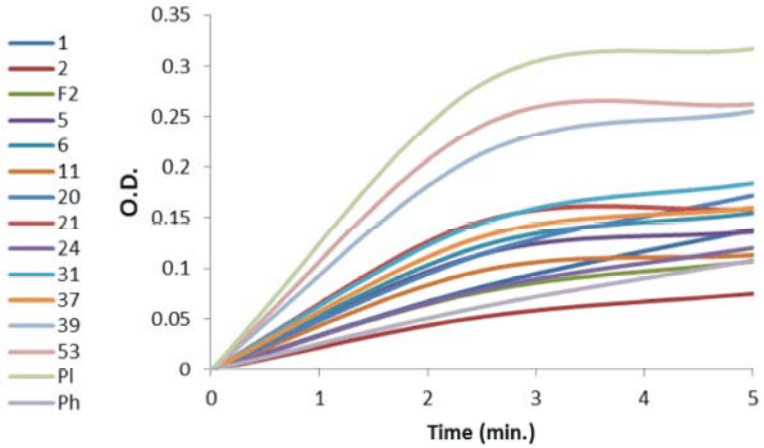

Fig. 1: Peroxidase assay results for the 15 fungal strains in MSM after 3-days

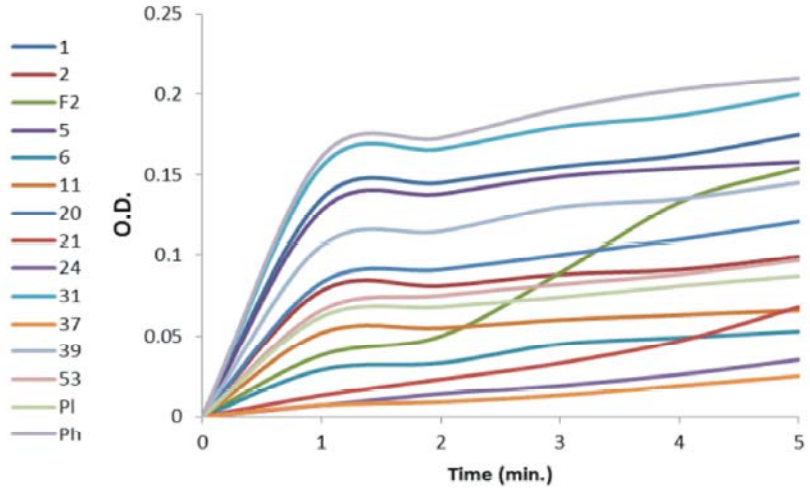

Fig. 2: Peroxidase assay results for the 15 fungal strains in MSM after 6-days

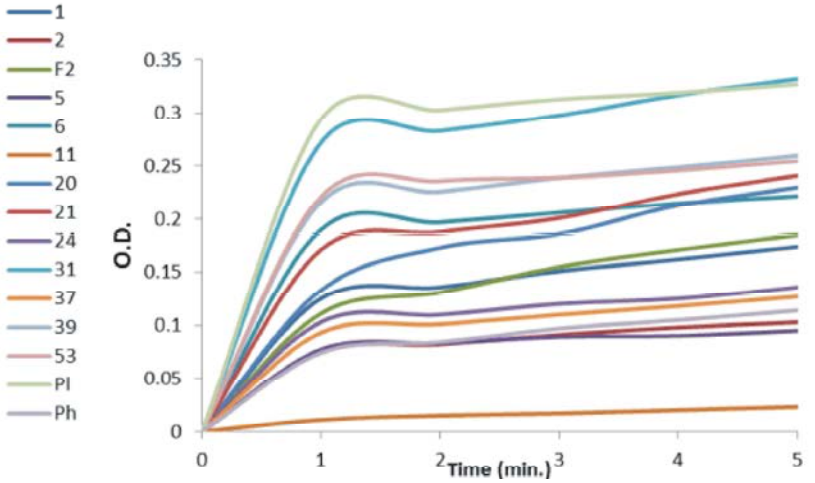

Fig. 3: Peroxidase assay results for the 15 fungal strains in MSM after 9-days

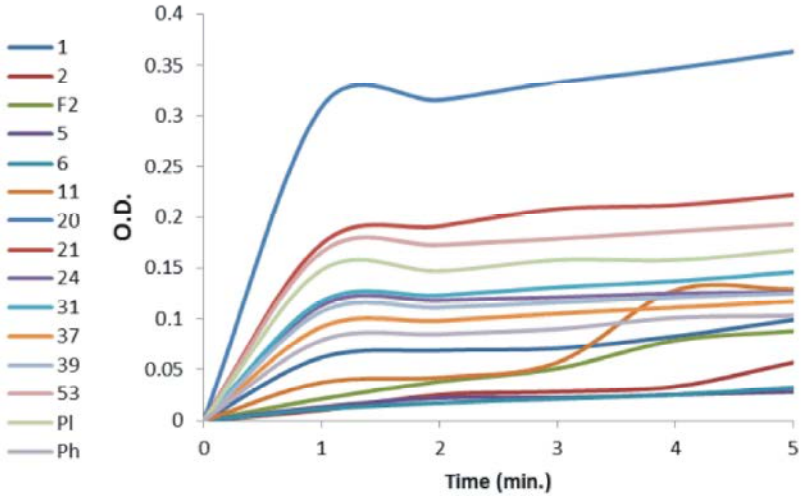

Fig. 4: Peroxidase assay results for the 15 fungal strains in MSM after 12-days

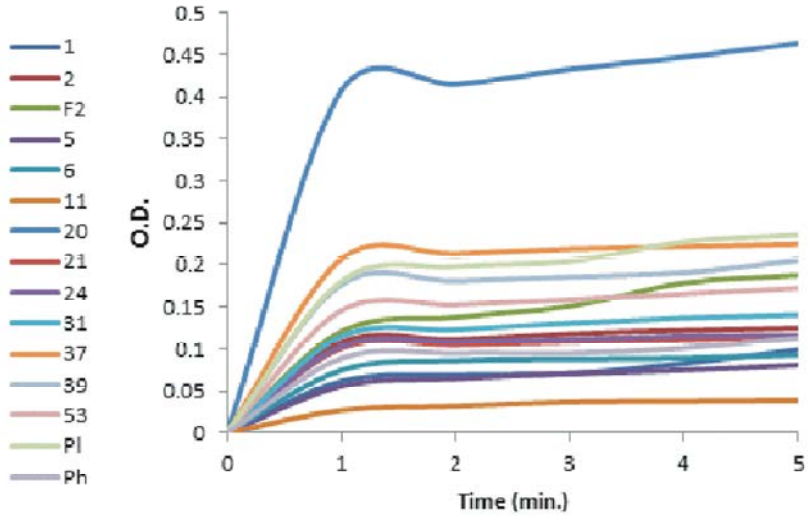

Fig. 5: Peroxidase assay results for the 15 fungal strains in MSM after 15-days

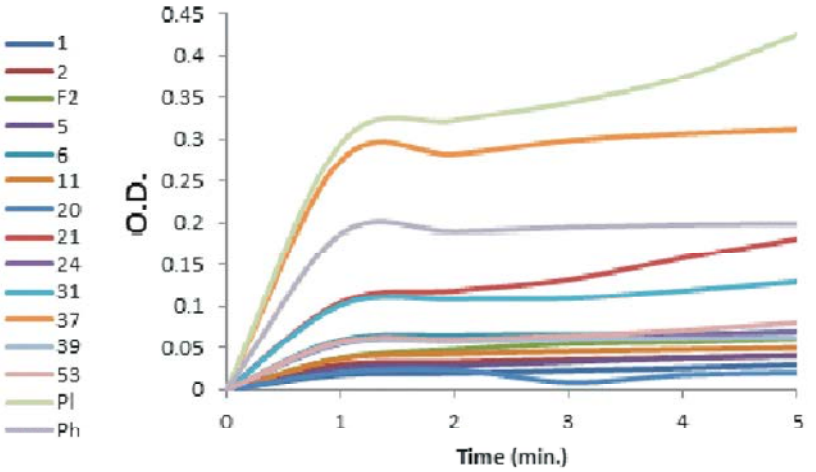

