



### Full Length Article

## Production and Application of Extracellular Laccase Produced by *Fusarium oxysporum* EMT

Esmail M. El-Fakharany<sup>1\*</sup>, Mohamed A. Hassan<sup>1\*</sup> and Tarek H. Taha<sup>2</sup>

<sup>1</sup>Protein Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research & Technological Applications (SRTA-City), New Borg El-Arab City, P.O. Box: 21934 Alexandria, Egypt

<sup>2</sup>Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research & Technological Applications (SRTA-City), New Borg El-Arab City, P.O. Box: 21934 Alexandria, Egypt

\*For correspondence: m\_adelmicro@yahoo.com; esmailelfakharany@yahoo.co.uk

### Abstract

Laccase enzymes are multi-copper oxidases mainly produced by fungi, and have the ability to oxidize various phenolic compounds and discharge of synthetic dyes which considered toxic, carcinogenic, neurotoxic and mutagenic for human and environment. In the current study, highly active extracellular laccase was produced by fungus isolated from soil and identified as *Fusarium oxysporum* EMT (KT182633) using 18S rRNA gene as a molecular identification tool. Laccase was active optimally at pH 4-5 and 60°C utilizing ABTS as an enzyme substrate. The decolorization of 3 synthetic dyes using direct fungal cultures was carried out at concentrations of 50 and 100 mg/L. The highest percentage of decolorization by laccase was exhibited for malachite green (98% and 96.8%) followed by Congo red (95% and 85%) and methyl orange (87.6% and 83%). The laccase was able to detoxify the cytotoxicity of the synthetic dyes against Caco-2 and fibroblast cells, which determined using a microculture tetrazolium assay (MTT) method. Additionally, the highest detoxification was revealed with malachite green that showed cell viability up to 100%. The outcomes demonstrated that laccase enzyme could be applied in environmental and agricultural applications. © 2016 Friends Science Publishers

**Keywords:** Laccase; Decolorization; Detoxification; Dyes; Caco-2 and fibroblast cells

### Introduction

Laccases are a group of enzymes found in certain plants, insects, and a variety of fungi (Giardina *et al.*, 2010; Kim *et al.*, 2010). They were first discovered in plants and subsequently observed in fungi and bacteria. The first record of laccases as fungal enzymes was reported in 1896 by both Bertrand and Laborde (Desai and Nityanand, 2011). Moreover, the first bacterial laccase was found in the plant root associated bacterium "*Azospirillum lipoferum*". However, the discovered bacterial laccases are few (Givaudan *et al.*, 1993). Laccases are produced as extracellular or intracellular, and all of them catalyzed the polymerization or depolymerization processes (Viswanath *et al.*, 2014). They are multi-copper enzymes belong to the blue oxidases group which particularly widespread in ligninolytic basidiomycetes (Hoegger *et al.*, 2006; Alfara *et al.*, 2013). However, they have an environmental interest of detoxification of pollutants and remediation of phenolic compounds (Viswanath *et al.*, 2014). Laccases can use oxygen as a final electron acceptor and oxidize aromatic substances such as diphenols, methoxy-substituted monophenols, and aromatic amines. They also can remove

the hydrogen atoms from the hydroxyl group of ortho- and para- substituted mono and polyphenolic substrates. However, laccases have the potentiality for industrial and biotechnological applications including pharmaceutical products (Thurston, 1994; Priyadarsinin *et al.*, 2011) and the purification of colored waste water (Ikehata *et al.*, 2004). Moreover, laccases could be applied in textile dye decoloration (Husain, 2006), pulp bleaching in the paper industry, oxygen cathode development for biofuel cells (Kalyani *et al.*, 2012) and inactivation of toxic environmental pollutants (Hwang *et al.*, 2007). Taha *et al.* (2015) reported that synthetic dyes have been decolorized using physical, chemical and biological methods. The remediation of synthetic dyes from waste effluents becomes an interest to remove their hazards components from the environment (Reffas *et al.*, 2010). The aim of this study is concerned with laccase enzyme production by a new fungus isolate which identified utilizing morphological and molecular tools. Besides, characterization of laccase enzyme was performed. Furthermore, decolorization of toxic dyes and determination of the probable cytotoxicity of residual treated dyes by laccase on cell lines were studied.

## Materials and Methods

### Isolation of Laccase Producing Fungi

Different soil samples and rotten tree barks were collected from various districts of Egypt (Wadi Alnatroon, El Behira, and Borg El-Arab City). One gram of each sample was serially diluted using sterile saline solution and spread over PDA plate supplemented with 0.5% (w/v) tannic acid as inducer (Kiiskinen *et al.*, 2004). The plates were incubated at 30°C for 5 days and the isolates, which able to form dark brown colors around their colonies were selected as presumptive laccase producers. The selected isolates were then purified using sterile PDA plates and checked for their actual laccase production. In order to confirm the production of laccase by selected fungi, positive fungal isolates were sub-cultured in PDA with and without 0.5% tannic acid (Forootanfar *et al.*, 2011). The potent laccase producing fungal isolate was preserved for further investigations.

### Genetic Identification of the Potent Fungal Isolate

**DNA extraction and PCR amplification of 18S rRNA gene:** The genomic DNA was extracted according to our designed method (Data not shown). The 18S rRNA gene was amplified following method described by White *et al.* (1990) with modified conditions according to Abol Fotouh *et al.* (2016) using universal 18S primers where the sequence of the forward primer was 5'-AACTTAAAGGAATTGACGGAAG-3', and the reverse primer was 5'-GCATCACAGACCTGTTATTGCCTC-3'. The reaction was carried out in 50 µL with the following components: 10 µL of 10 X buffer including 10 mM MgCl<sub>2</sub>, 10 pmol of each primer, 10 mM dNTPs, 1 µL of 1 u/µL Taq polymerase and 1 µL of 50 ng DNA. The PCR was carried out under the following conditions: a denaturation step at 95°C for 5 min, followed by 35 cycles; of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR product was analyzed using 1.5% agarose gel, visualized and photographed using gel documentation system. The PCR product was eluted from the gel using gel purification kit (Qiagen, USA) and purified for DNA sequencing. The DNA sequencing was carried out using enzymatic chain terminator technique designed by Sanger *et al.* (1977). Similarity analysis of the nucleotides was performed by basic local alignment search tool (BLAST) and a phylogenetic tree was constructed using MEGA 5 software version 5.1 (Tamura *et al.*, 2011).