Fig. 6: Peroxidase assay results for the 15 fungal strains in PDB after 3-days

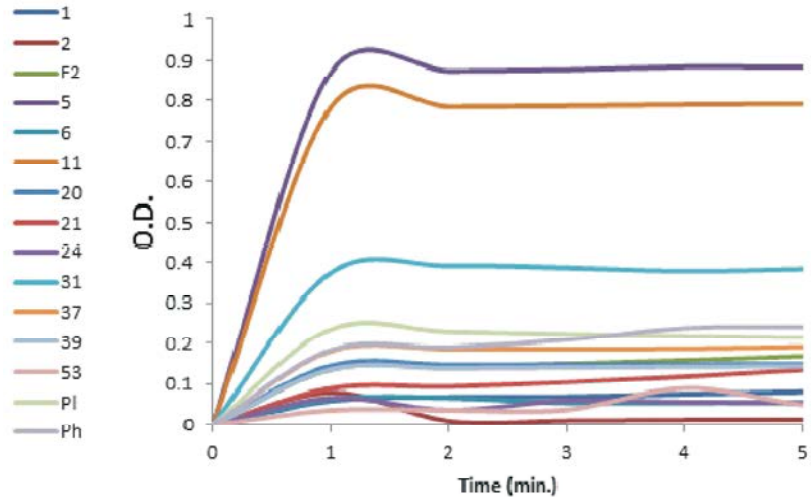

Fig. 7: Peroxidase assay results for the 15 fungal strains in PDB after 6-days

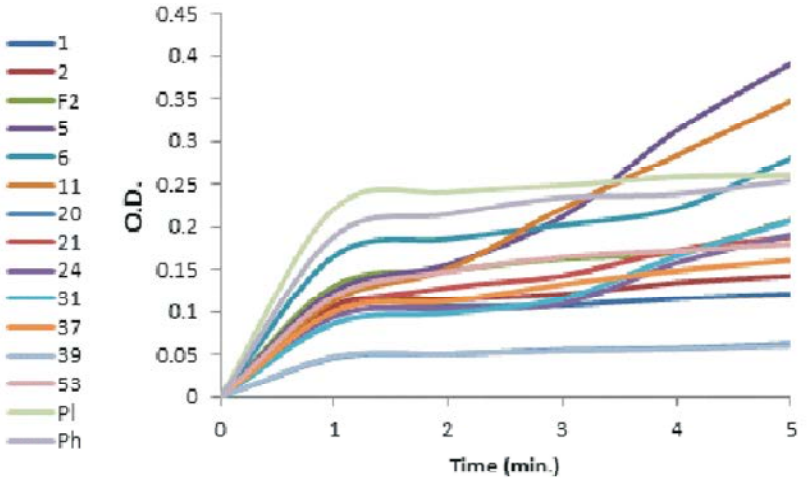

Fig. 8: Peroxidase assay results for the 15 fungal strains in PDB after 9-days

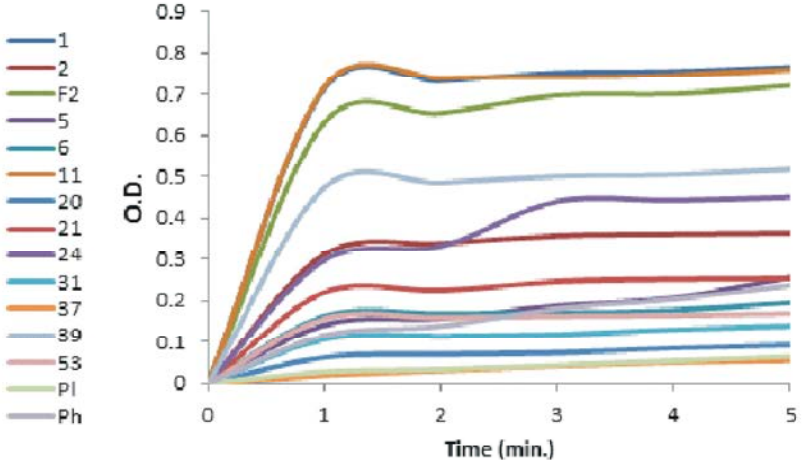

Fig. 9: Peroxidase assay results for the 15 fungal strains in PDB after 12-days

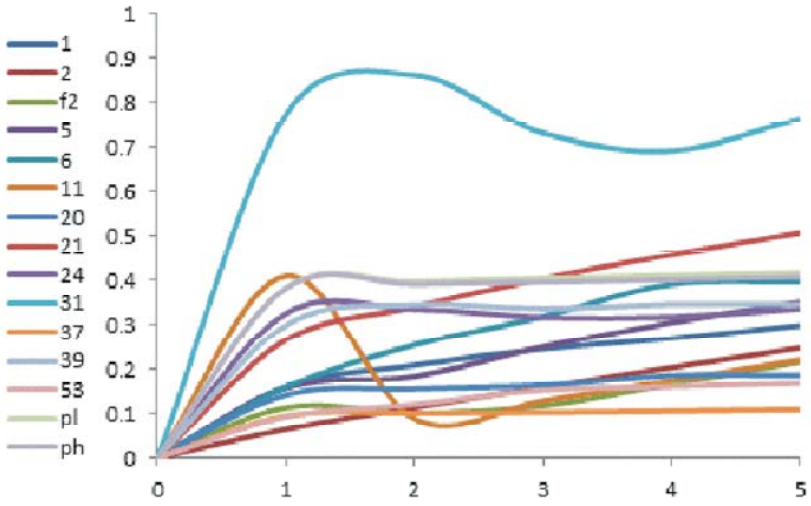

Fig. 10: Peroxidase assay results for the 15 fungal strains in PDB after 15-days

Table 3: Laccases production by fifteen fungal strains in MSM (units*/L)

	Incubation time (days)				
Fungal strains number	3	6	9	12	15
1	9.86	0.95	3.11	2.03	2.97
2	20.68	2.03	1.62	4.19	6.08
F2	4.73	0.68	0.68	1.22	1.35
5	13.92	0.27	0.68	0.81	0.68
6	8.38	0.41	0.54	0.54	0.41
11	2.30	2.97	0.27	1.08	1.76
20	5.27	2.03	0.68	1.08	1.35
21	16.49	1.00	2.03	0.81	1.08
24	27.70	1.22	0.41	0.27	0.54
31	18.51	0.27	4.73	0.95	1.35
37	7.16	1.62	0.41	2.03	3.11
39	13.92	2.43	0.41	0.41	0.27
53	4.05	2.03	1.49	2.84	4.05
Pleurotus ostreatus	19.86	2.84	1.76	7.43	10.41
Phanerochaete chrysosporium	4.32	1.22	3.11	1.62	5.00

*One unit was expressed as the enzyme amount that catalyzes oxidation of 1µM, DMP/min.