### Production of Extracellular Laccase in Submerged Culture

The extracellular laccase was produced by potent fungal isolate using a specific submerged culture medium. The production medium was prepared according to Udayasoorian and Prabu (2005) with some modifications components in g/L were; glucose 10; peptone 2; KH<sub>2</sub>PO<sub>4</sub>

0.8; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5; Na<sub>2</sub>HPO<sub>4</sub> 0.4; CaCl<sub>2</sub>·2H<sub>2</sub>O 0.06; FeSO<sub>4</sub>·7H<sub>2</sub>O 0.005; CuSO<sub>4</sub>·5H<sub>2</sub>O 0.005; MnSO<sub>4</sub>·H<sub>2</sub>O 0.005; ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.001; and finally 1 mM Gallic acid or 1 mM Guaiacol as an inducer and the pH was adjusted to 5±0.2. For enzyme production by selected fungal isolate; a single disc of 5 days old culture (5 mm in diameter) from the solid medium was inoculated into 500 mL Erlenmeyer flasks with final incubation conditions at 30°C and 150 rpm for 14 days. The fungal mycelia were then removed by centrifugation at 10000 rpm for 30 min followed by filtration using what-man filter papers, and the obtained supernatant was used for further investigations of the crude enzyme.

### Assay of Laccase Activity

The obtained supernatant included with the laccase enzyme was used for determination of the crude enzyme activity by measuring the oxidation of 2, 7-azino bis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt (ABTS) according to Shin and Lee (2000) and Niku-Paavola *et al.* (1988) with some modifications. An aliquot of the supernatant was incubated in 1300 µL of 67 mM sodium acetate buffer, pH 4.5 supplemented with 1.54 mM ABTS as a substrate, while the enzyme blank was including boiled crude enzyme. The mixture was submitted to vortex for 30 sec and incubated at 50°C for 10 min. After incubation, the reaction was stopped using 50 µL of 10% trichloroacetic acid (TCA) solution followed by centrifugation at 10000 rpm for 10 min and finally the optical density of the supernatant was measured at 405 nm. The enzyme activity was expressed as international unit (IU), where; 1 IU can be defined as the amount of enzyme that can oxidize 1 micromole of the substrate per minute (Han *et al.*, 2005). One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405 nm of one per min per ml of the reaction mixture under the standard conditions.

### Zymography of Laccase

Zymogram technique was carried out according to El Fakharany *et al.* (2009, 2010) to confirm the extracellular production of laccase by selected fungal isolate. Separating gel of 12% SDS-Polyacrylamide gel electrophoresis was performed in 30% (w/v) acrylamide using Tris-glycine buffer, pH 8.3 as running buffer (Laemmli, 1970). The samples were mixed with 5 X sample buffer without reducing agent ( $\beta$ -mercaptoethanol or Dithiothreitol) and without boiling. After running the gel at 80 V, the gel was stripped off from the gel plate followed by washing twice in distilled H<sub>2</sub>O to remove SDS. Then, the gel was soaked in 2.5% triton X-100 for 30 min with the change of the solution every 15 min for removing SDS and renaturing the enzymes (Hassan *et al.*, 2013). Finally, the gel was soaked in sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS to visualize laccase activity on the gel.

### Effect of pH and Temperature on Laccase Activity

Different aliquots of the crude containing laccase were submitted to activity estimation using different pH and temperature conditions to determine the optimum pH and temperature of enzyme activity. The used ABTS substrate was prepared in 1.54 mM concentration in different buffers with different pH values ranging from 2 to 9. The prepared buffers were glycine HCl, pH 2-3 (0.1 M), acetate buffer, pH 4-5 (0.1 M), sodium phosphate, pH 6-8 (0.1 M) and glycine/NaOH pH 9-10 (0.1 M). The incubation conditions and activity determination factors were performed as mentioned above. In addition, the enzyme activity was also measured at different temperatures ranging from 10 to 90°C. The buffers were prepared at pH 4.5 with 1.54 mM ABTS, 50 µL of the crude enzyme were added, and the mixtures were incubated at the mentioned temperature ranges for 10 min in a shaking water bath. The optical density was then measured at 405 nm and the highest enzyme activity as unit/mL was recorded according to Shin and Lee (2000) method.

### De-colorization of Different Synthetic Dyes

The fungal isolate was pre-cultured on PDA agar medium plates as described above. One fungal disc with diameter of 5 mm from the solid medium was inoculated into 250 mL Erlenmeyer flasks contained 50 mL liquid media with the following components per liter; (10 g; glucose, 2 g; peptone, 0.8 g; KH<sub>2</sub>PO<sub>4</sub>, 0.5 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g; Na<sub>2</sub>HPO<sub>4</sub>, 0.06 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.005 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.005 g; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.005 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.001 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, and the pH was adjusted to 5.5) with incubation conditions at 30°C and 150 rpm for 7 days. Then; different three dyes including malachite green, Congo red and methyl orange at concentrations of 50 and 100 mg/L were added into the 5 days cultures and incubated at 30°C with shaking at 150 rpm continuously. Aliquots (3 mL) of each flask were withdrawn at different time intervals (1, 2, 3 and 4 days) and the optical density was measured at 618 nm for malachite green, 462 nm for methyl orange and 485 nm for Congo red. The percentage of dye decolorization was calculated according to the following formula:

$$\text{The decolorization rate (\%)} = [(S_i - S_t)/S_i] * 100$$

Where, S<sub>i</sub>: initial absorbance of the dye and S<sub>t</sub>: absorbance of the dye after incubation time.

### Detoxification Assays

**Cytotoxicity of crude laccase:** Different volumes of the crude laccase enzyme were tested for their cytotoxicity against Caco-2 and fibroblast cell lines using (MTT) Thiazolyl Blue Tetrazolium Bromide method according to Almahdy *et al.* (2011) and El Fakharany *et al.* (2013). In brief, Caco-2 and fibroblast cells (10×10<sup>3</sup>) were cultured in a 96 well plate for overnight at 37°C, 5% CO<sub>2</sub> and 88%

humidity. The total volume of used DEMEM supplemented medium and crude enzyme supernatant was 200 µL, where the supernatant of the enzyme was added with volumes 20, 40, 60 and 80 µL in triplicate with the required adequate volume of the medium up to 200 µL total volume. The plate was incubated at 37°C, 5% CO<sub>2</sub> for 3 days. After incubation, debris, and dead cells were removed by washing three times with fresh medium. Twenty µL of MTT solution (5 mg/mL of MTT in PBS buffer) was added to each well and shook for 5 min at 150 rpm to thoroughly mix the MTT into the media. The cells were incubated at 37°C and 5% CO<sub>2</sub> for 3-5 h to metabolize MTT by viable cells. 200 µL dimethylsulfoxide (DMSO) was added to each well and shook again for 5 min at 150 rpm, and then the viability of the cells was calculated by measuring the optical density at 630 nm subtracted from optical density at 570 nm (Mosmann, 1983). The percentage of viability cells was calculated by comparison with control cells (without adding the crude enzyme to the cells) using the equation of: (A) test/ (A) control × 100.

### Cytotoxicity of Treated Dyes by Laccase

After decolorization of the three mentioned dyes (malachite green, Congo red and methyl orange) by fungal isolate, they were tested for their toxicity against both of Caco-2 and fibroblast cells compared with dyes containing water with final concentrations of 5, 10, 15, 20 and 25 mg/L. Caco-2 and fibroblast cells (10×10<sup>3</sup>) were incubated in a 96 well plate for overnight at 37°C, 5% CO<sub>2</sub> and 88% humidity. Decolorized dyes were added to cells at concentrations of 5, 10, 15, 20 and 25 mg/L in triplicate with the required appropriate volume of the supplemented DMEM medium up to 200 µL total volume. After incubation for 3 days, the relative cell viability (%) compared to control wells containing cell culture medium without dyes or decolorized dyes was calculated as described above.

## Results

### Isolation and Molecular Identification of Laccase-producing Fungal Isolate

The laccase producing fungi were preliminarily screened from soil samples; 10 fungal isolates were found to produce brown halos on PDA plates containing tannic acid as a laccase production indicator. One isolate was selected to complete the study as it showed a biggest dark brown colored zone around its colony after 7 days of incubation at 30°C as shown in Fig. 1. For identifying the potent laccase producing isolate, the amplified and sequenced 18S rRNA gene of the isolate was compared with the deposited sequences in GenBank (NCBI GenBank) using BLAST tools. The fungal strains exhibited 99% identity to *Fusarium oxysporum*, *Gibberella fujikuroi* and *Hypocreales* sp. The fungal isolate was identified as *F. oxysporum* after

morphological and microscopical examinations. The sequence was submitted to NCBI GenBank and deposited as *F. oxysporum* EMT with accession number (KT182633). The phylogenetic relationship between *F. oxysporum* EMT (KT182633) and other related microorganisms found in the GenBank database was constructed using the neighbor-joining method and based on Jukes-Cantor distances in MEGA 5.1 software (Tamura *et al.*, 2011) as shown in Fig. 2.

### Extracellular Laccase Production by *F. oxysporum* EMT and Zymography of Produced Laccase

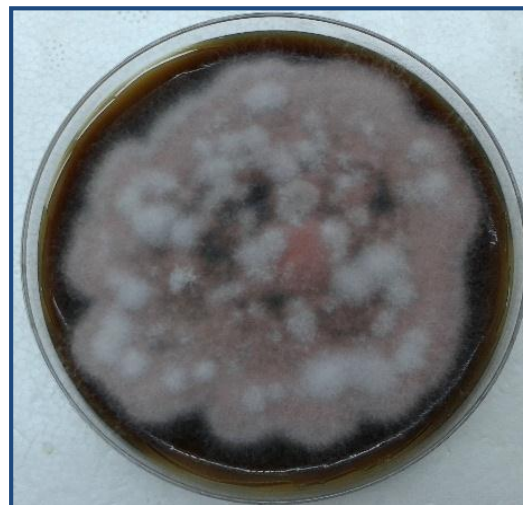
The selected positive strain [*F. oxysporum* EMT (KT182633)] was cultured in submerged medium to produce laccase extracellularly. Extracellular laccase producing strain was initially cultivated in medium containing 1 mM Gallic acid or Guaiacol as inducers sources. The Gallic acid was observed as the better inducer to produce laccase at a high level than Guaiacol. The extracellular laccase was produced by *F. oxysporum* EMT in the presence of Gallic acid and Guaiacol, and it was investigated using zymogram analysis on SDS-PAGE. Fig. 3 shows the activity of laccase on SDS-PAGE and the laccase enzyme was successfully oxidizing ABTS substrate to produce two dark green bands for enzyme zymography.

### Effects of pH and Temperature on Laccase Activity

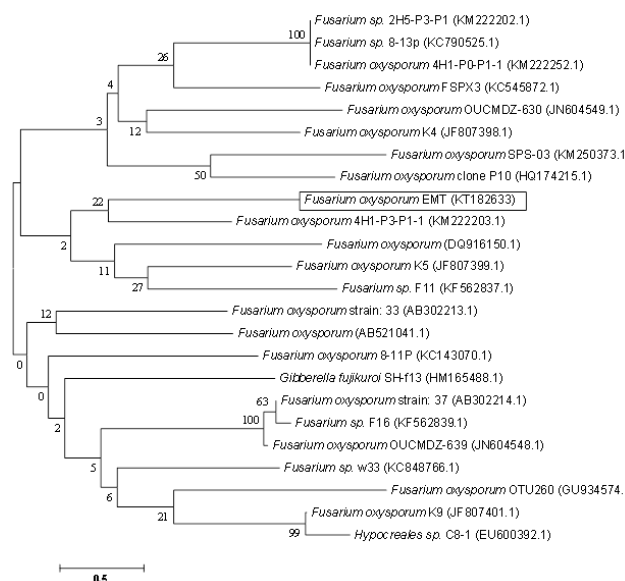
The crude laccase solution was obtained from the filtrate of a submerged culture medium incubated at 30°C with shaking at 150 rpm for 14 days. The optimum pH of laccase was determined to be 4.0 using ABTS substrate, the activity of laccase increased steadily from pH 2.0 to the highest activity between pH 4.0 and 5.0 while, the enzyme activity decreased to become 12% at pH 9.0 as shown in Fig. 4A. The optimum temperature for laccase was 60°C; the activity of laccase increased from 20% at a temperature of 10°C to a relative activity of 100% at temperature 60°C while the activity decreased to become 30% at 90°C as presented in Fig. 4B.