Table 4: The laccases production by fifteen fungal strains in PDB (units/L)

	Incubation time (days)				
Fungal strains number	3	6	9	12	15
1	0.68	1.22	1.89	3.24	204.86
2	3.51	0.41	66.62	36.89	306.62
F2	4.46	0.00	2.84	2.03	1.35
5	0.68	0.27	0.54	2.16	0.81
6	12.30	0.00	1.89	34.59	12.38
11	2.70	0.95	65.27	77.70	216.08
20	19.73	9.19	21.49	8.24	3.92
21	2.30	0.41	2.30	19.86	0.68
24	0.54	2.84	16.89	1.49	77.16
31	0.54	0.14	16.76	19.32	165.68
37	0.95	0.14	0.95	0.27	0.41
39	0.68	3.11	7.57	1.49	85.41
53	0.81	0.41	0.95	1.62	0.68
Pleurotus ostreatus	117.30	2.84	1.22	4.59	303.92
Phanerochaete chrysosporium	206.89	5.41	201.08	1.89	0.81

*One unit was expressed as the enzyme amount that catalyzes oxidation of 1µM DMP/min

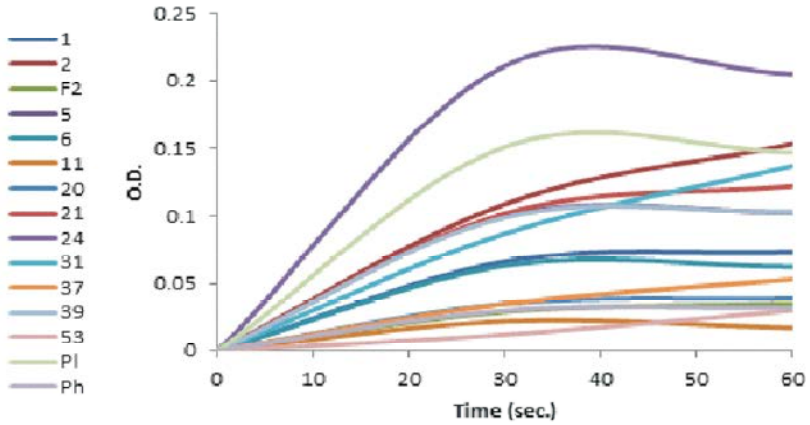

Fig. 11: Laccases assay results for the 15 fungal strains in MSM after 3-days

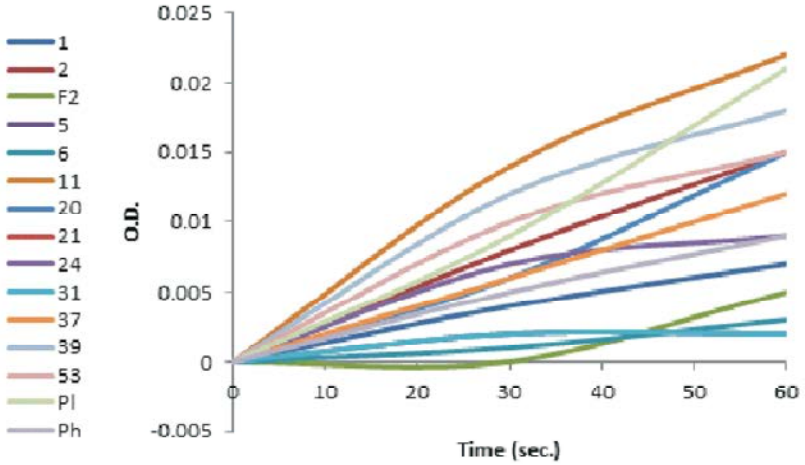

Fig. 12: Laccases assay results for the 15 fungal strains in MSM after 6-days

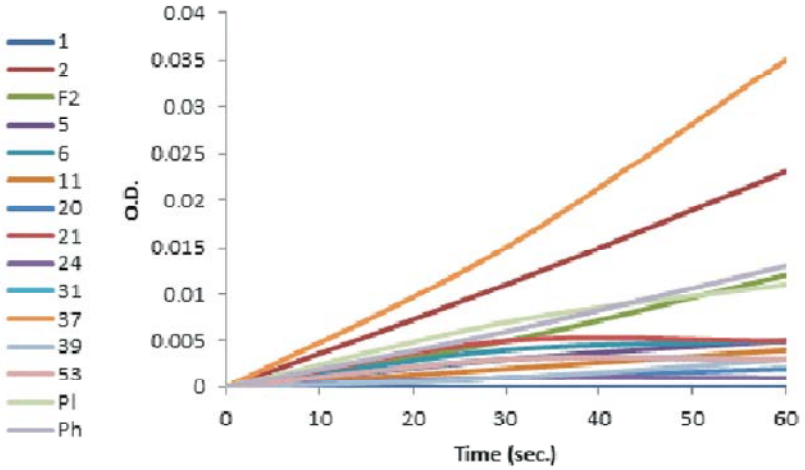

Fig. 13: Laccases assay results for the 15 fungal strains in MSM after 9-days

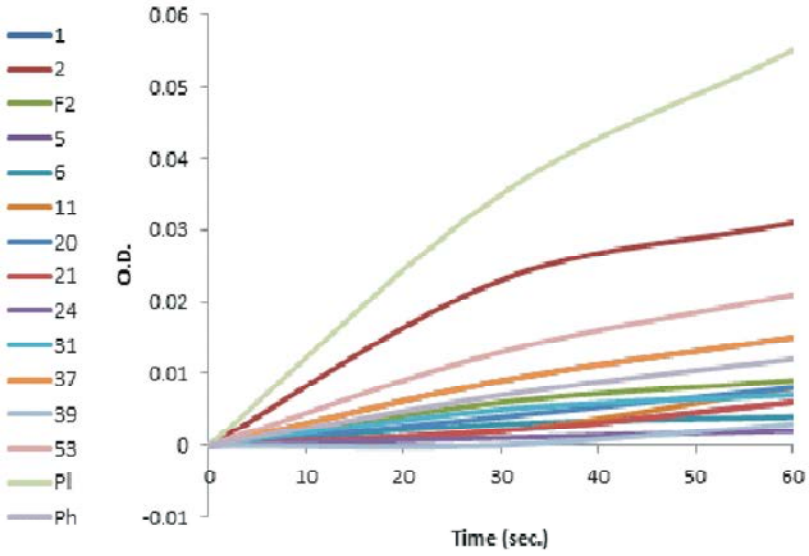

Fig. 14: Laccases assay results for the 15 fungal strains in MSM after 12-days