### Decolorization of Synthetic Dyes by *F. oxysporum* EMT Strain

*F. oxysporum* EMT with accession number (KT182633) was pre-cultured on PDA plates containing 0.5% tannic acid at 30°C for 7 days. One fungal disc from the PDA culture was inoculated into flasks containing 50 mL liquid submerged medium without adding any inducers, and the pH was adjusted to 5.5. After incubation of *F. oxysporum* EMT strain in triplicate at 30°C in a shaking incubator at 150 rpm for 7 days, different dyes (malachite green, methyl orange and Congo red) were added at concentrations of 50 and 100 mg/L and incubated at 30°C and 150 rpm continuously. Control cultures also were included without adding any dye to culture media. An

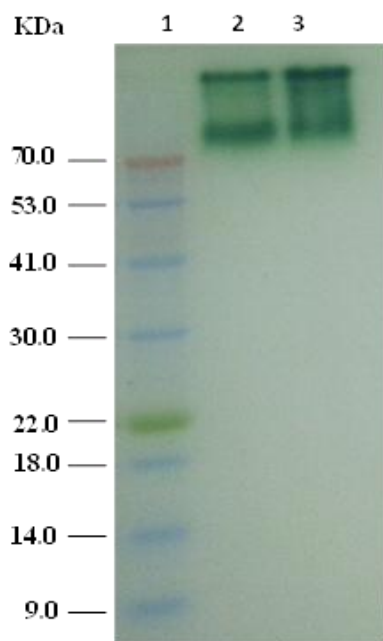


**Fig. 1:** Sporulated culture and colony morphology of *F. oxysporum* EMT (KT182633) in tannic acid supplemented PDA solid media. The dark brown color under and around the fungus culture in the PDA media containing tannic acid is indicated to a positive reaction for the production of laccase



**Fig. 2:** Phylogenetic position of *F. oxysporum* EMT within the genus *Fusarium*. The branching pattern was generated by neighbor-joining tree method with 1000 bootstrap and the Genbank accession numbers of the 18S rRNA nucleotide sequences are indicated in brackets

aliquot (3 mL) from each culture medium was withdrawn at different time intervals, and the decolorization was estimated by measuring the absorbance of the culture supernatant after centrifugation at 618, 462 and 485 nm for malachite green, methyl orange and Congo red respectively.



**Fig. 3:** Detection of the production of extracellular laccase by *F. oxysporum* EMT using zymogram. Lane 1 is protein marker, lane 2 is crude enzyme from submerged medium containing Guaiacol as inducer and lane 3 is crude enzyme from submerged medium containing Gallic acid as inducer. ABTS was used as laccase substrate to produce the dark green zone

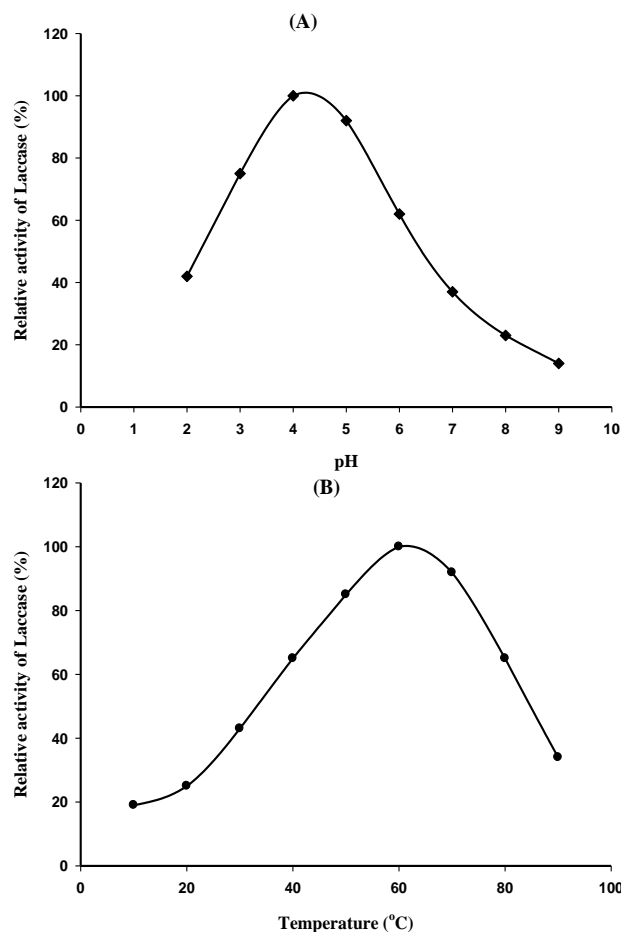
The decolorization of each dye was expressed as relative dye decolorization (%) and calculated as described above in details. The results of this study indicated that laccase can decolorize the used dyes efficiently. Fig. 5 shows that malachite green at concentrations of 50 and 100 mg/L were decolorized up to 98% and 96.8%, respectively by *F. oxysporum* EMT strain within 4 days. Methyl orange at a concentration of 50 mg/L was decolorized up to 87.6% more efficiently than at concentration of 100 mg/L that decolorized up to 83%. However, Congo red at a concentration of 50 mg/L was decolorized up to 95% and at a concentration of 100 mg/L to 85% as shown in Fig. 5.

#### ***In vitro* Cytotoxicity Effect of Laccase on Mammalian Cells**

The cell viability (%) was investigated *in vitro* by MTT-assay using Caco-2 and fibroblast cell lines after conducting crude supernatant including laccase enzyme. As given in Fig. 6, crude samples did not exhibit any cytotoxicity on the Caco-2 and fibroblast cells and the cell viability was around 99 (%) for all used concentrations.

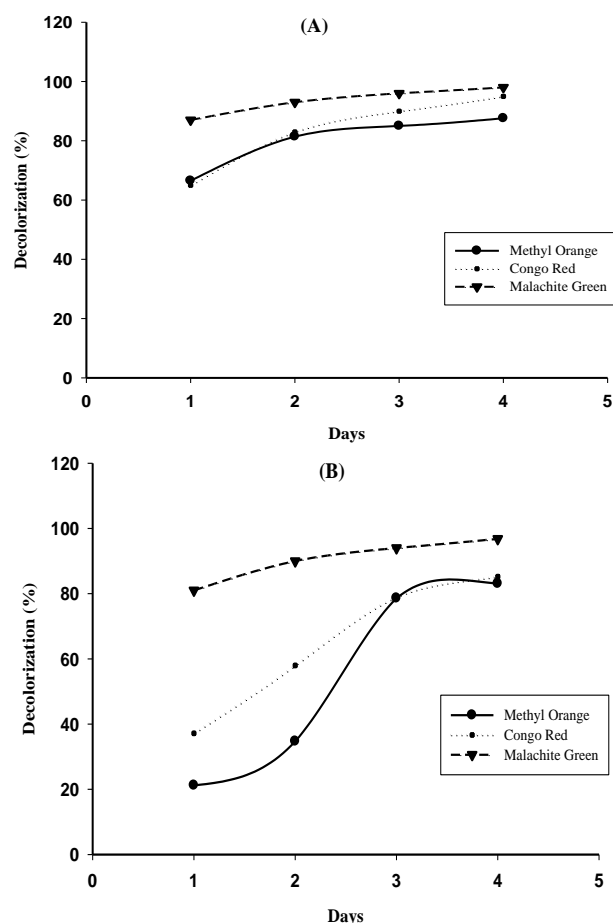
#### **Cytotoxicity Effect of the Decolorized Dyes on Mammalian Cells**

The cytotoxicity effect of the synthetic dyes (malachite



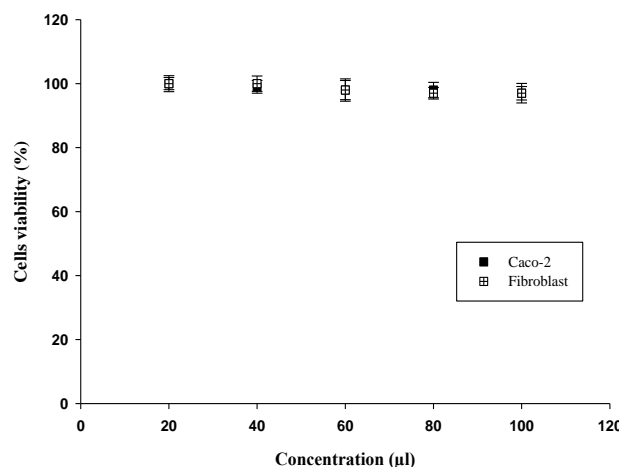
**Fig. 4:** Effect of pH (A) and temperature (B) on activity of laccase produced by *F. oxysporum* EMT towards ABTS as a substrate. The activity was assessed by the standard assay method and the activities are expressed as percentages of maximum activity

green, methyl orange and Congo red) before and after decolorization by whole fungal culture containing laccase treatment was assayed using MTT assay test. The relative activities of the malachite green dye on the viability of Caco-2 cells at concentrations of 5, 10, 15, 20 and 25 mg/L were reported to be 34, 24, 16, 10 and 8% respectively. Additionally, the relative activities of the malachite green dye on the viability of fibroblast cells at concentrations of 5, 10, 15, 20 and 25 mg/L were reported to be 14, 11, 10, 9 and 8%, respectively. While after the decolorization of the malachite green by laccase, the viability of the Caco-2 and fibroblast cells increased significantly up to 97-100% and 93-100% respectively, according to the initial concentration of the decolorized dye as shown in Tables 1 and 2. The results indicated that the relative activities of the Congo red dye on the viability of Caco-2 cells at concentrations of 5, 10, 15, 20 and 25 mg/L were reported to be 42, 40, 36, 30 and 20%, respectively. Also, the relative activities of the



**Fig. 5:** Decolorization percentage of malachite green, Congo red and methyl orange at concentration of 50 mg/L (A) and 100 mg/L (B) at different time intervals (days) using laccase enzyme produced by *F. oxysporum* EMT strain

Congo red dye on the viability of fibroblast cells at concentrations of 5, 10, 15, 20 and 25 mg/L were reported to be 29, 26, 22, 20 and 18%, respectively. However, the laccase enzyme has the ability to detoxify the Congo red dye after treatment, and the viability of the Caco-2 cells ranged from 90% to 96% according to the initial concentration of the detoxified dye (Table 1). On the other hand, fibroblast cells viability ranged from 87% to 95% as shown in Table 2. In addition, our results demonstrated that the relative activities of the methyl orange dye on the viability of Caco-2 cells at concentrations of 5, 10, 15, 20 and 25 mg/L to be 58, 46, 41, 38 and 31%, respectively. Also, the relative sensitivities of the methyl orange dye on the viability of fibroblast cells at concentrations of 5, 10, 15, 20 and 25 mg/L were reported to be 33, 30, 25, 23 and 21%, respectively. The results revealed that the laccase enzyme has the ability to remove the toxicity of the methyl orange dye after treatment and the percentage of viability of the Caco-2 cells ranged from 95 to



**Fig. 6:** *In vitro* cells viability assay against different concentrations of crude laccase enzyme produced by *F. oxysporum* EMT strain using Caco-2 and Fibroblast cell lines

99% and the percentage of viability of fibroblast cells ranged from 94 to 98% as shown in Tables 1 and 2.