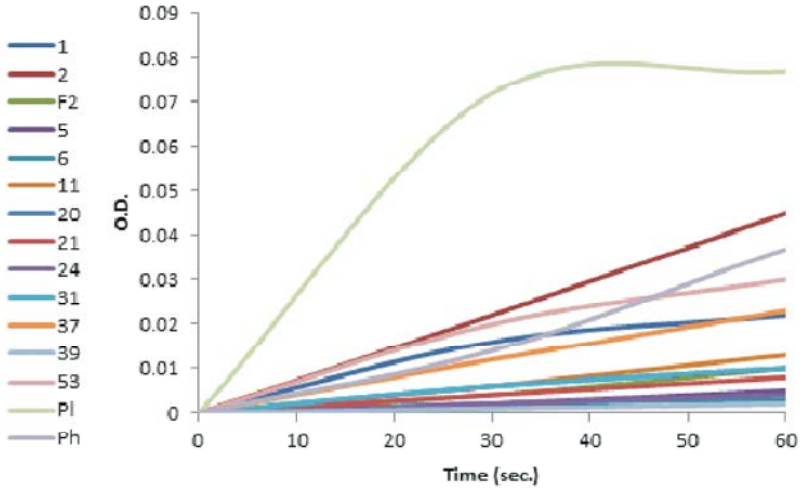

Fig. 15: Laccases assay results for the 15 fungal strains in MSM after 15-days

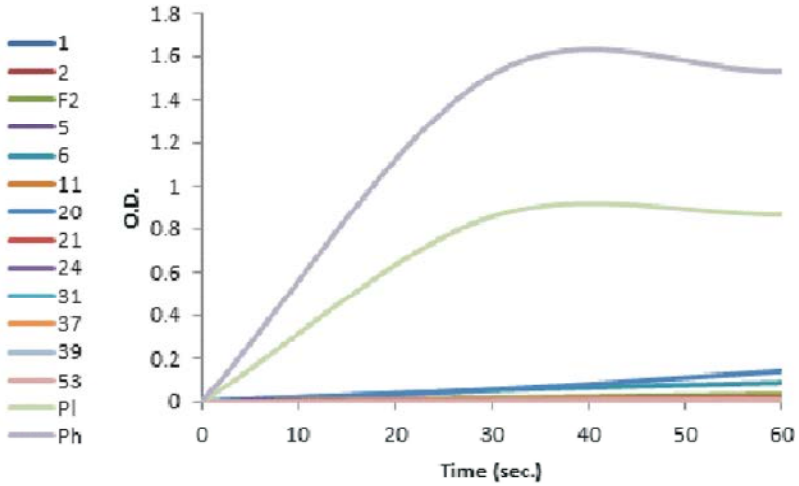

Fig. 16: Laccases assay results for the 15 fungal strains in PDB after 3-days

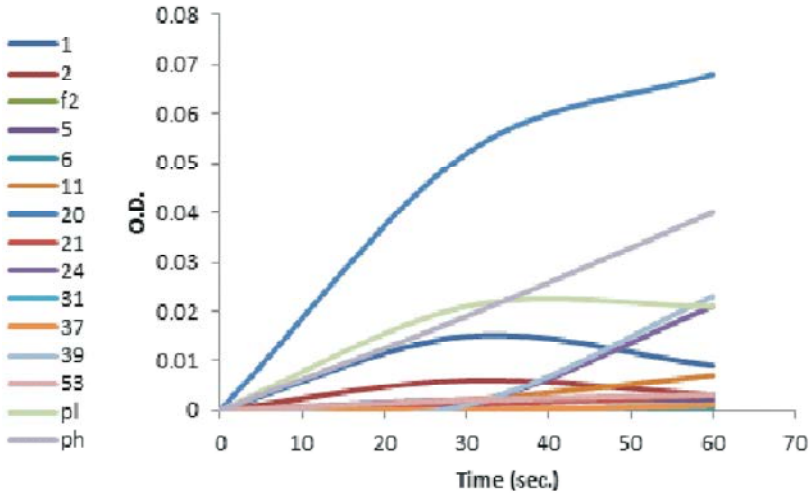

Fig. 17: Laccases assay results for the 15 fungal strains in PDB after 6-days

Laccases Production: The laccases production by the fifteen fungal strains was studied in both MSM supplemented with the dye and PDB throughout the incubation period that lasted for 15 to compare the production efficacy of each fungal isolate under the two nutritional conditions the growth media. The levels of enzyme production was monitored by measuring spectrophotometrically, the changes in dopachrome concentration every 10 seconds interval since the start of the reaction throughout 50 consecutive seconds to allow the enzyme reaction to reach the maximum and stabilize. Figures (11-15) show the results for laccases assay in MSM fungal growth medium, while Figures (16-20) show the assay results for laccases in PDB medium. The laccases production (U/L) by the tested fungal isolates is presented in Tables 3 and 4.