## Discussion

Synthetic dyes are known to be used as reactive textile dyes and other various industries such as printing, pulp and paper, cosmetics and pharmaceuticals which lead to the production of more dangerous dyes (Ayed *et al.*, 2011; Tang *et al.*, 2011). It is worth that the removal of all dyes wasn't occurred by enzymatic treatment of waste water, and this treatment could help to reduce the consumption of water (Abadulla *et al.*, 2000). Most studied laccases are extracellular glycoproteins secreted into the medium by fungi (Agematu *et al.*, 1993), bacteria and insects (Martins *et al.*, 2002). Laccases are generally produced during the secondary metabolism and can be used to remove dangerous synthetic dyes. The total of ten fungal strains isolated from soil samples were reported to produce laccase enzyme, and *F. oxysporum* EMT (KT182633) strain showed high activity on plates containing tannic acid as a color indicator. *F. oxysporum* is a vascular wilt fungal pathogen for some important crops (Beckman, 1987; Di Pietro *et al.*, 2003). Plant pathogenicity of *F. oxysporum* and other fungi depends on a number of laccase isozymes (Cañero and Roncero, 2008). In the present study, *F. oxysporum* was succeeded to produce laccase extracellularly using submerged medium containing 1 mM Gallic acid and appropriate nutrients with shaking at 150 rpm. The activity of produced laccase in fungal cultures affected by some conditions which including fermentation and inducer used in the production process (Forootanfar *et al.*, 2011). Zouari-Mechichi *et al.* (2006) produced laccase from *Trametes trogii* in the presence of  $\text{CuSO}_4$  as an inducer and Forootanfar *et al.* (2011) produced laccase from *Paraconiothyrium variabile* in the presence of copper and



**Table 1:** Cytotoxic effect of synthetic and decolorized dyes by laccase on the viability of Caco-2 cells

Dye	Dye concentration (mg/L)	Cells viability in presence of crude dye (%)	Cells viability in presence of Decolorized dye (%)
Malachite green	5	34±0.1	100±0.05
	10	24±0.15	100±0.1
	15	16±0.19	100±0.24
	20	10±0.05	100±0.05
	25	8±0.16	97±0.14
Congo red	5	42±0.22	96±0.08
	10	40±0.09	95±0.75
	15	36±0.13	93±0.02
	20	30±0.25	90±0.18
	25	20±0.07	90±0.23
Methyl orange	5	58±0.14	99±0.05
	10	46±0.04	97±0.09
	15	41±0.18	96±0.08
	20	38±0.1	95±0.12
	25	31±0.13	95±0.13

The data are the mean of three determinations and  $\pm$  is standard deviation (SD)

**Table 2:** Cytotoxic effect of synthetic and decolorized dyes by laccase on the viability of fibroblast cells

Dye	Dye concentration (mg/L)	Cells viability in presence of crude dye (%)	Cells viability in presence of Decolorized dye (%)
Malachite green	5	14±0.2	100±0.12
	10	11±0.05	98±0.15
	15	10±0.04	95±0.16
	20	9±0.08	93±0.13
	25	8±0.2	93±0.2
Congo red	5	29±0.13	95±0.09
	10	26±0.12	93±0.07
	15	22±0.18	90±0.04
	20	20±0.25	88±0.12
	25	18±0.04	87±0.2
Methyl orange	5	33±0.15	98±0.23
	10	30±0.18	96±0.18
	15	25±0.1	96±0.08
	20	23±0.11	94±0.07
	25	21±0.07	94±0.06

The data are the mean of three determinations and  $\pm$  is standard deviation (SD)

2,5-xyldine. The crude laccase activity showed optimum activity at pH 4.0-5.0 and temperature of 60°C using ABTS as a substrate. In general, laccase showed optimum activity at pH ranging from 3.0-5.0 and temperature ranging from 60-70°C. Our results are consistent with Desai *et al.* (2011) who reported that the optimum pH of laccase from the *Tricoderma* spp. was 5.0. In contrast, laccase extracted from *Trametes versicolor* exhibited optimum activity at pH of 3.0 and temperature of 50°C (Han *et al.*, 2005). One of the most important applications of laccase enzyme is synthetic dye decolorization. Here, malachite green, methyl orange and Congo red at concentrations of 50 and 100 mg/L were treated with fungal culture producing laccase to decolorize these synthetic dyes for 4 days. Malachite green dye was decolorized more efficiently than Congo red and methyl orange with decolorization percentage up to 98%. Laccase was also able to decolorize methyl orange with percentage up to 87.6%. While, Congo red was decolorized with percentage up to 95%. Our results are in agreement with Zhuo *et al.* (2011) results that revealed *Ganoderma* sp. En3 could decolorize malachite green, methyl orange and crystal violet at a concentration of 50 mg/L up to 98.9%, 96.7% and 75.8% respectively, within 3 days. A previous study by Liu

*et al.* (2004) reported that *Fomes lignosus* was able to decolorize malachite green at a concentration of 15 mg/L with decolorization percentage up to 66.5% within 10 days. Moreover, *T. versicolor* showed decolorization activity up to 99.5% for malachite green at a concentration of 15 mg/L within 10 days. Zhuo *et al.* (2011) demonstrated that the rate of decolorization was decreased with increasing the concentration of malachite green to 70 and 100 mg/L with decolorization 13% and 6%, respectively. These results indicated that malachite green at higher dye concentration have a fungal toxicity against *Kocuria rosea* MTCC 1532 (Parshetti *et al.*, 2006). However, *F. oxysporum* EMT (KT182633) could tolerate high concentration of malachite green, Congo red and methyl orange up to 100 mg/L.

*In vitro* cytotoxicity effect test is the principle property of any compound being surrounded by living cells or organisms. Hassan *et al.* (2010) proved the importance of cytotoxicity test for biotechnological products purified from microorganisms. *In vitro*, cytotoxicity toward the crude laccase at different concentrations from synthetic dye culture media free and decolorized were tested by MTT-assay in control with synthetic dye using Caco-2 and fibroblast cell lines. Here, crude laccase couldn't show

cytotoxicity effect at used concentrations and cells viability reached around 99%. These results are in agreement that laccase purified from mushroom (*Pleurotus ostreatus*) had not any cytotoxicity effect on HepG2 cells (El Fakharany *et al.*, 2010). However, malachite green, Congo red and methyl orange at concentrations of 5-25 mg/L showed high cytotoxicity effect on both Caco-2 and fibroblast cells and reduced the cell viability to become below 50%. However; the decolorized dyes using laccase from *F. oxysporum* EMT showed almost non-cytotoxic effects against both of Caco-2 and fibroblast cell lines. In addition, cytotoxicity and microtoxicity of synthetic dyes and their degraded products by laccase on human cervix cells (HeLa) have been demonstrated reducing in toxicity of the end degraded compounds (Younes *et al.*, 2012). There are other biological assays were performed to measure cytotoxicity effect of decolorized dye, such as phytotoxicity studies on plant seeds (*Oryza sativa* or *Triticum aestivum*) performed by measuring root and shoot lengths after and before exposure to decolorized dye by enzymatic treatment (Zhuo *et al.*, 2011). Whereas, Ashrafi *et al.* (2013) demonstrated that the toxicity of the decolorized synthetic dyes products by laccase on growth inhibition of bacterial strains showed a significant decrease in the growth inhibition percentage of applied microbe. The degradation and decolorization of synthetic azo dyes by laccases enzymes depends on a high formation of a nonspecific free radical mechanism to form nontoxic phenolic compounds and avoid the formation of toxic aromatic amines (Chivukula and Renganathan, 1995; Wong and Yu, 1999). Additionally, Abdou and Hassan (2014) explained the harmful effects of these free radicals on kidney, liver causing hepatotoxicity and nephrotoxicity.

## Conclusion

The laccase enzyme was produced by *F. oxysporum* EMT (KT182633) isolated from the rotten tree and identified using 18S rRNA. The ability of laccase to remove and decolorize hazard synthetic dyes such as malachite green, Congo red and methyl orange was studied. The cytotoxicity effect of decolorized dyes using MTT method exhibited a significant nontoxic behavior and higher cell viability on Caco-2 and fibroblast cells than synthetic dyes. The cytotoxicity results revealed that the purified water from dyes using laccase could become safe to be used in agricultural applications.

## Conflict of Interest

The authors have no conflict of interest to declare.

## References

- Abadulla, E., T. Tzanov, S. Costa, K. Robra, A. Cavaco-Paulo and G.M. Gübitz, 2000. Decolorization and detoxification of textile dyes with a Laccase from *Trametes hirsute*. *Appl. Environ. Microbiol.*, 66: 3357–3362
- Abdou, H. and M.A. Hassan, 2014. Protective role of Omega-3 polyunsaturated fatty acid against lead acetate-induced toxicity in liver and kidney of female rats. *Biom. Res. Intern. J.*, 2014, 435857: 1–11
- Abol Fotouh, D.M., R.A. Bayoumi and M.A. Hassan, 2016. Production of thermoalkaliphilic lipase from *Geobacillus thermoleovorans* DA2 and application in leather industry. *Enzy. Res.*, 2016, 9034364: 1–9
- Agematu, H., T. Tsuchida and K. Kominato, 1993. Enzymatic dimerization of penicillin X. *J. Antibiotic*, 46: 141–148
- Alfarra, H.Y., N.H.M. Hasali and M.N. Omar, 2013. A lignolytic fungi with laccase activity isolated from Malaysian local environment for phytochemical transformation purposes. *Int. Res. J. Biol. Sci.*, 2: 51–54
- Almahdy, O., E.M. EL-Fakharany, E. EL-Dabaa, T.B. Ng and E.M. Redwan, 2011. Examination of the activity of camel milk casein against hepatitis C virus (Genotype-4a) and its apoptotic potential in hepatoma and HeLa cell lines. *Hepat. Mon.*, 11: 724–730
- Ashrafi, S.D., S. Rezaei, H. Forootanfar, A.H. Mahvi and M.A. Faramarz, 2013. The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*. *Int. Biodeterior. Biodegr.*, 85: 173–181
- Ayed, L., A. Mahdhi, A. Cheref and A. Bakhrouf, 2011. Decolorization and degradation of azo dye methyl red by an isolated *Sphingomonas paucimobilis*: biotoxicity and metabolites characterization. *Desalination*, 274: 272–277
- Beckman, C.H., 1987. *The Nature of Wilt Diseases of Plants*, p: 175. Handbook of American Phytopathological Society, APC Press, St. Paul, Minnesota, USA
- Cañero, D.C. and M.I.G. Roncero, 2008. Functional Analyses of laccase Genes from *Fusarium oxysporum*. *Amer. Phytop. Soc.*, 98: 509–518
- Chivukula, M. and V. Renganathan, 1995. Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. *Appl. Environ. Microbiol.*, 61: 4347–4377
- Desai, S.S. and C. Nityanand, 2011. Microbial laccases and their applications: a review. *Asian J. Biotechnol.*, 3: 98–124
- Desai, S.S., G.B. Tennali, N. Channur, A.C. Anup, G. Deshpande and B.P. Murtuza, 2011. Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnol. Bioinf. Bioeng.*, 1: 543–549
- Di Pietro, A., M.P. Madrid, A. Caracuel, J. Delgado-Jarana and M.I.G. Roncero, 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Mol. Plant Pathol.*, 4: 315–325
- El-Fakharany, E.M., B.M. Haroun, T.B. Ng and E.M. Redwan, 2010. Oyster mushroom laccase inhibits hepatitis C virus entry into peripheral blood cells and hepatoma cells. *Prot. Pept. Lett.*, 17: 1031–1039
- El-Fakharany, E.M., L. Sánchez, H.A. Al-Mehdar and E.M. Redwan, 2013. Effectiveness of human, camel, bovine and sheep lactoferrin on the hepatitis C virus cellular infectivity: comparison study. *Viro. J.*, 10: 199
- El-Fakharany, E.M., E.A. Serour, A.M. EL-Rahman, H. Bakry and E.M. Redwan, 2009. Purification and characterization of camel (*Camelus dromedarius*) milk amylase. *Prep. Biochem. Biotechnol.*, 39: 105–123
- Forootanfar, H., M. Faramarzi, A.R. Shahverdi and M.T. Yazdi, 2011. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. *Bioresour. Technol.*, 102: 1808–1814
- Giardina, P., V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle and G. Sannia, 2010. Laccases: a never-ending story. *Cell. Mol. Lif. Sci.*, 67: 369–385
- Givaudan, A., A. Effosse, D. Faure, P. Potier, M.L. Bouillant and R. Bally, 1993. Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS Microbiol. Lett.*, 108: 205–210
- Han, M.J., H.T. Choi and H.G. Song, 2005. Purification and characterization of laccase from the white rot fungus *Trametes versicolor*. *J. Microbiol.*, 43: 555–560