Laccases Production in MSM: All tested fungal strains showed the maximum laccases production after 3-days of incubation, except only two strains (2 and *P. chrysosporium*) which gave the maximum production after 6 and 15-days, respectively.

Laccases Production in PDB: After 3-days of incubation, three fungal strains (F2, 37 and *P. chrysosporium* showed the maximum laccases production (4.4, 0.95 and 206.9 U/L, respectively). None of the strains showed maximum laccases production after 6-dayes, whereas only one strain (21) showed its maximum production (21.5 U/L) after nine days of incubation. After 12-days of incubation, four fungal strains (5, 6, 21 and 53) showed their maximum laccases production (21.2, 34.6, 19.9 and 1.6 U/L, respectively). After 15-days, the remaining fungal strains (1, 2, 11, 24, 31, 39 and *Pleurotus ostreatus*) gave showed large amounts of laccases production (204.9, 306.7, 216, 77.2, 165.7, 85.4 and 303.9 U/L, respectively).

In summary the maximum production of peroxidases and laccases regardless the time required to reach these maximums are presented in Table (5). As has been previously shown the maximum production of both enzymes by the fungal strains was greatly affected by the medium composition, as it greatly vary from MSM and PDB media for the same fungal strain.

The peroxidase production by fungal strain1 on PDR medium was five folds more than that produced on in MSM. The same phenomena were also observed with laccases; where laccases production was increased from 9.8 on MSM medium to 204.8U/L on PDB. The same trend was also noticed for fungal strains 2, 11, 21, 24, 31, 39, *Pleurotus ostreatus* and *Phanerochaete chrysosporium;* where both peroxidases and lacccases production were higher in PDB than in MSM. The incubation time required to reach the maximum enzyme production was also varied from one medium to another (Tables 1-4).

The wide variations in enzyme production on the two different media may be due to that the higher nutritional value of PDB, as it contains several growth factors such as vitamins, which are absent in the MSM. The presence of vitamins and elements in the PDB may function as co-enzymes which enhance the performance of both peroxidases and laccases enzymes. The same was observed with peroxidase production by the fungal strains F2, 5, 11 and 37 which were higher in the PDB than in MSM. On the contrary the laccases production by the fungal strains 5, 37 and 53 was higher in the MSM medium than in PDB. This may be due to the specific induction of laccases in these strains in presence of the proper substrate. Therefore, its production was enhanced by presence of the green dye) in the MSM. From the results it is clear that, the highest peroxidase production (47.8 U/L) on MSM medium was obtained by the fungus (20); while on the PDB the highest production was 102.1 U/L (the maximum in all tested cases) by the fungus (5). These maximum values for both strains were obtained after 6-days of incubation Table (2).

The highest laccases production (27.7 U/L) In the MSM was obtained from the fungus (24) after 3-days of incubation; while in PDB it was 306.6 U/L from the fungus (2) after 15-days of incubation. This value was the highest laccases production obtained in this study followed by 303.9 U/L obtained by *Pleurotus ostreatus* in the PDB after 15-days of incubation for the both fungi.

Fungal Cell Microstructure Changes Due to Exposure to Direct Dye Residues: The integrity of fungal cell component is crucial for the function and performance of complex biological system of the cells including the strong enzymatic machinery contributing to different aspects of fungal life and the environment surrounding it. It is documented that the major enzymes of fungal cells are usually attached to endo-plasmatic membranes existing in the cell and to the permeability of cell membranes particularly for the exo-enzymes released from the cell wall to the outer ecosystem. The study of the fungal cell microstructure changes is therefore, important to better understand the correlation between the activities of enzymes involved in dye biodegradation.