- Hassan, M.A., A.A. Amara, A.T. Abuelhamd and B.M. Haroun, 2010. Leucocytes show improvement growth on PHA polymer surface. *Pak. J. Pharm. Sci.*, 23: 332–336
- Hassan, M.A., B.M. Haroun, A.A. Amara and E.A. Serour, 2013. Production and characterization of keratinolytic protease from new wool-degrading *Bacillus* species isolated from Egyptian ecosystem. *Biom. Res. Intern. J.*, 2013, 175012: 1–14
- Hoegger, P.J., S. Kilaru, T.Y. James, R.J. Thacker and U. Kues, 2006. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.*, 273: 2308–2326
- Husain, Q., 2006. Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. *Crit. Rev. Biotechnol.*, 60: 201–221
- Hwang, H.M., X. Hu and X. Zhao, 2007. Enhanced bioremediation of polycyclic aromatic hydrocarbons by environmentally friendly techniques. *J. Environ. Sci. Heal. C Environ. Carcinog. Ecotoxicol. Rev.*, 25: 313–352
- Ikehata, K., I.D. Buchanan and D.W. Smith, 2004. Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. *J. Environ. Eng. Sci.*, 3: 1–19
- Kalyani, D., S.S. Dhiman, H. Kim, M. Jeya, I. Kim and J. Lee, 2012. Characterization of a novel laccase from the isolated *Coltricia perennis* and its application to detoxification of biomass. *Proc. Biochem.*, 47: 671–678
- Kiiskinen, L.L., M. Ratto and K. Kruus, 2004. Screening for novel laccase-producing microbes. *J. Appl. Microbiol.*, 97: 640–646
- Kim, J., S. Park and D. Kim, 2010. Heterologous expression of a tannic acid inducible laccase3 of *Cryphonectria parasitica* in *Saccharomyces cerevisiae*. *BMC Biotechnol.*, 10: 1–9
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685
- Liu, W.X., Y.P. Chao, X.Q. Yang, H.B. Bao and S.J. Qian, 2004. Biodecolorization of azo, anthraquinonic and triphenylmethane dyes by white-rot fungi and a laccase secreting engineered strain. *J. Ind. Microbiol. Biotechnol.*, 31: 127–132
- Martins, L.O., C.M. Soares, M.M. Pereira, M. Teixeira, T. Costa, G.H. Jones and A.O. Henriques, 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. Biol. Chem.*, 277: 18849–18859
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65: 55–63
- Niku-Paavola, M.L., E. Karhunen, P. Salola and V. Raunio, 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biol. J.*, 254: 877–884
- Parshetti, G., S. Kalme, G. Saratale and S. Govindwar, 2006. Biodegradation of malachite green by *Kocuria rosea* MTCC 1532. *Acta Chim. Slov.*, 53: 492–498
- Pierce, J., 1994. Colour in textile effluents: the origins of the problem. *J. Soc. Dy. Colou.*, 110: 131–134
- Priyadarsinin, I.R., V. Bhuraneswari and S.K. Kumar, 2011. Isolation, Identification and phylogenetic analysis of white rot fungus and heterologous expression of gene encoding laccase. *J. Appl. Sci. Environ. Sanit.*, 6: 69–83
- Reffas, A., V. Bernardet, B. David, L. Reinert, L.B. Lehocine, M. Dubois, N. Batisse and L. Duclaux, 2010. Carbons prepared from coffee grounds by HPO activation: Characterization and adsorption of methylene blue and Nylosan Red N-2RBL. *J. Haz. Mater.*, 175: 779–788
- Sanger, F., S. Nicklen and A.R. Coulson, 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.*, 74: 5463–5467
- Shin, K.S. and Y.J. Lee, 2000. Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*. *J. Arch. Biochem. Biophys.*, 384: 109–115
- Spadaro, J.T., I. Lorne and V. Renganathan, 1994. Hydroxyl radical mediated degradation of azo dyes: evidence for benzene generation. *Environ. Sci. Technol.*, 28: 1389–1393
- Taha, N.A., T.H. Taha, M.A. Abu-Saied, M.E. Youssef and C. Štefan, 2015. New study for cationic dye removal using prepared SPGMA polymer combined with an isolated bacteria. *Jök. J.*, 65: 138–160
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28: 2731–2739
- Tang, W., R. Jia and D. Zhang, 2011. Decolorization and degradation of synthetic dyes by *Schizophyllum* sp. F17 in a novel system. *Desalination*, 265: 22–27
- Thurston, C.F., 1994. The structure and function of fungal laccases. *Microbiology*, 140: 19–26
- Udayasoorian, C. and P.C. Prabu, 2005. Biodegradation of phenols by lignolytic fungus *Trametes versicolour*. *J. Biol. Sci.*, 5: 824–827
- Viswanath, B., B. Rajesh, A. Janardhan, A.P. Kumar and G. Narasimha, 2014. Fungal laccases and their applications in bioremediation. *Enz. Res.*, 163242: 1–21
- White, T.J., T.D. Bruns, S. B. Lee and J.W. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR- Protocols and Applications- A Laboratory Manual*, pp: 315–322. Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White (eds.). Academic Press, San Diego, California, USA
- Wong, Y. and J. Yu, 1999. Laccase-catalyzed decolourization of synthetic dyes. *Water Res.*, 33: 3512–3520
- Younes, S.B., Z. Bouallagui and S. Sayadi, 2012. Catalytic behavior and detoxifying ability of an atypical homotrimeric laccase from the thermophilic strain *Scytalidium thermophilum* on selected azo and triarylmethane dyes. *J. Mol. Catal. B: Enzy.*, 79: 41–48
- Zhuo, R., L. Ma, F. Fan, Y. Gong, X. Wan, M. Jiang, X. Zhang and Y. Yang, 2011. Decolorization of different dyes by a newly isolated white-rot fungi strain *Ganoderma* sp. En3 and cloning and functional analysis of its laccase gene. *J. Haz. Mater.*, 192: 855–873
- Zouari-Mechichi, H., T. Mechichi, A. Dhoub, S. Sayadi, A.T. Martinez and M.J. Martinez, 2006. Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme. *Enzy. Microbiol. Technol.*, 39: 141–148

(Received 18 January 2016; Accepted 24 May 2016)