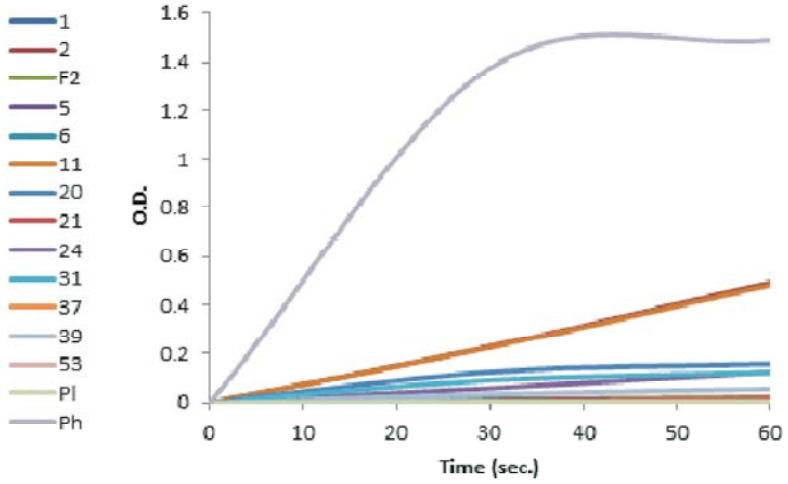

Fig. 18: Laccases assay results for the 15 fungalstrains in PDB after 9-days

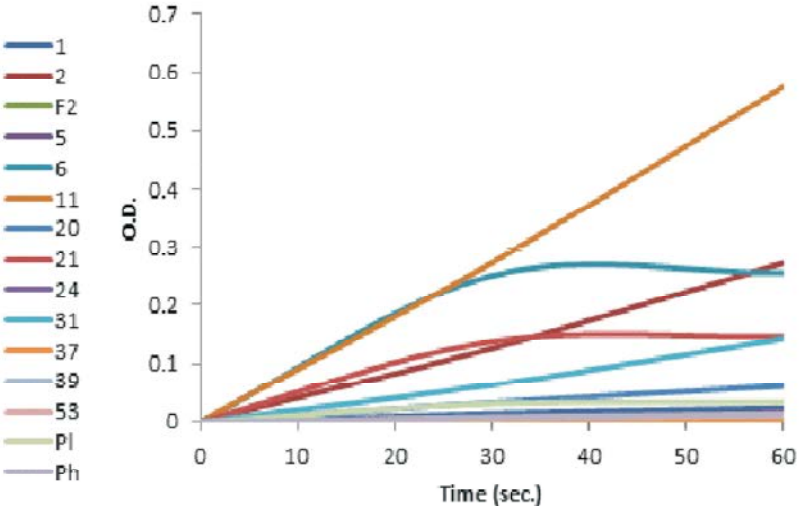

Fig. 19: Laccases assay results for the 15 fungal strains in PDB after 12-days

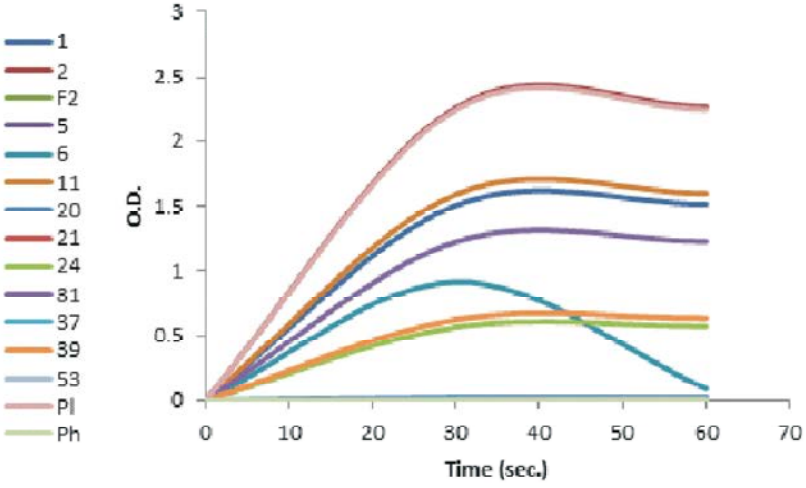

Fig. 20: Laccases assay results for the 15 fungal strains in PDB after 15-days

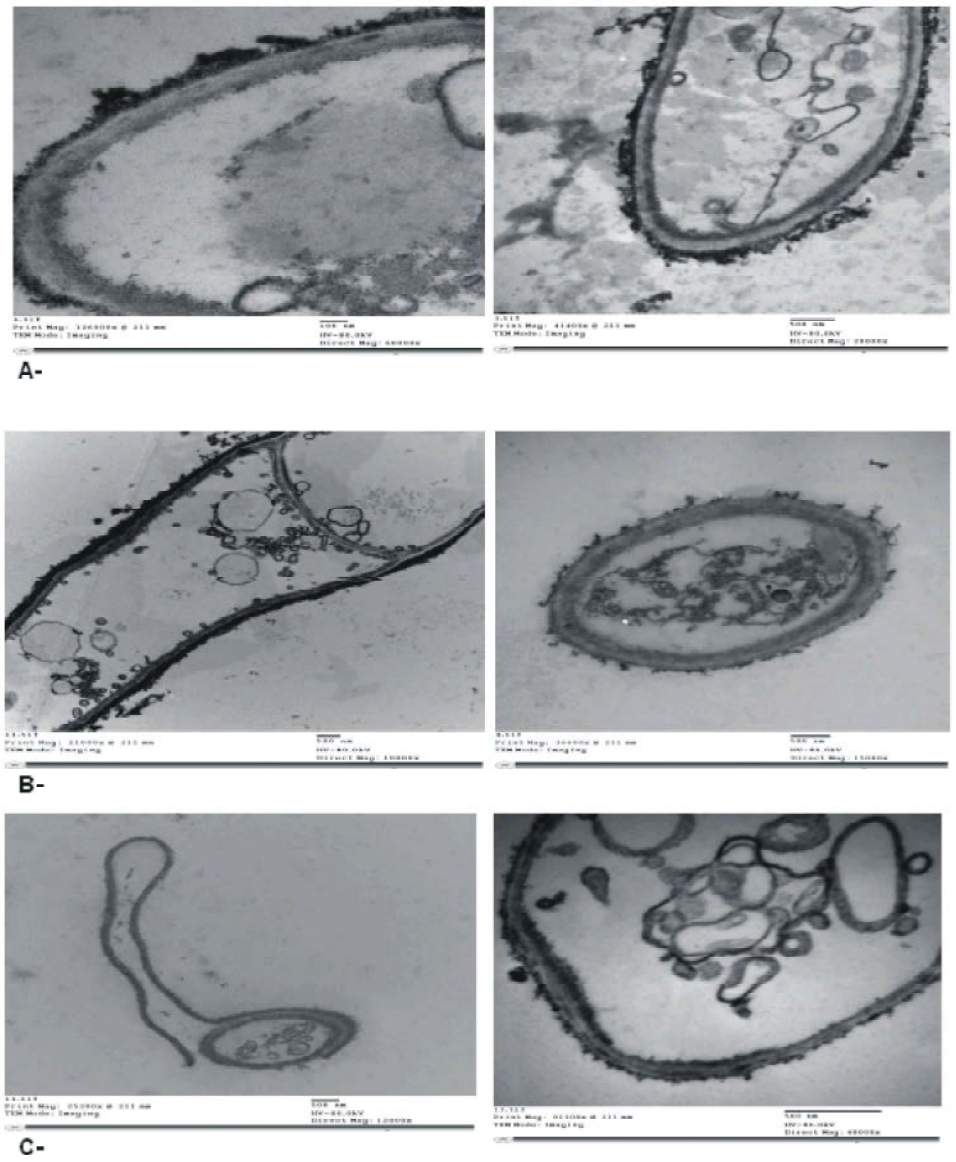

Fig. 21: Scanning electron micrograph of *A. tubingensis* fungal cells at magnification of 10000, 60000, 40000, 20000, 15000 and 12000 X, respectively: A: Cells 48h after treated with dye. B: Cells 24h after treated with dye. C: Control dye free

The study was designed to follow the changes in the fungal cell before and after the exposure to dye during the process of removing dye. Following the changes of microstructure of the cell components was done using scanning electron microscopy analyses. The electron micrographs showed obvious differences in the cell wall and most of the cell components. The cell wall after contact with the dye solution was almost doubled; moreover new components inside the cells were formed it might be due to induction of specific enzymes such as ligninolytic ones. The dye in high concentration was likely to enhance the formation of specific cellular bodies, which may play role in reducing the reported toxic effect of the dye. The cellular changes in pathogen fungi were reported with using toxic chemicals. The changes in the fungal cell before and after the exposure to dye during the process of removing dye are shown in the following figures;

- Figure a :*A. tubingensis* after exposure to Direct dye for 48 h.
- Figure b :*A. tubingensis* after exposure to Direct dye for 24 h.

- Figure c :*A. tubingensis* control with no exposure to dye.

The surface morphology of the *A. tubingensis* mycelia without dye exposure is presented in the scanning electron micrograph is in Fig. 21. As shown in the SEM micrograph, the fungal myceliahave rough and porous surface, this surface property should be considered as a factor providing an increase in the total surface area. Also these properties are important to allow the fungi to interact with the surrounding media elements, weather for nutrition purposes and/or for bioremediation of the media to reduce the risk threatening the fungal life. Here, the enzyme activity plays the significant role in the media modification.

DISCUSSION

Large number of industrial applications for fungal laccases has been proposed and they include paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation ofdye and their precursors, enzymatic conversion of chemical intermediates and production of chemicals from lignin. Before laccases can be commercially implemented for potential applications, however, an inexpensive enzyme source needs to be made available [18]. Two of the most intensively studied areas inthe potential industrial applications of laccases are the delignification and pulp bleaching and the bioremediation of contaminating environmental pollutants [7].

In this work the peroxidases and laccase senzymes production by the fifteen fungal strains was studied in both MSM supplemented with the dye and PDB throughout the incubation period that lasted for 15 days to compare the production efficacy of each fungal isolate under the two nutritional how conditions the growth media. The same results were optioned by Buddolla how showed that the laccase production by fungi depend markedly on the composition of the cultivation medium. The optimum time for maximum peroxidases and laccases production varied depending on the fungal strain and the medium type. Buddolla [19] found that out of 12 cultures tested, six cultures were found to be laccase-positive with *Stereumostrea* and *Phanerochaete chrysosporium* being the best potential cultures. Laccase production on 5 different liquid media was compared using these two white rot fungi. The results indicated that all tested fungal strains under this study showed the highest laccases production after 3-days of incubation, except only two strains. The same trend has been previously observed for a number of fungal laccases [20]. Also Takao [21] proposed that the peroxidase used as remover of aromatic pollutants from process streams, but laccase has the distinct advantage that, unlike peroxidase, On the other hand three fungal strains showed the maximum peroxidase production after 6-days of incubation. The wide variations in enzymes production on the two different media may be due to that the higher nutritional value of PDB, as it contains several growth factors such as vitamins.

CONCLUSION

The identification of the highest fungal peroxidase and laccases produces is important for potential extraction of these enzymes required for azo dyes degradation, particularly for developing enzymatic bioremediation technology instead of using fungal intact cells. The laccase production by the 15 fungal strains showed the highest records on PDB medium as compared with MSM medium. It was noticed that the production was generally higher in late sampling (12 and 15 days of incubation) than in early sampling. The most promising laccase producers are strains 1, 2, 11, 31 and *Pleurotus ostreatus. The production of* peroxidase enzyme was achieved after different inoculation period depended on the strain tested. The general trend shows that the peroxidase production on PDB medium was more than that on MSM media particularly after 6 day of inoculation.

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